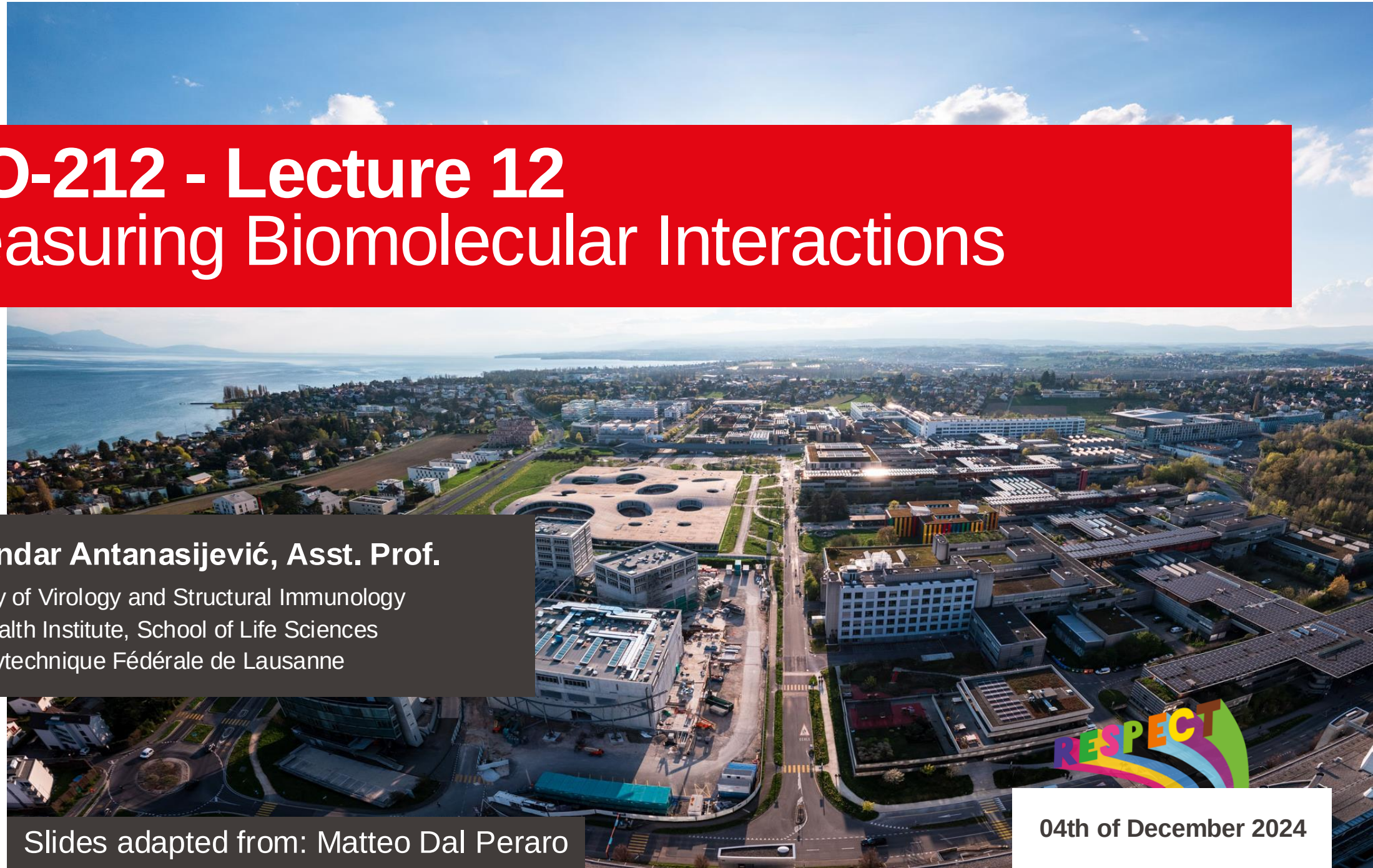
BIO-212 - Lecture 12
Measuring Biomolecular Interactions



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Slides adapted from: Matteo Dal Peraro

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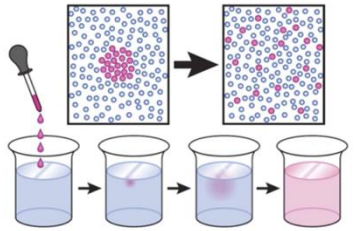
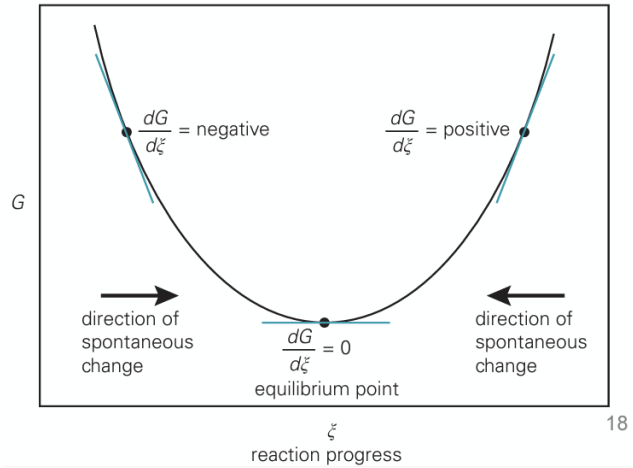
École polytechnique fédérale de Lausanne

Lecture 11 - Summary

• Gibbs free energy and equilibrium



$$dG = dH - TdS$$



18

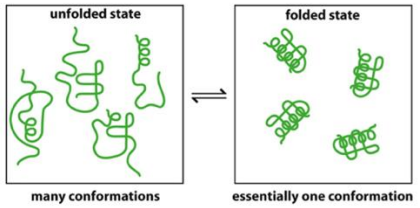
• Equilibrium in different contexts

Chemical reaction

$$ATP + H_2O \rightarrow ADP + P_i$$

$$K = \frac{[ADP][P_i]}{[ATP]}$$

Protein Folding



$$K_{folding} = \frac{[F]}{[U]}$$

Acid-Base Eq.

$$HA \rightleftharpoons H^+ + A^-$$

$$K_a = \frac{[H^+][A^-]}{[HA]}$$

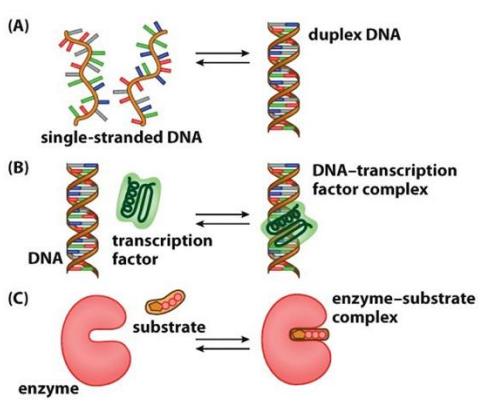
• Equilibrium constants

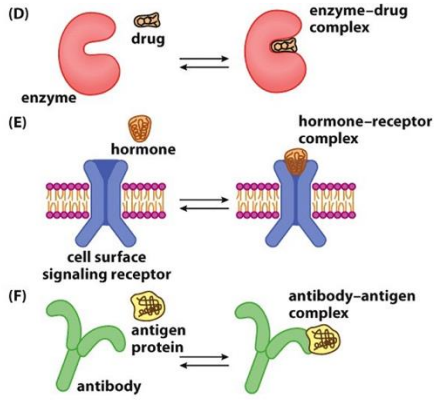
$$\upsilon_A A + \upsilon_B B \rightleftharpoons \upsilon_C C + \upsilon_D D$$

$$K_{eq} = \frac{[C]_{eq}^{\upsilon_C}\,[D]_{eq}^{\upsilon_D}}{[A]_{eq}^{\upsilon_A}\,[B]_{eq}^{\upsilon_B}} \qquad \Delta G^o = -RT \ln K_{eq}$$

Reaction quotient at chemical equilibrium

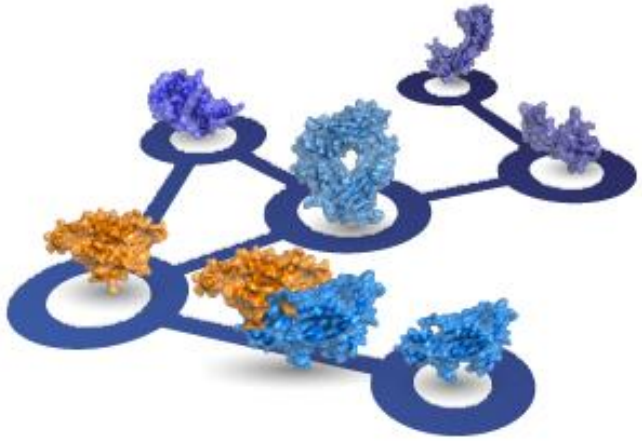
• Molecular interactions





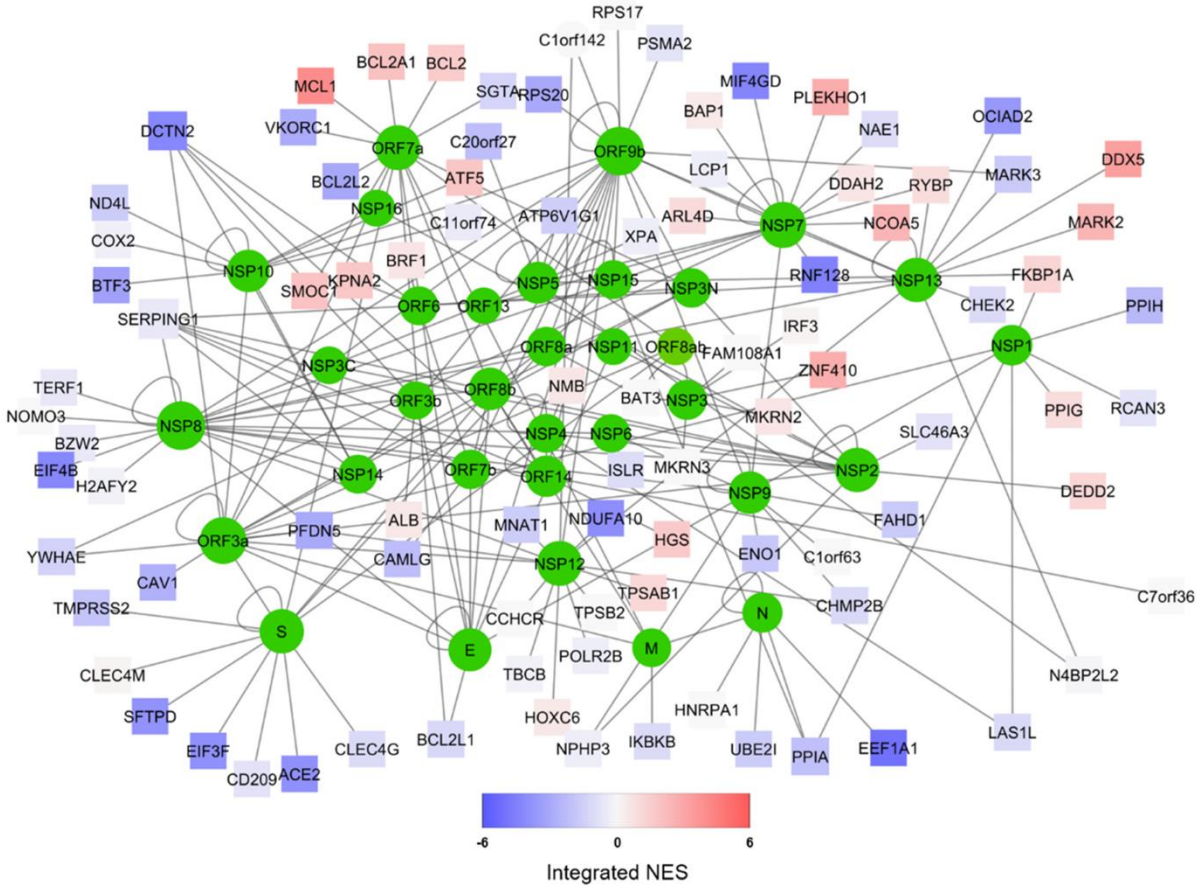
Bimolecular interactions in different contexts

- The function of a biomolecule is partially defined by the interactions that it makes with other biomolecules



Each line depicts possible interaction between these molecules

Systems biology - A very active and important field of research that studies interactomes and their impact on complex biological systems (e.g., cells and tissues)



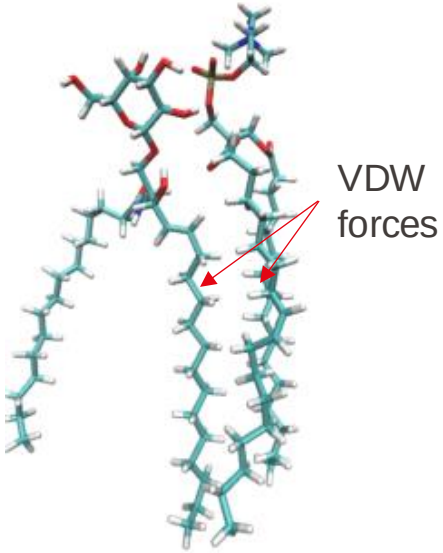
Interactome of the SARS-CoV-2 virus proteins (green)

How strong are biomolecular interactions?

- Gibbs free energy (ΔG) and dissociation constants (K_d) are used to define the strength of interaction

Weak transient interactions

e.g., lipid-lipid

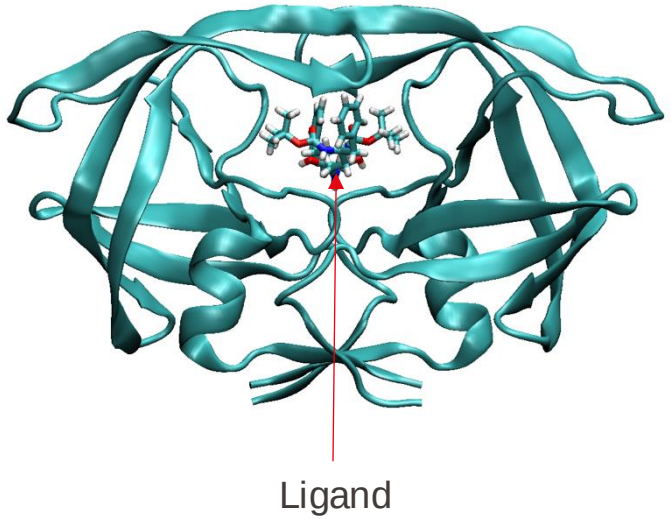


K_d ~10^{-3}-10^{-5} M

ΔG ~16-30 kJ/mol

Intermediate transient interactions

e.g., protein-ligand

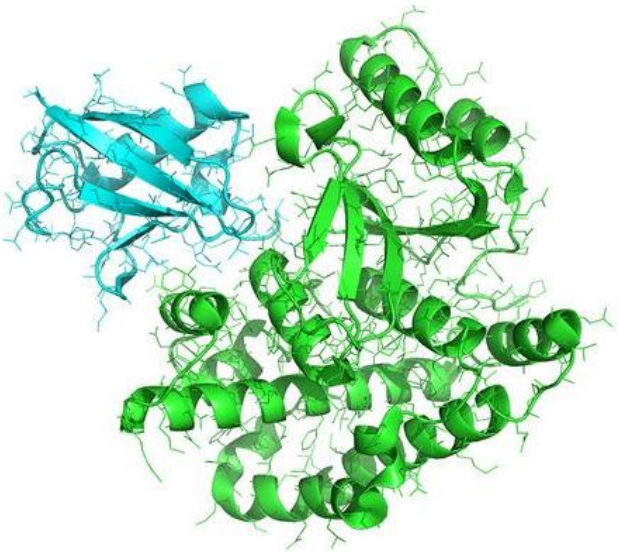


K_d ~10^{-5}-10^{-7} M

ΔG ~30-40 kJ/mol

Strong interactions
(sometimes permanent)

e.g., protein-protein



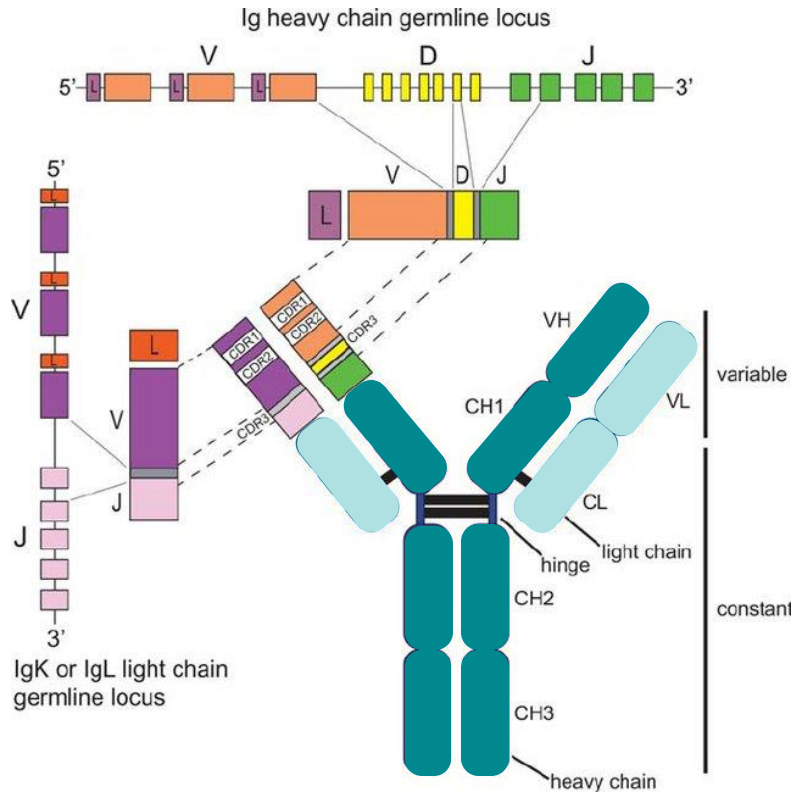
K_d ~10^{-7}-10^{-12} M

ΔG ~40-65 kJ/mol

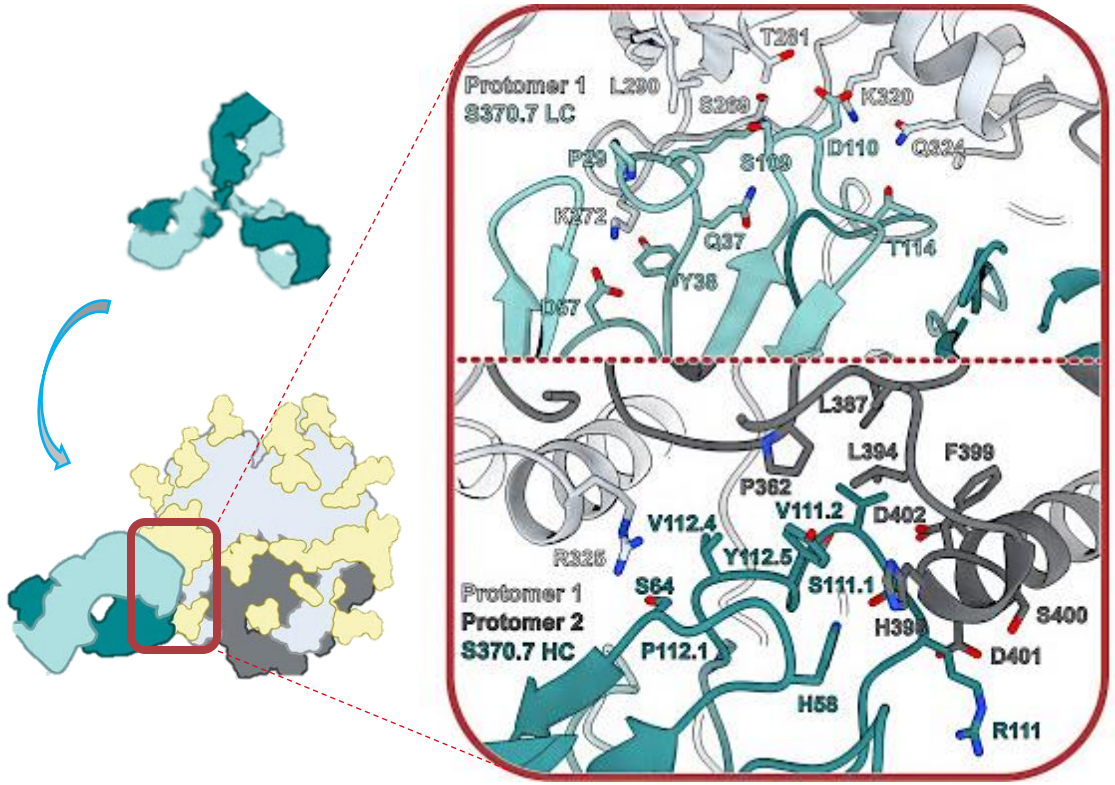
Antibodies: Professional binders

• Antibodies are produced by B cells which undergo **somatic hypermutation** to develop high affinity to foreign material (i.e., antigens).

~10^{11} B-cells in the human body
~10^{4}-10^{6} antigen-specific antibodies induced by immunization or infection
~10^{-7}-10^{-12} M dissociation constants (Kd)



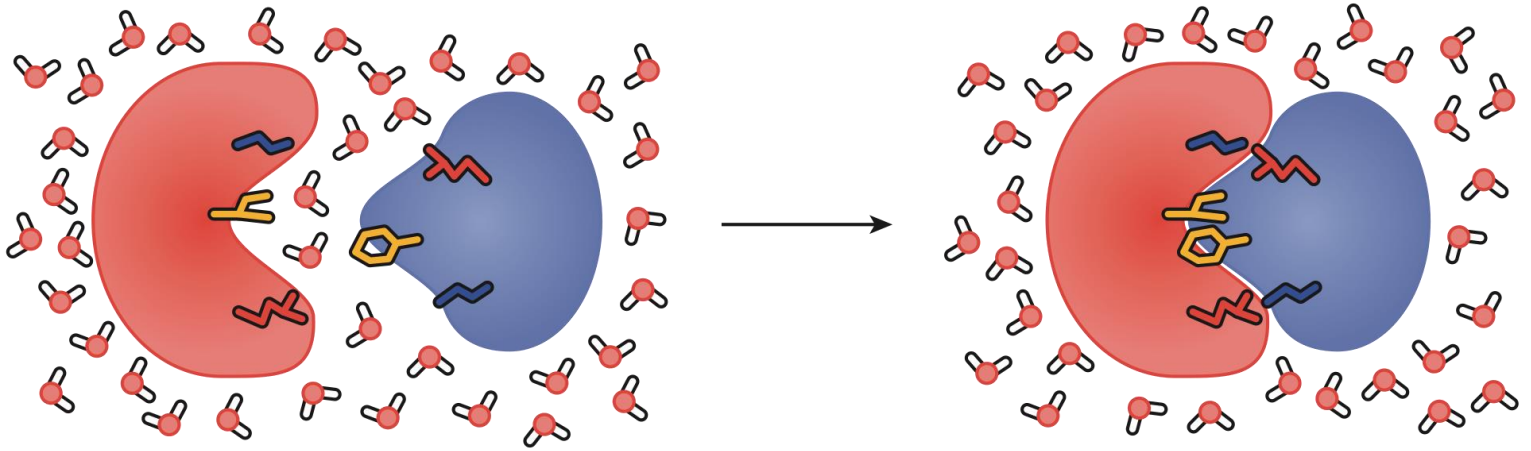
Binding is achieved through complementatity determining regions (CDR)

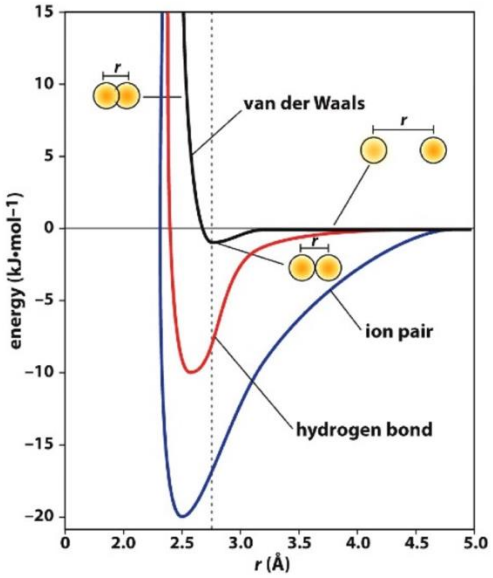


Adapted from Boyd et al., 2014

Origins of biomolecular interactions

- Interface for PPI is similar to the protein core: there are VDW interactions, hydrophobic effect, Hydrogen bonds and salt bridges (ionic interactions
- Interface has usually a small hydrophobic core (which contributes to increase affinity) and other polar interactions (that are important factors for developing specificity)

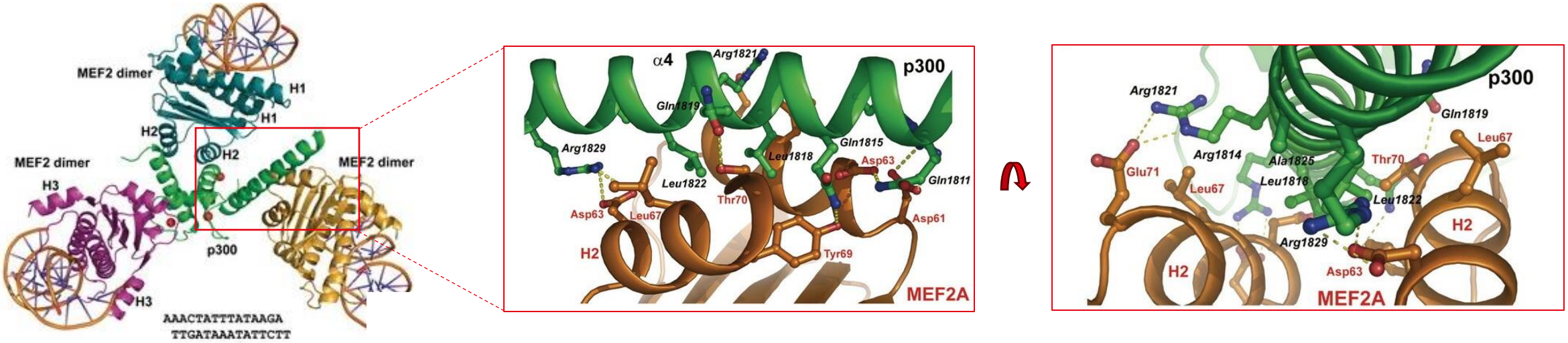




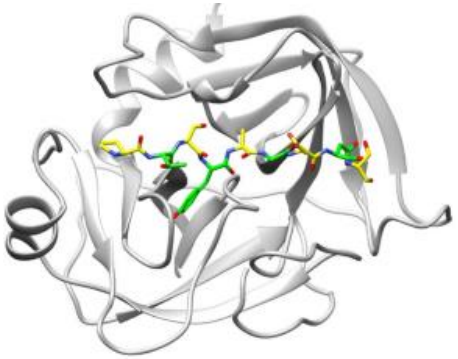
- Matching geometric (shape) and chemical (interaction) properties between the binding molecules
- Small energy terms from individual atomic interactions positively and negatively contribute to the ΔG

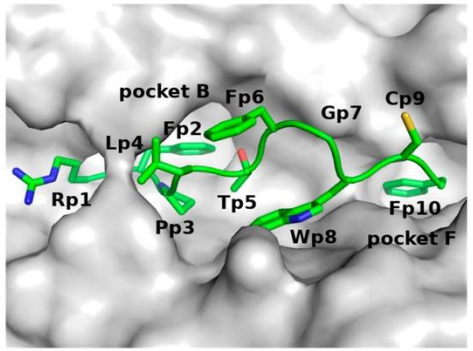
Examples of protein-protein interactions

- Protein-Protein interaction via broader molecular surface (sometimes created by multiple protein domains)



- Protein-Protein interaction via a flexible loop (as a single linear peptide)

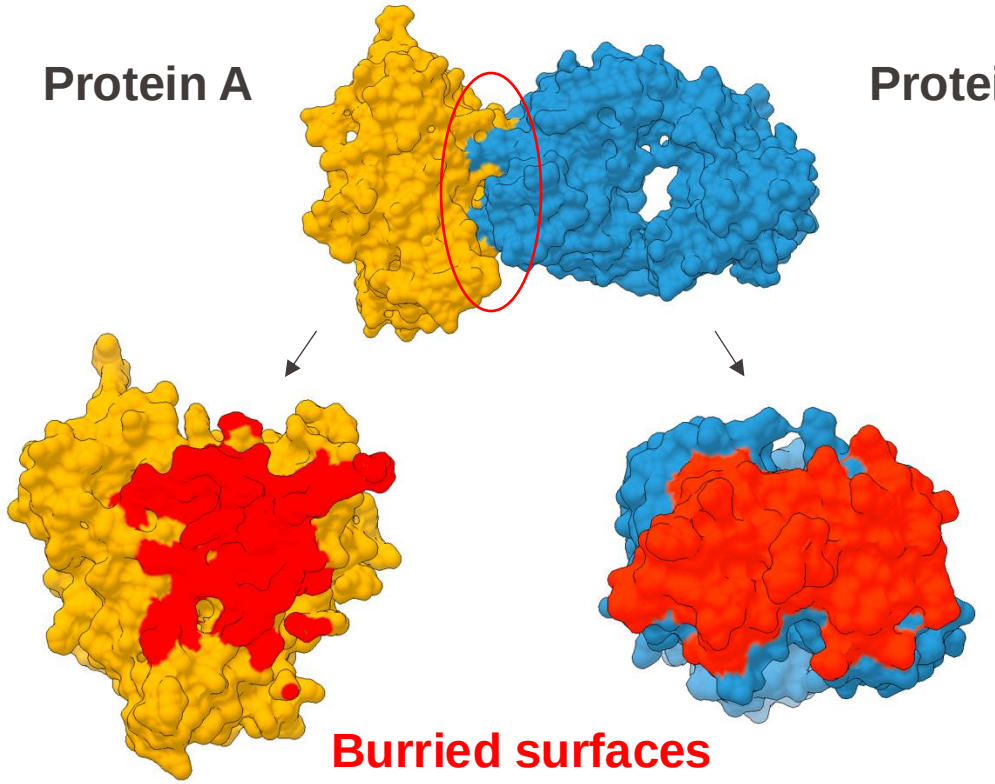




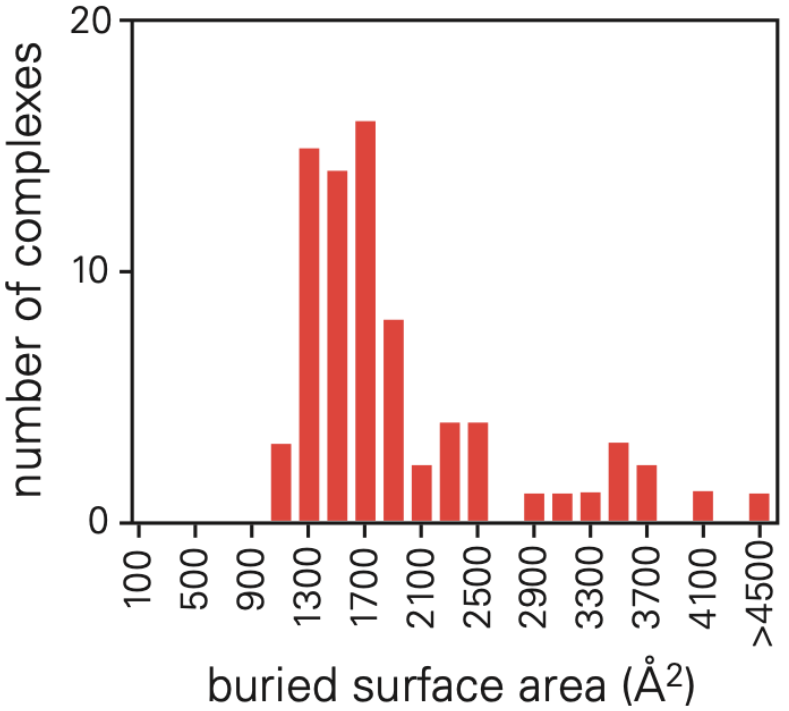
- Usually created by multidomain proteins
- Up to 40% of all protein-protein interactions in cells are mediated through linear peptide loops

The size of the binding interface

- **Buried surface area** provides a quantitative readout of the interacting surface footprint created by the binding between two molecules



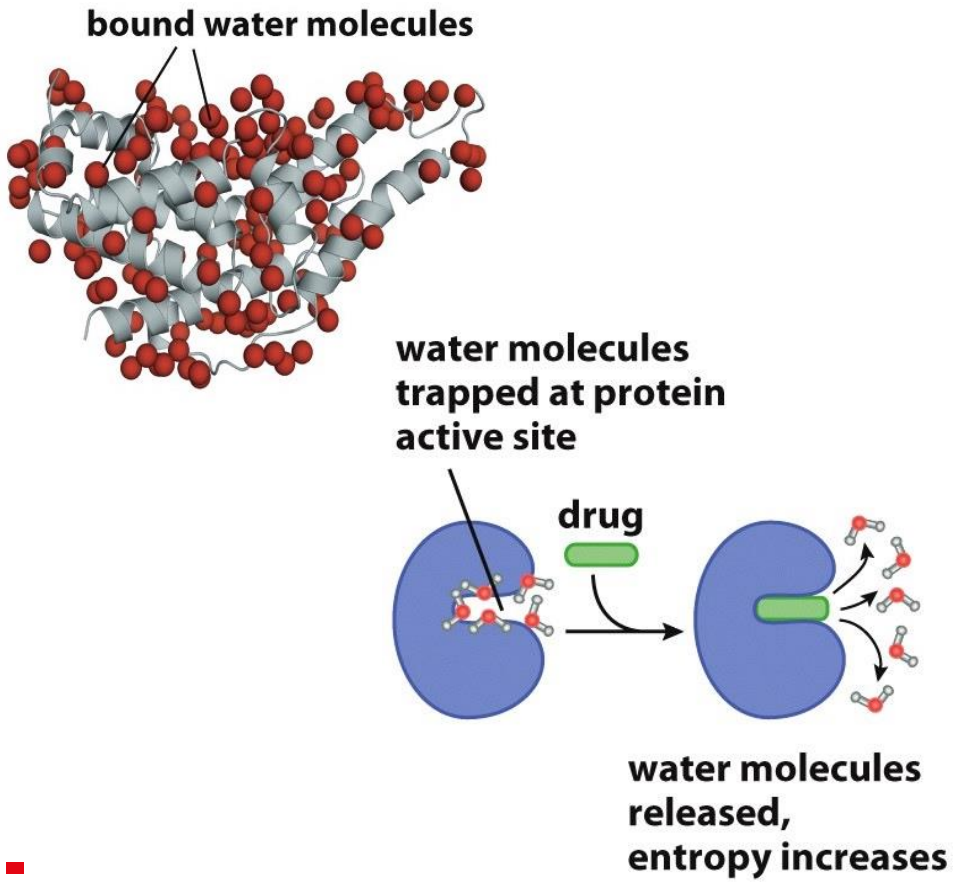
Calculated as sum for both molecules

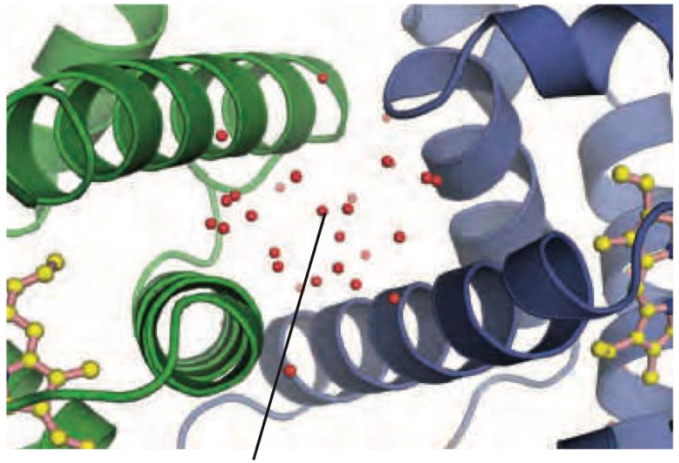


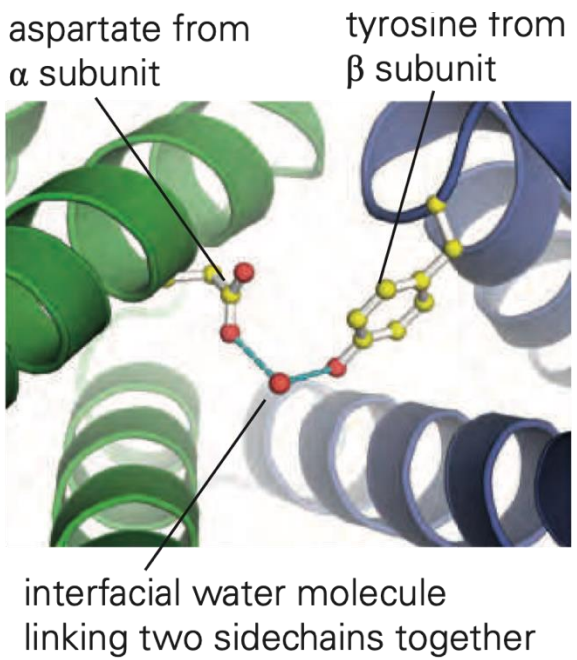
- For each binding partner, the contribution is on average ~700-800 Å^2 , which is a fraction of the total surface, and around 30 residues are involved in the interactions

Water molecules play an important role in binding

- Another important aspect of biomolecule interactions is the **desolvation energy**: the energy cost to remove the water from a surface when you form a complex between two molecules.
- The release of water increases the entropy of the system which has positive impact on binding energy
- Also, ~10-20 water molecules can be trapped at the interface and contribute to binding by bridging H-bonds

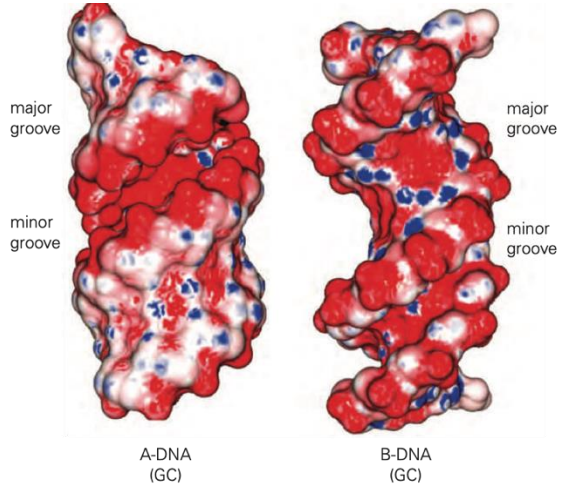




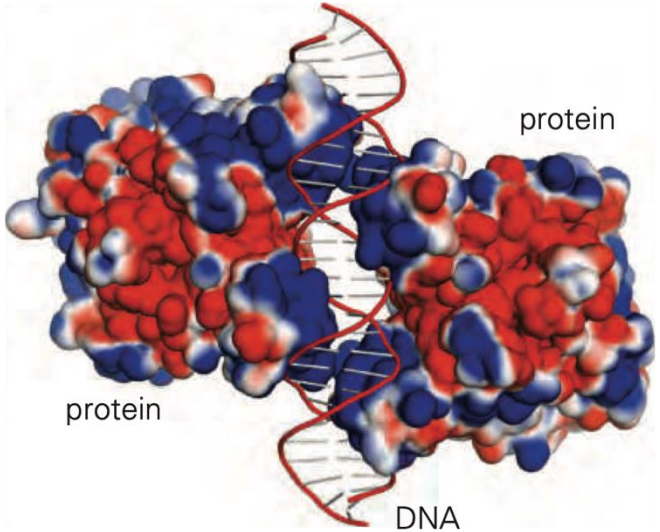


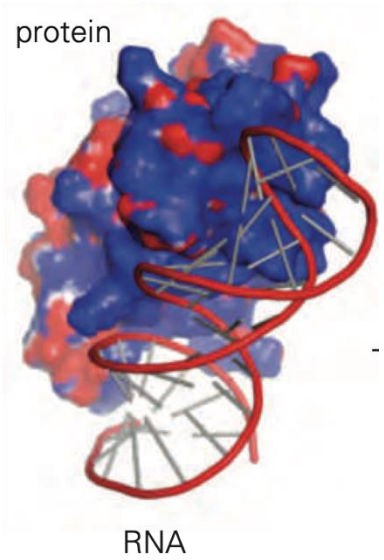
$$dG = dH - TdS$$

Protein-nucleic acid interactions



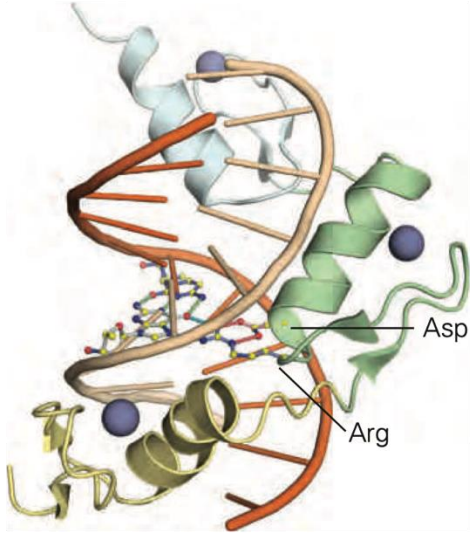
- DNA and RNA are mainly negatively charged due to phosphate backbone
- Thus, electrostatics plays a key role in protein binding, along with shape complementarity

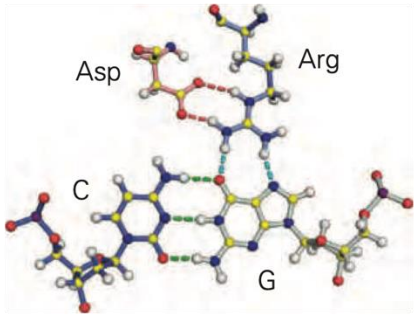




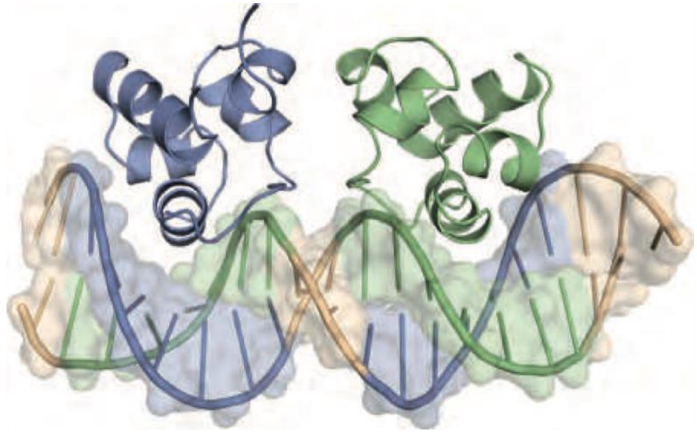
- Binding interfaces are rich in Lys an Arg and depleted from Asp and Glu
- Most interactions are of polar nature (~60%)
- Large desolvation energy penalty

Principles of protein-nucleic acid interactions





- Polar interactions are key for developing specificity for the DNA target
- Specificity comes from interactions with the bases in the major and minor groove of DNA



5′ AGTACAAACTAGTTTGTACT 3′

3′ TCATGTTTGATCAAACATGA 5′

Cro repressor– DNA complex

- Affinity can be enhanced by using a dimeric complex binding mode

$$\Delta G^{o}_{\text{dimer}} = 2\Delta G^{o}_{\text{monomer}} = 2RT\ln(K_{\text{D,monomer}}) = RT\ln(K^{2}_{\text{D,monomer}})$$

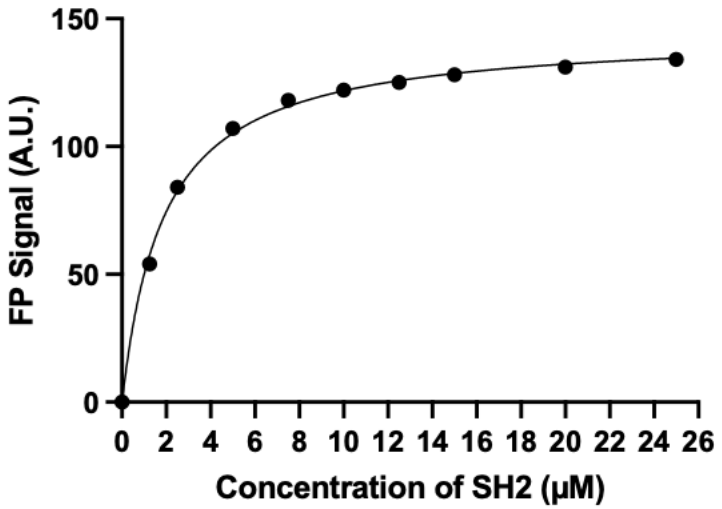
$$K_{\text{D,dimer}} = K^{2}_{\text{D,monomer}}$$

- This type of multivalent interaction is called **avidity**
- Protein dimers bind palindromic DNA sequences

Characterization of biomolecular interactions

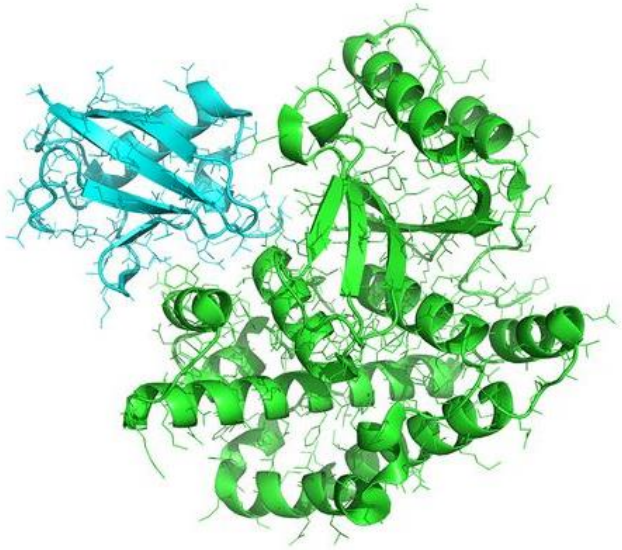
• The strength of interaction

Determine **Kd, Ka or ΔG**



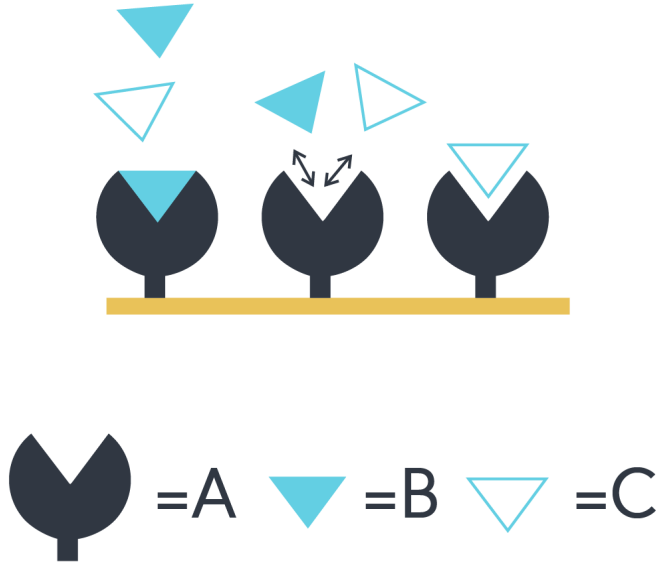
Interaction quantification methods:
- Isothermal Titration Calorimetry
- Surface Plasmon Resonance
- Fluorescence Polarization
- Nuclear Magnetic Resonance

• The location of the binding site



Structural biology, computational predictions, and mutagenesis approaches.

• Competition with other molecules

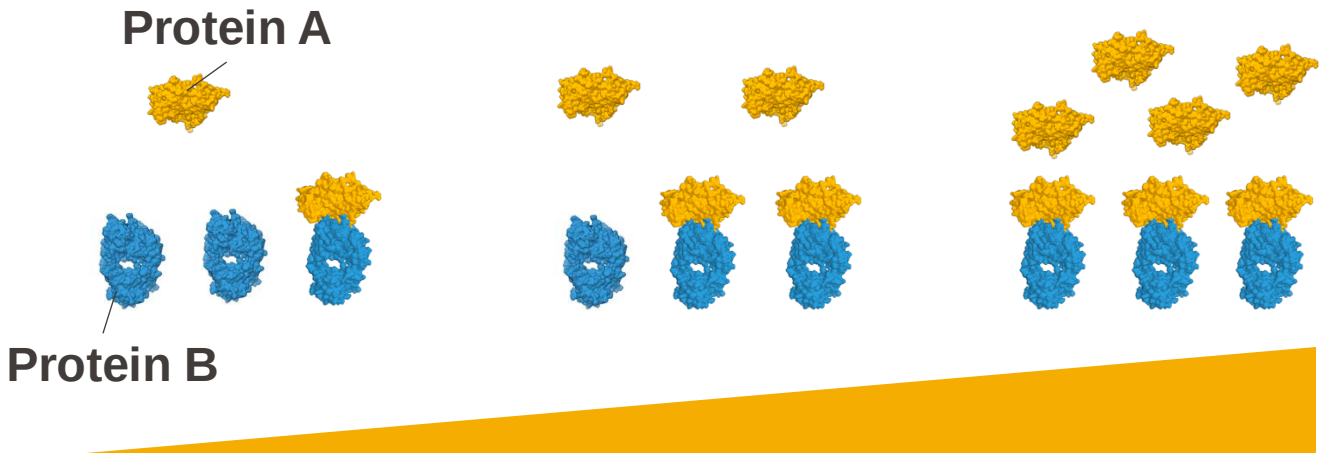


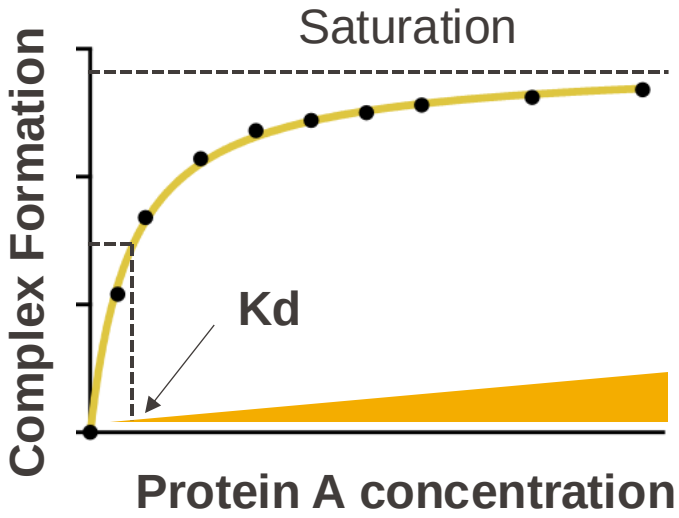
Interaction quantification methods, structural methods, functional (e.g., enzymatic) assays

Experimental methods to measure biomolecular interactions

General assay principles

- The assays typically involve keeping one molecule at constant concentration and gradually adding (titrating) the other molecule which results in formation of increasing number of complexes
- Complex formation is monitored using different spectroscopic or calorimetric methods





- The experimentally measured complex formation data is plotted as a function of concentration which results in **binding curve (sometimes referred as isotherm)** which is used for the calculation dissociation and association constants, kinetic and/or thermodynamic parameters

Two different views on binding

Thermodynamic approach

What is the difference in free energy between the unbound and bound state?

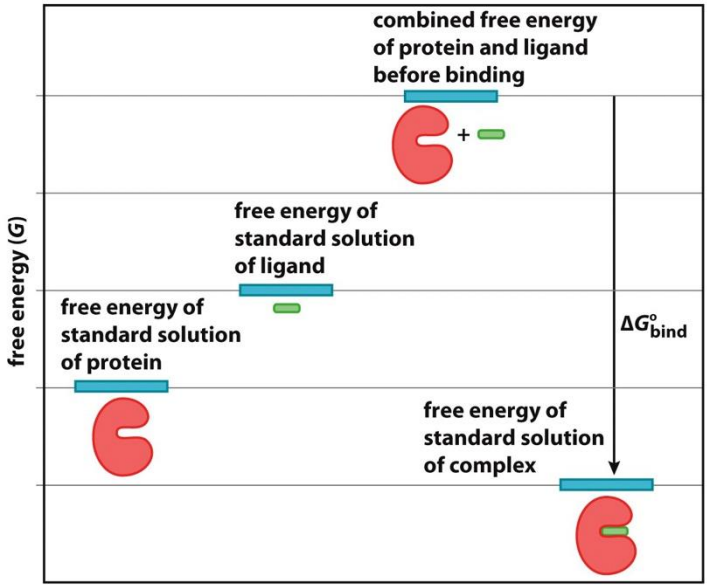


Figure 12.3 The Molecules of Life (© Garland Science 2013)

Measuring the energy change at steady state to calculate K_d

$$\Delta G^{\circ} = RT \ln K_d$$

Kinetic approach

How fast does the ligand bind the protein?
How fast does the complex dissociate?

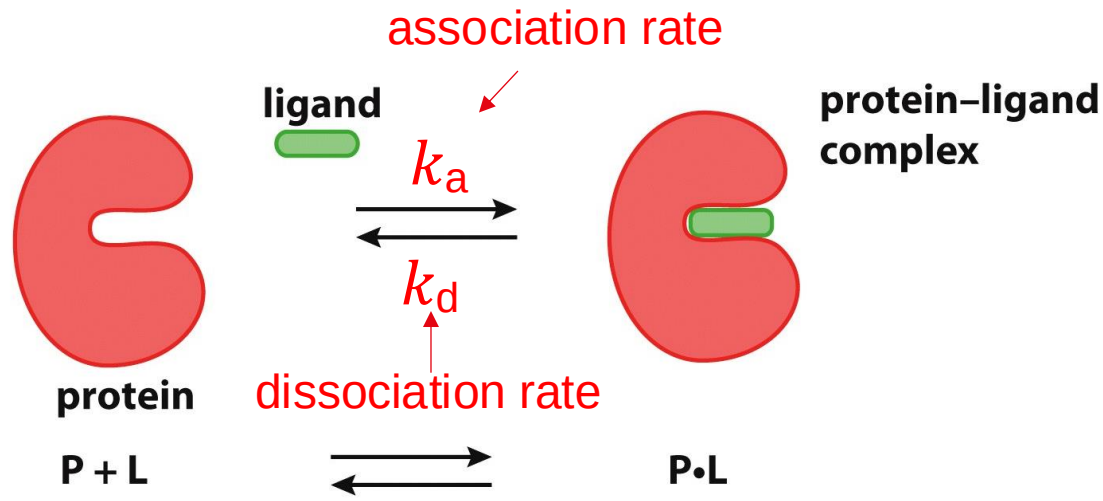


Figure 12.2a The Molecules of Life (© Garland Science 2013)

Measuring the rates at which system achieves equilibrium (k_a and k_d) to calculate K_d

$$\frac{k_d}{k_a} = \frac{k_{off}}{k_{on}} = \frac{[P][L]}{[P \bullet L]} = K_D = \frac{1}{K_A}$$

Isothermal Titration Calorimetry (ITC)

- Binding characterization requires not only to know K_d but also to know how enthalpy and entropy changes balance - **$\Delta G = \Delta H - T\Delta S$ -** ITC permits to measure this

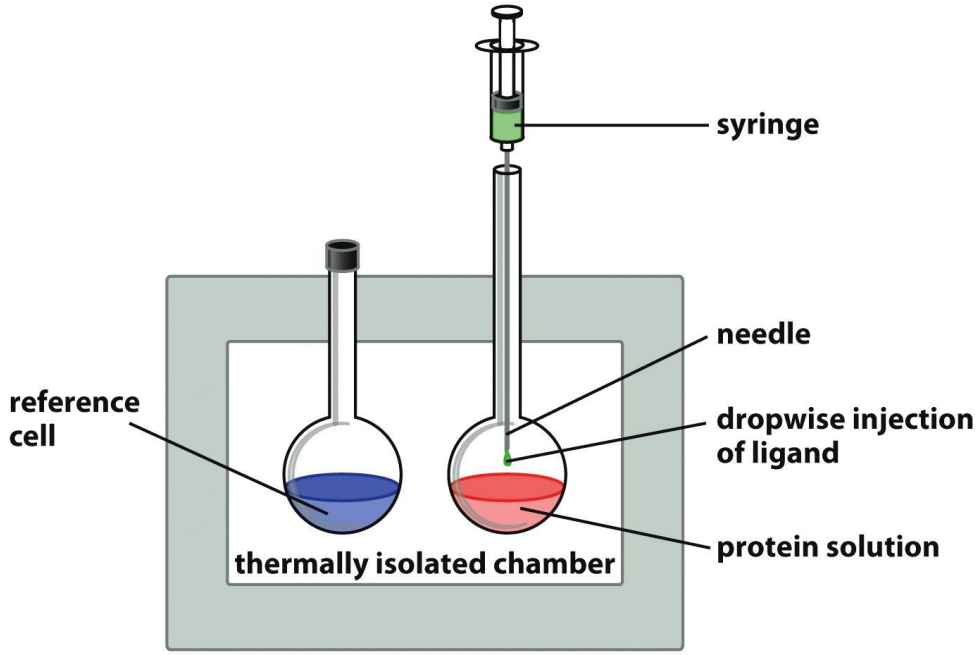


Figure 12.2.1a The Molecules of Life (© Garland Science 2013)

Example device

- Load one biomolecule to the solution while **maintaining constant temperature**
- Titrate small aliquots of the biomolecular binding partner
- Record changes in heat upon binding of ligand to protein

Isothermal Titration Calorimetry (ITC)

- ITC relies on direct measurement of the heat released upon binding
- Heat is released if the reaction is exothermic

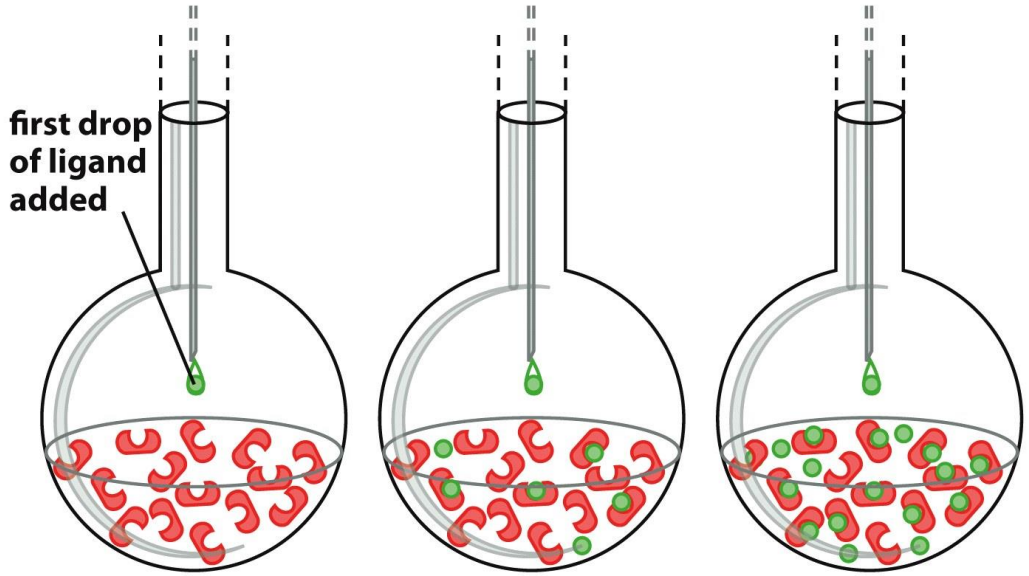


Figure 12.2.1b The Molecules of Life (© Garland Science 2013)

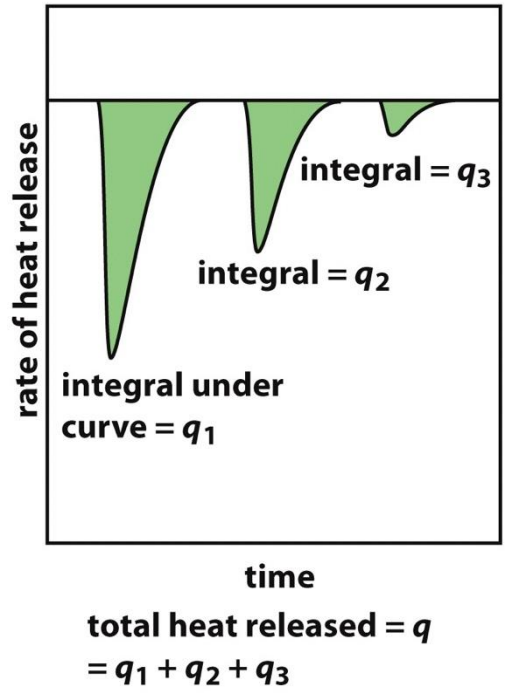
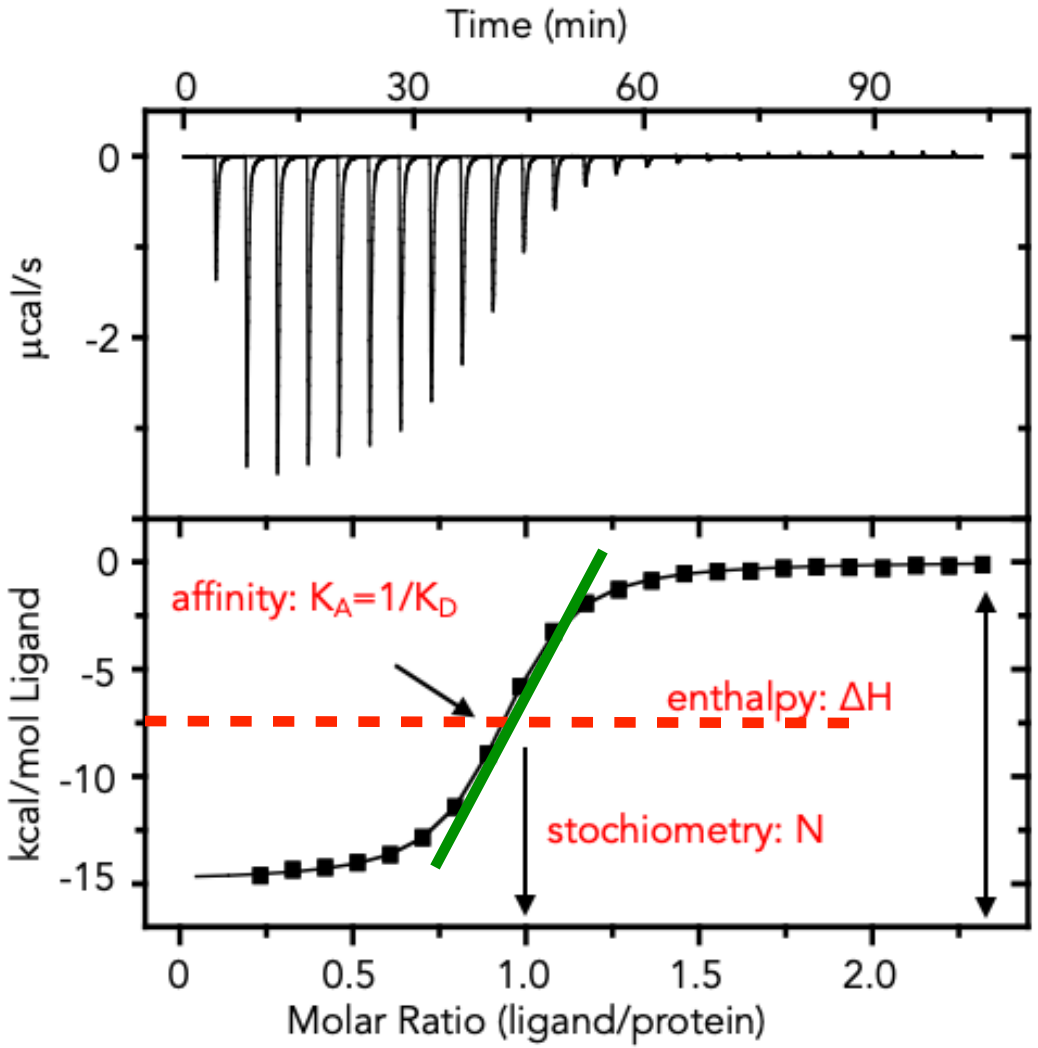


Figure 12.2.2 The Molecules of Life (© Garland Science 2013)

- The more biomolecule is titrated the less heat is released as less binding partners are available for interaction until full saturation is reached.
- In rare cases, the reaction does not release or takes up heat, no enthalpy changes and ITC cannot be used to measure binding

ITC - Data analysis

- ITC allows to determine different thermodynamic properties for a specific interaction

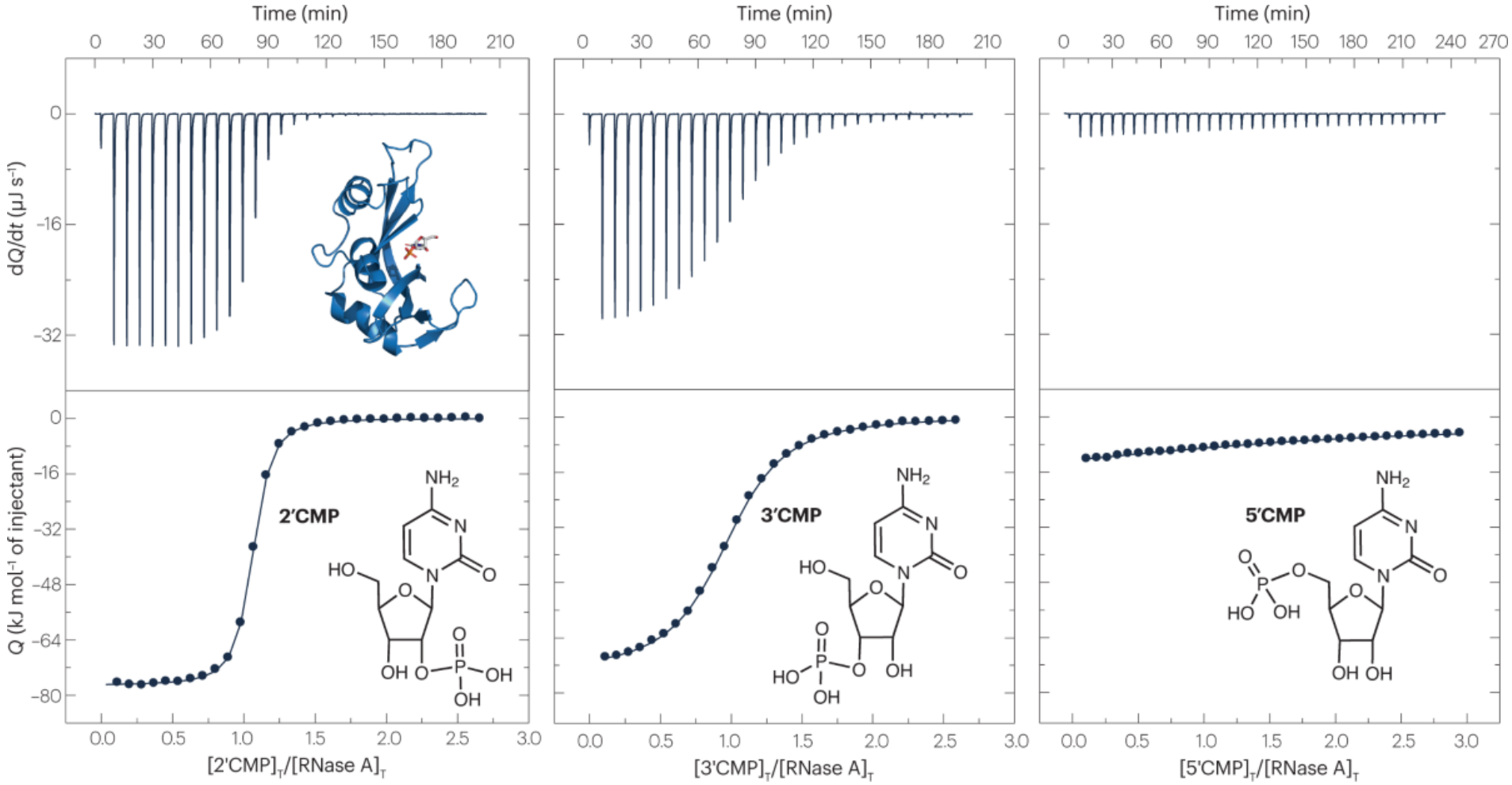


$$\Delta G = RT \ln K_D = \Delta H - T\Delta S$$

- Measure change in heat (Enthalpy: ΔH)
- Fit integrated heat of single injections to obtain titration curve: slope is K_A
- Determine stoichiometry of interaction (*n*)
- Calculate entropy (ΔS) and Gibbs free energy (ΔG)

ITC Example - High vs Low Affinity

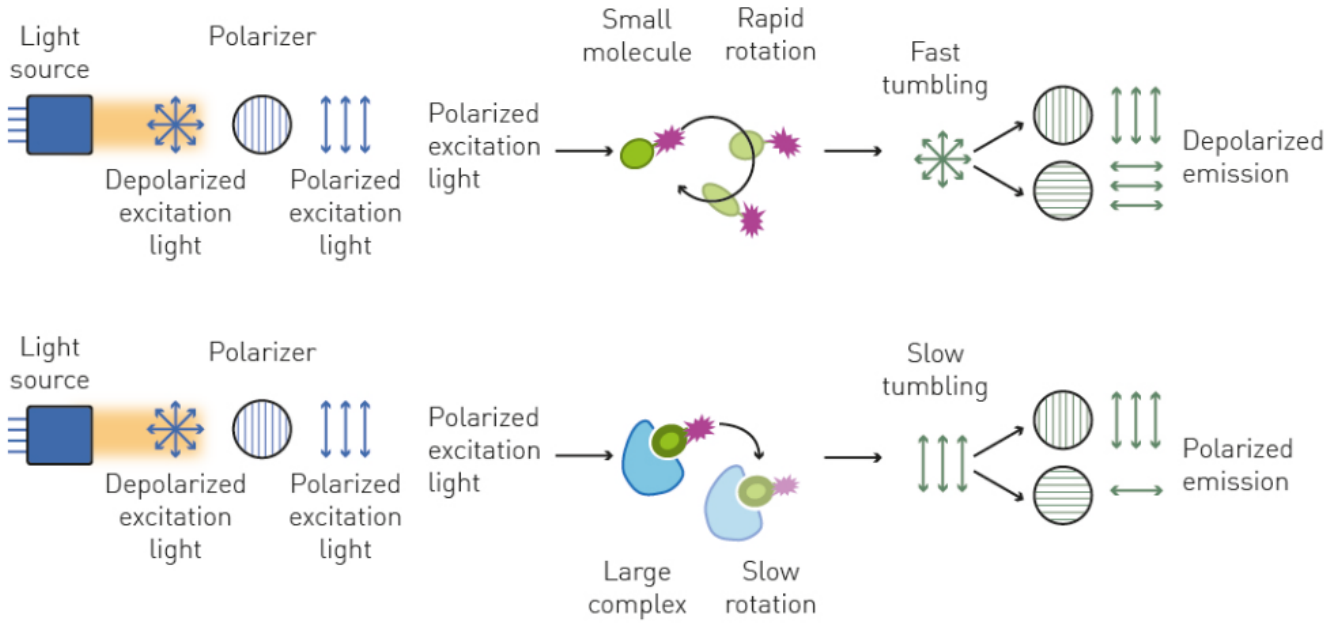
- Very similar compounds exhibiting different binding affinities to the target protein (blue)

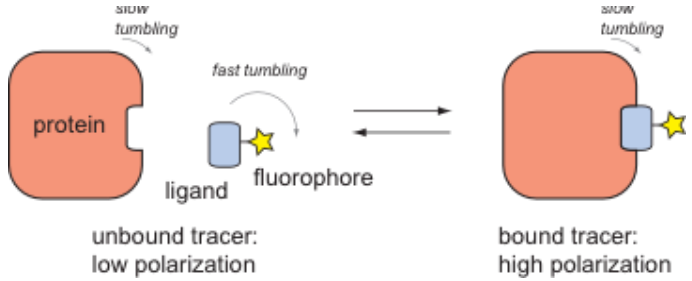


High **Intermediate** **Low**

Fluorescence polarization

- Fluorescence polarization or anisotropy is a phenomenon where the light emitted by a fluorescent molecule has different intensities in different polarization planes
- This effect is dependent on the rotational and translational speed of molecules (correlation time) which is a function of molecular weight.
- Small molecules tumble very fast in solution and give weak signal with plane-polarized light, showing no or low FP
- Large molecules tumble much slower and give high FP signal



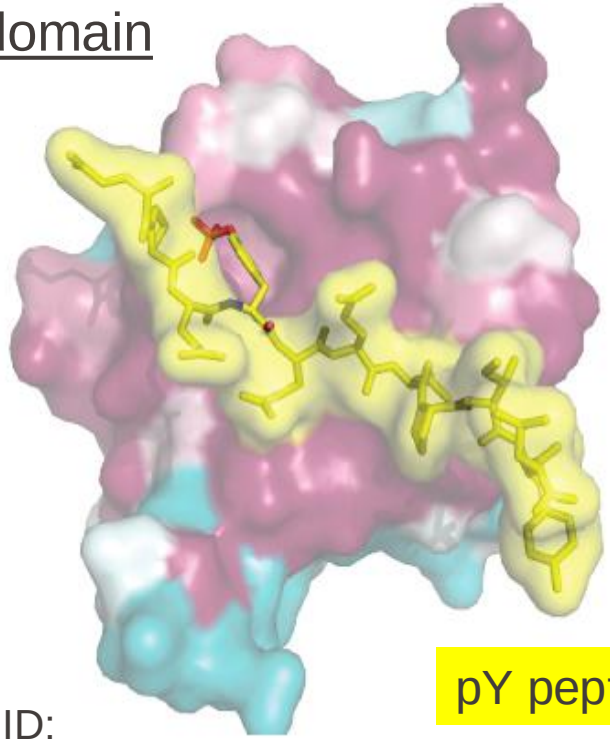


- Fluorescent labeling of at least one binding partner is **necessary** for this experiment
- Usually done by chemical attachment

Fluorescence polarization - Binding Isotherm

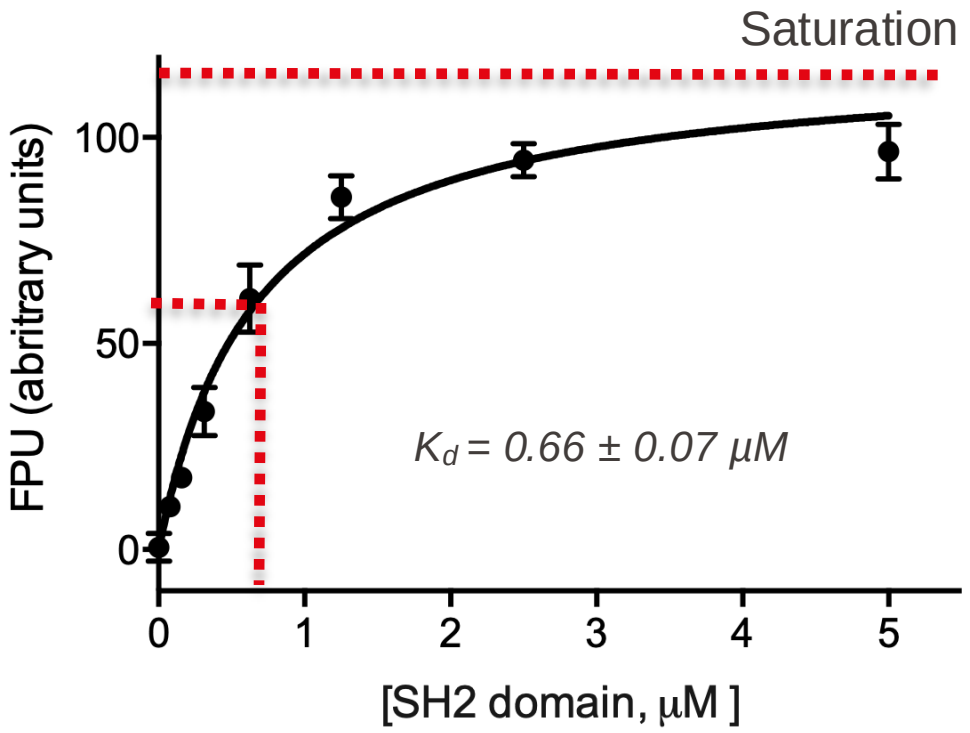
- Binding of phospho-tyrosine (pY) peptide with fluorescent dye at N-terminus to SH2 domain
- Measure FP at different SH2 domain concentrations (pY peptide concentration constant)
- SH2 **concentration at which half-maximum FP is obtained corresponds to K_d** of this binding reaction.

SH2 domain



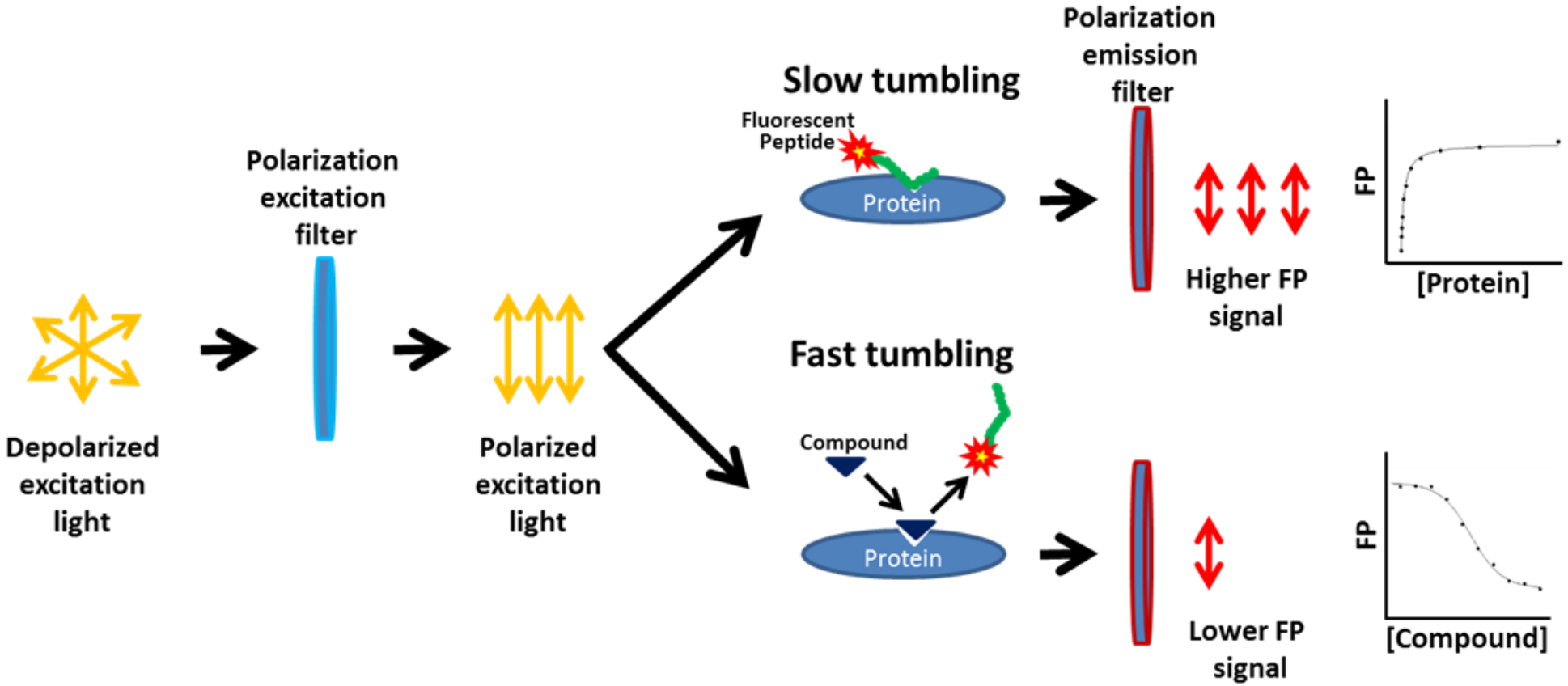
pY peptide

PDB ID:
1LCJ



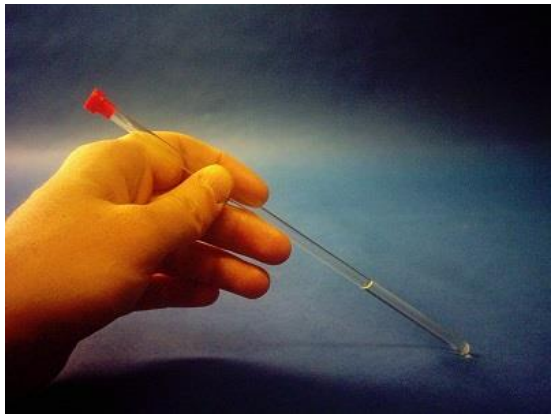
Fluorescence polarization - Example applications

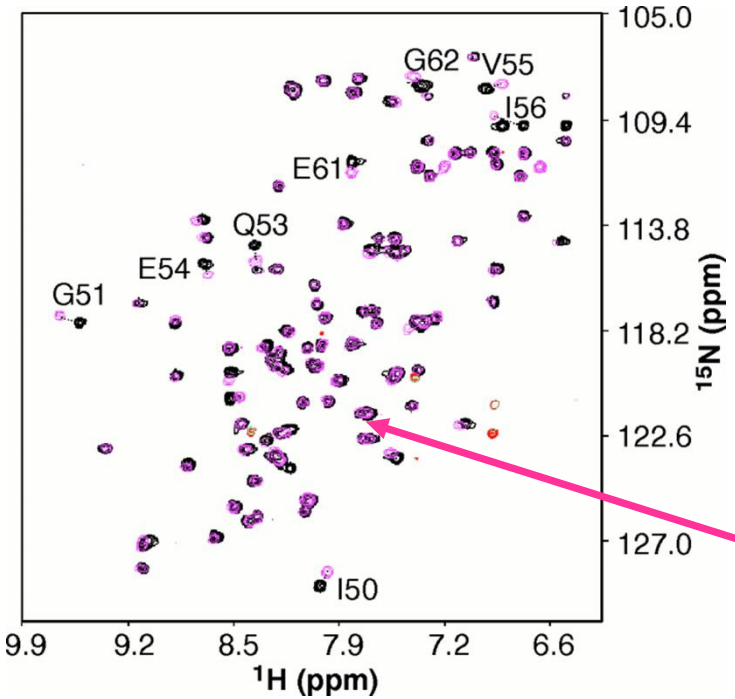
- FP can be used for two different assays:
  - To determine binding affinities of a binding partner labeled with fluorescent dye
  - To measure affinities of a non-labeled ligand/compound/drug that competes with a fluorescently labeled binding partner



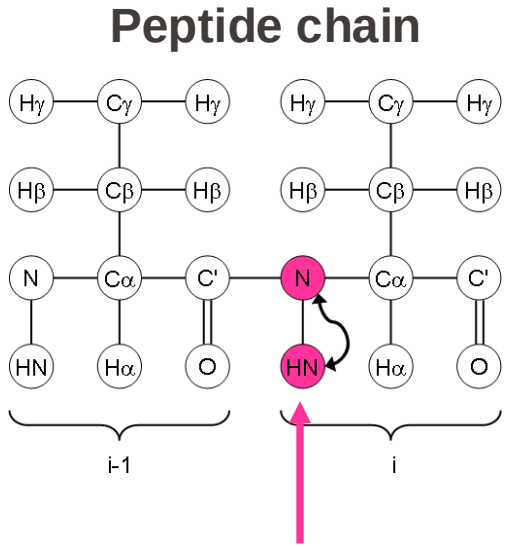
Nuclear Magnetic Resonance for binding analyses

- NMR is a structural biology method (see lecture 5), which is used to study molecular parameters of biomolecules while in solution
- Individual atoms are separated in NMR spectra based on their local chemical environment and this is measured as chemical shifts (δ) expressed in parts-per-million (ppm)





^{1}H-^{15}N HSQC spectrum

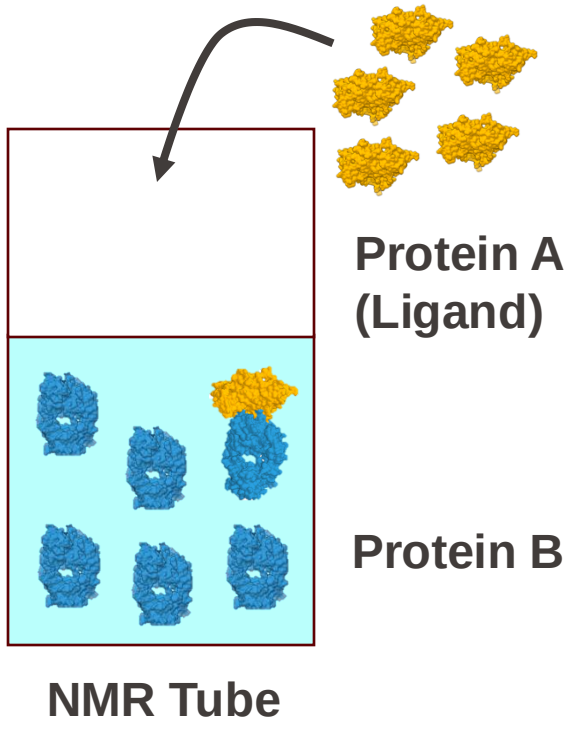


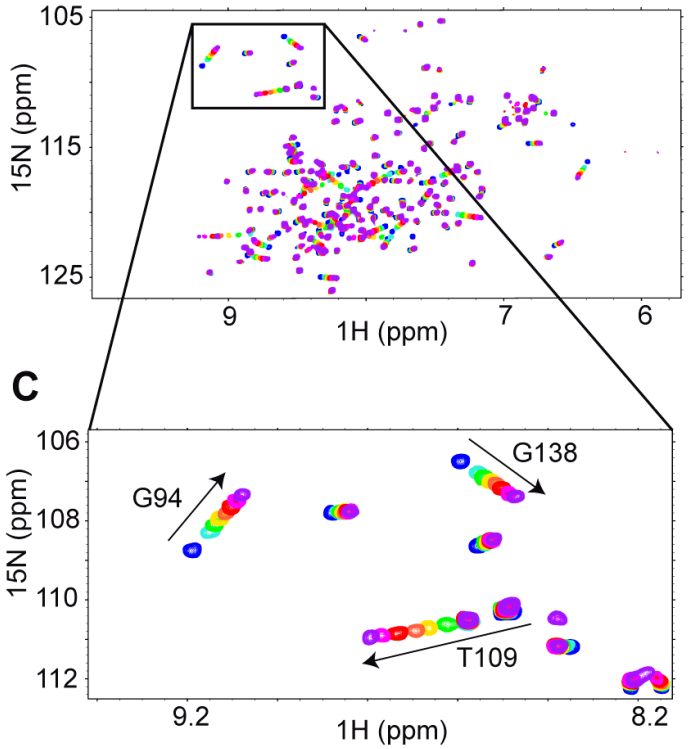
Each peak corrresponds to one covalently bound N-H pair

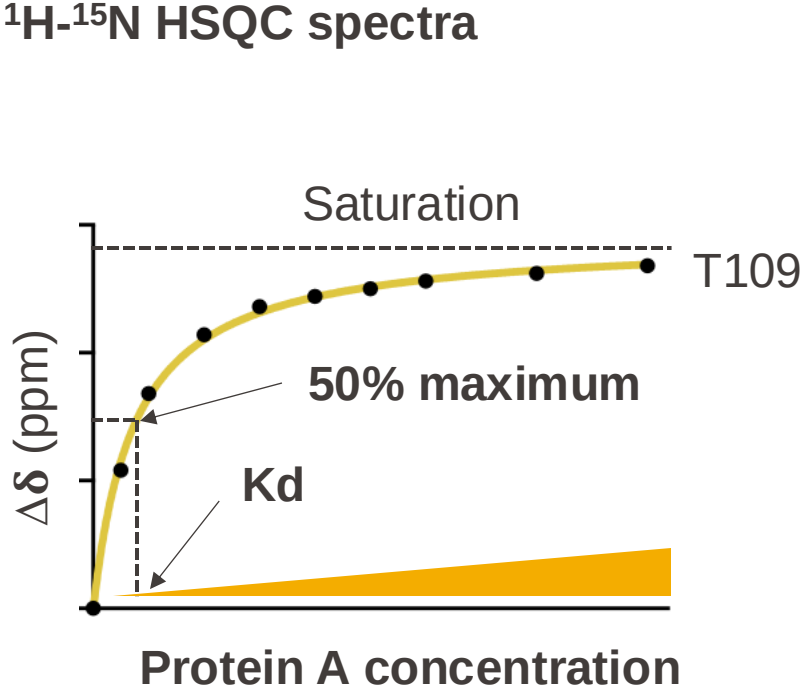
- Binding evens cause the perturbation of local chemical environment which can be used for qualitative (i.e., structural) and quantitative (i.e., dissociation constant) characterization of binding

Nuclear Magnetic Resonance for binding analyses

- One binding partner (e.g., Protein B) can be isotope labeled with ^{15}N and used for collection of ^{1}H-^{15}N HSQC spectra while gradually adding the binding partner (e.g., Protein A, unlabeled)
- Measure chemical shift changes ($\Delta\delta = \delta_{A+B} - \delta_{B}$) of H-N functional groups in amino-acids



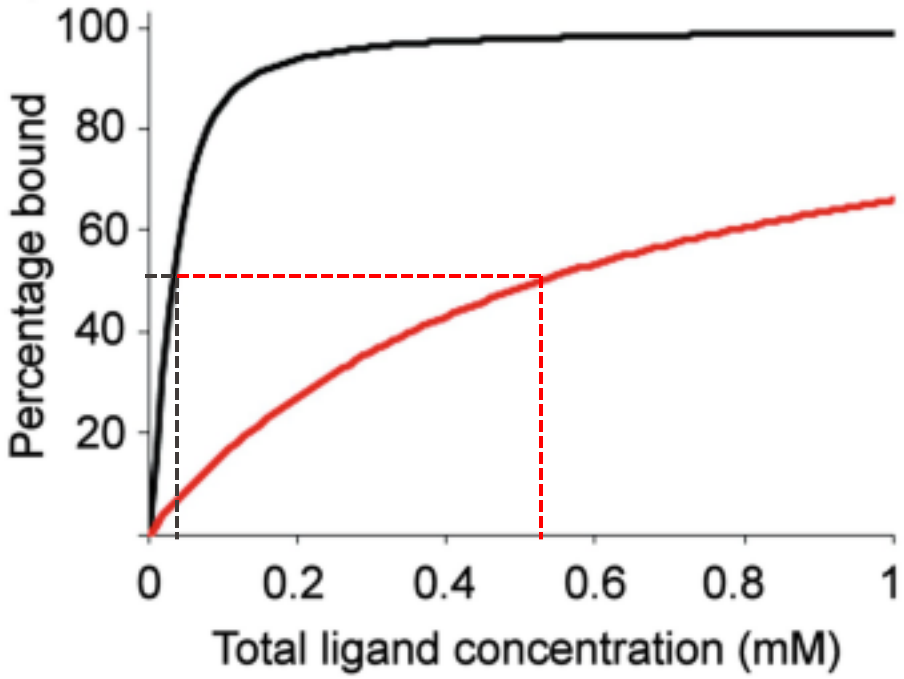




- The binding isotherm is a plot of $\Delta\delta$ over Protein A concentration
- The concentration at which half-maximum $\Delta\delta$ is reached corresponds to K_d

NMR - Examples of high and low affinity curves

- Maximum signal intensity is not the main determining factor when it comes to binding. Its role is to provide a dynamic range where complex formation can be readily detected
- The initial slope of the curve shows how quickly the system reaches saturation in response to different concentration of the ligand and is a better indicator of strong/weak binding



Kd ~ 0.04mM
(Higher Affinity)

Kd ~ 0.53mM

(Lower Affinity)

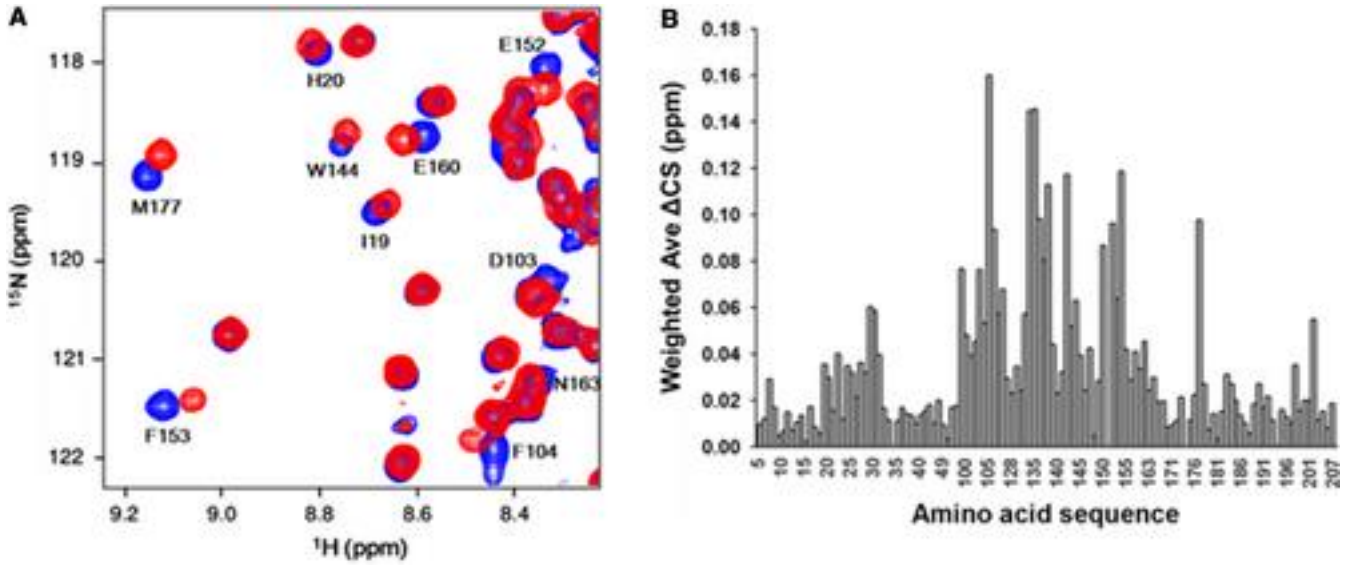
- Similar rationale regarding the slope can be applied to FP and ITC measurements

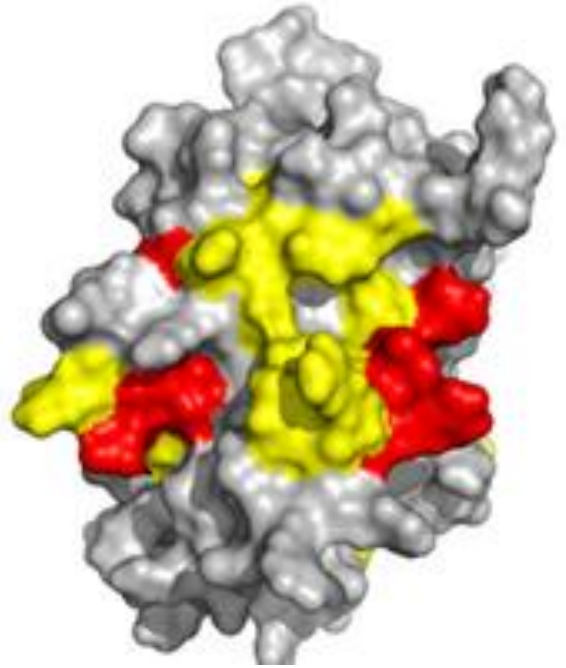
NMR also allows to determine molecule binding sites

- By completely assigning each peak in an NMR spectra to the corresponding amino acid, one can identify which parts of the protein are being perturbed the most due to binding (in terms of the chemical shift change)

^{1}H-^{15}N HSQC spectra

- Ligand
+ Ligand



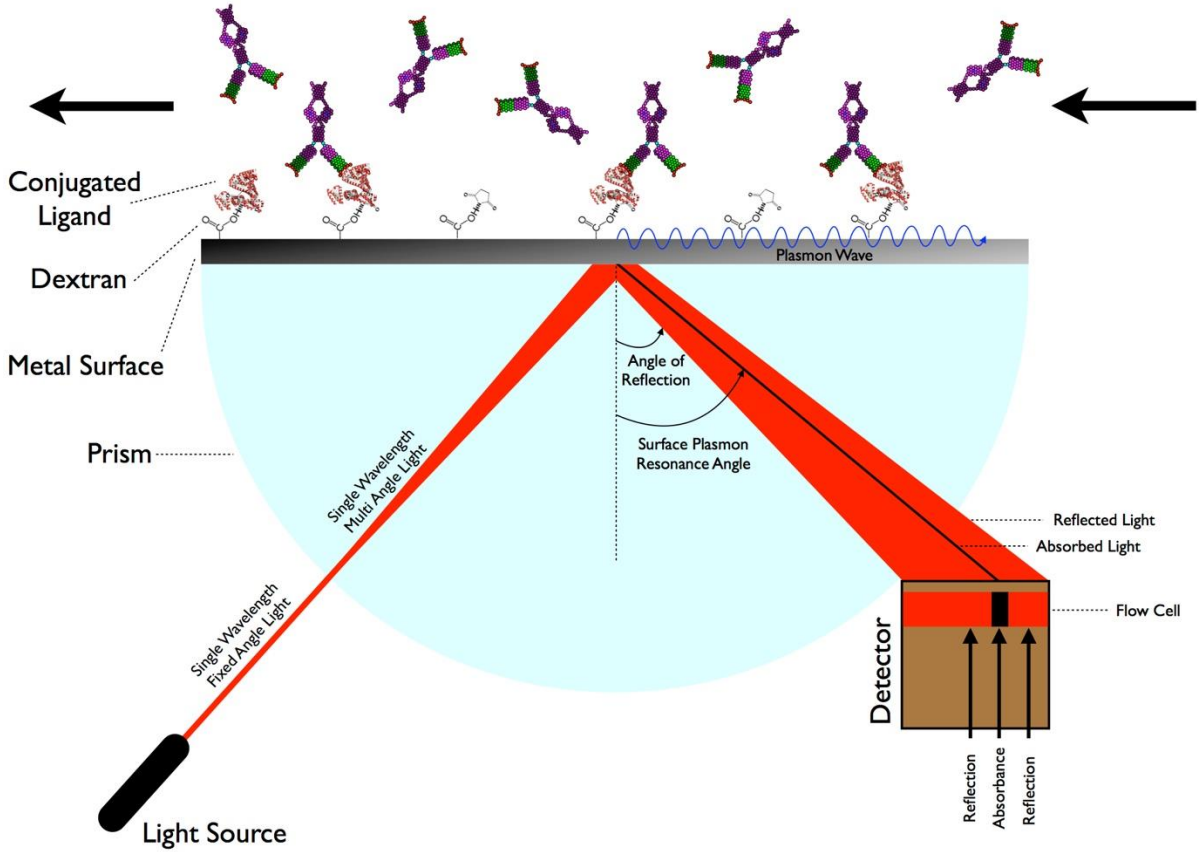


Gray = Not perturbed
Yellow = Weakly perturbed
Red = Strongly pertubed

- This provides the basis to identify the most likely binding site on the protein surface, although in practice the analysis can be complicated by long-range effects of interactions

Surface Plasmon Resonance

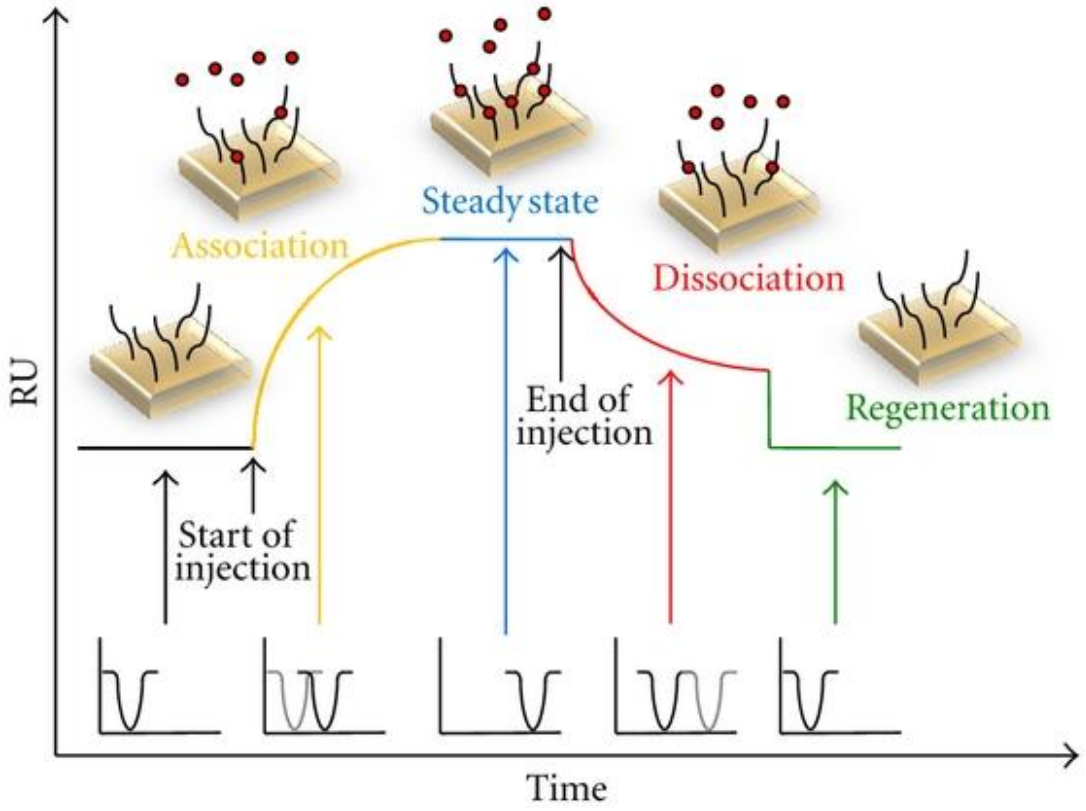
- SPR is a kinetic method for measuring association and dissociation processes between molecules
- SPR-based instruments use an optical method to measure the refractive index near (within ~300 nm) a sensor surface

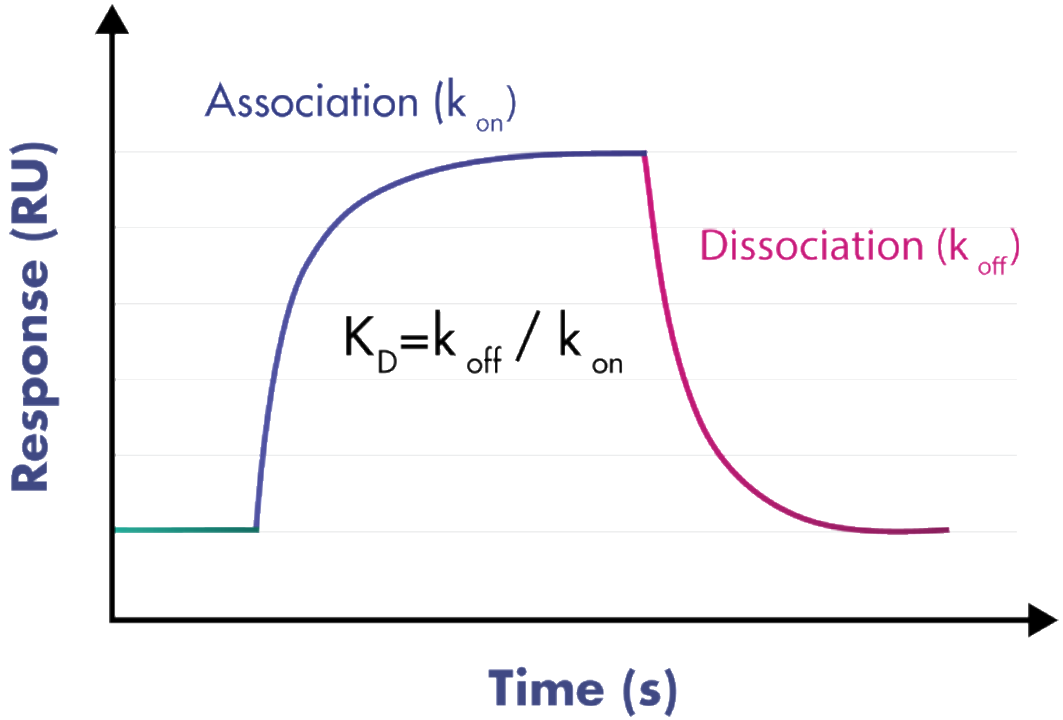


- One binding partner immobilized on surface of flow cell (constant concentration)
- The second binding partner continuously flown over
- Binding results in increase in refractive index, which is measured in real time
- Result plotted as resonance units (RUs) versus time (the sensogram)

SPR - Experimental workflow and output

- SPR experiment goes in phases where different components are loaded at different times
- Following the immobilization of the first molecule, its' binding partner is loaded over the SPR chip, causing the signal to increase (association phase) reaching saturation after some time.
- Then a solution without any binding partner is run over the chip which causes the bound molecule to gradually dissociate from the chip (dissociation phase)



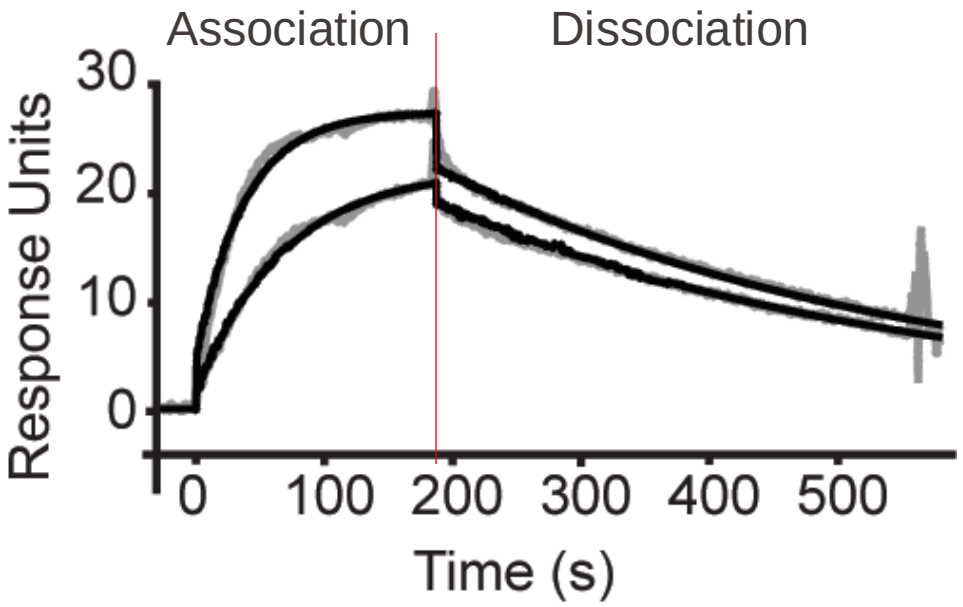


SPR Sensogram - Example

- Example: Binding of immobilized SH2 domain protein to engineered monobody protein
- Fast association and very slow dissociation rates result in high affinity interaction

- Kinetic rate constants:
  - $k_a \equiv k_{on}$ is the **association rate constant ($M^{-1}s^{-1}$)**
  - $k_d \equiv k_{off}$ is the **dissociation rate constant (s^{-1})**

$$\frac{k_d}{k_a} = \frac{k_{off}}{k_{on}} = \frac{[P][L]}{[P \bullet L]} = K_D = \frac{1}{K_A}$$



$k_a = 3.79 \times 10^5\ M^{-1}s^{-1}$

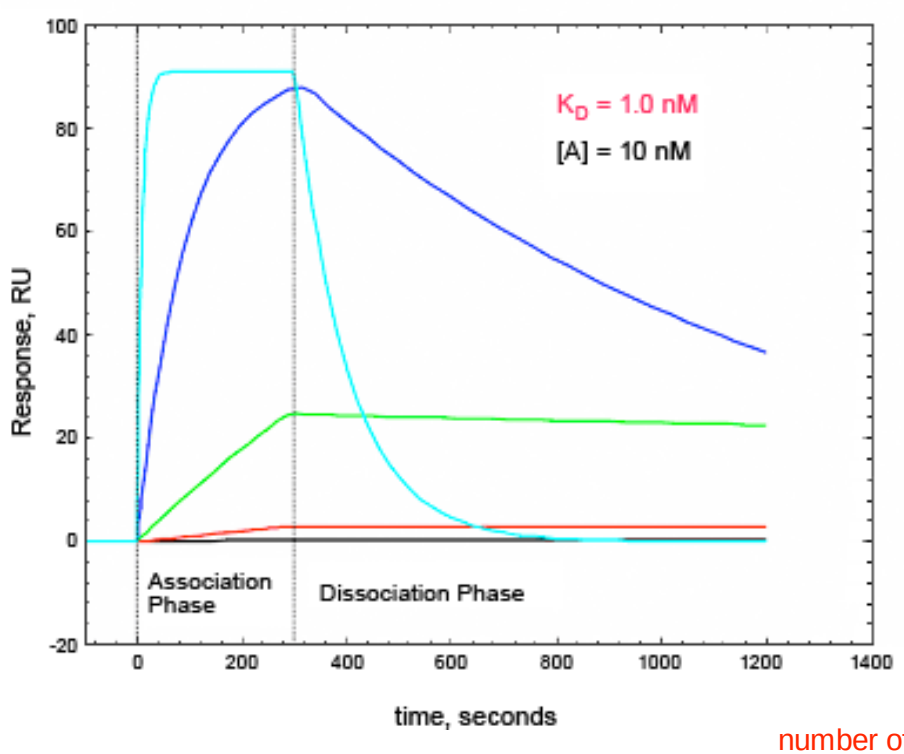
$k_d = 2.72 \times 10^{-3}\ s^{-1}$

$K_d = 7.2$ nM

SPR: Same affinity but different binding kinetics

- For most drugs: dissociation rates are optimized to be very slow to ensure long residence times and continuous selective inhibition.

$$K_D = k_d / k_a$$



5 Interactions
Different k_a and k_d
Same K_D

k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})
1.00E+03	1.00E-06
1.00E+04	1.00E-05
1.00E+05	1.00E-04
1.00E+06	1.00E-03
1.00E+07	1.00E-02

number of complexes formed per second in 1M solution

fraction of complexes that decays per second. eg 10^{-2} s^{-1} means that 1% of complexes decay per second

Comparison of different methods

ITC

Pros:
- Label and tag free
- Measures ΔG, ΔH, ΔS
- Measuring affinities in the mM -> pM range
- Inexpensive hardware
- Can be used with small molecules

Cons:
- High quantity of sample required
- Only 1 sample at once
- Dependent on heat change upon binding
- Does not provide kinetic constants

FP

Pros:
- Straightforward to use
- High-throughput
- Can be applied with small molecules
- Easy to implement for competition assays

Cons:
- Limited applications
- Fluorophores can impact binding
- Requires significant change in MW to detect
- Does not provide kinetic constants

NMR

Pros:
- Provides information on binding sites
- Can be applied with small molecules
- Additional structural insights on the molecule

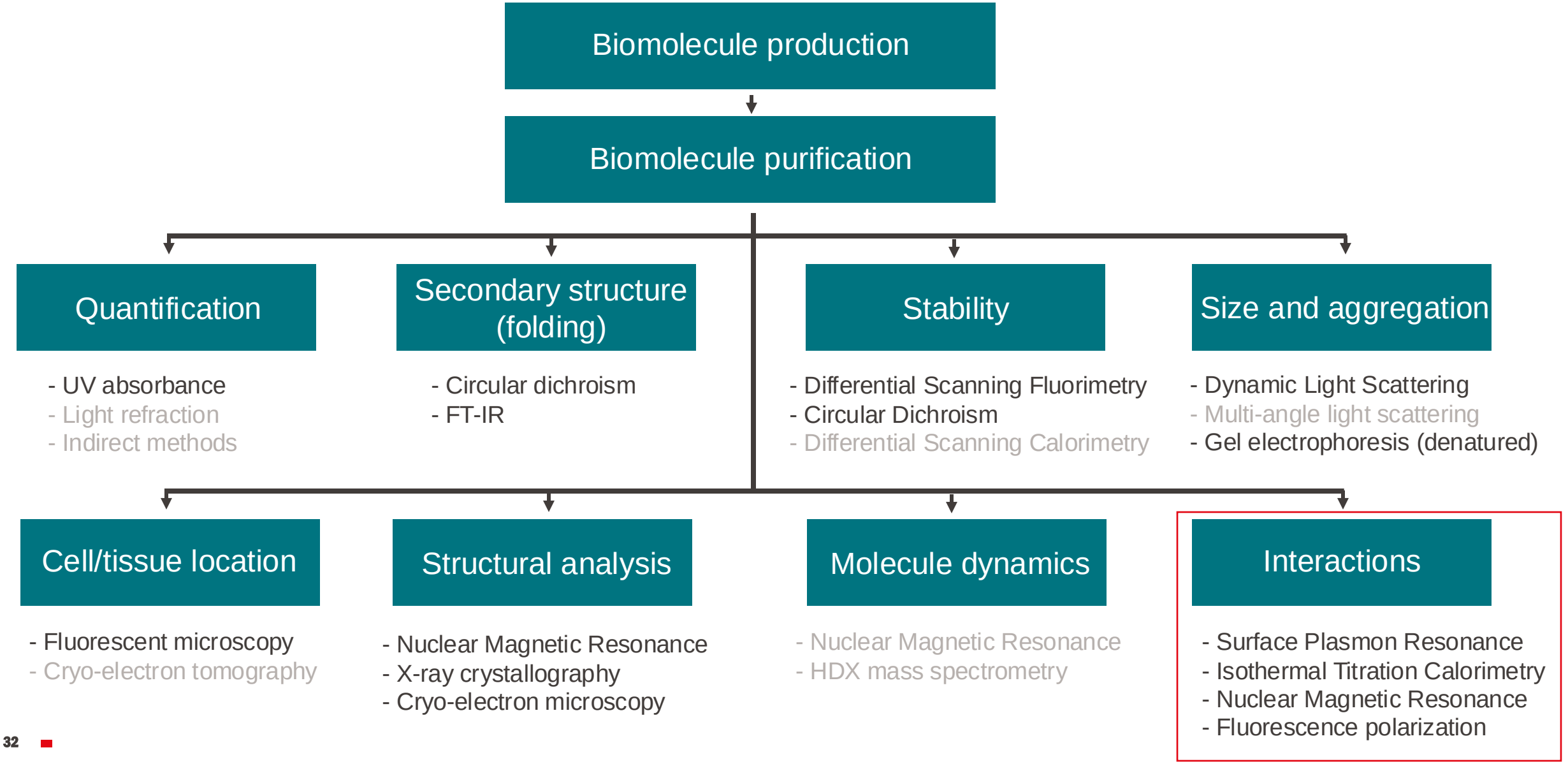
Cons:
- Needs labeling with isotopes
- Limited to low MW biomolecules
- Applicable to low-affinity interactions (µM-mM)
- Does not provide kinetic constants

SPR

Pros:
- Label-free
- Measurements of kinetic parameters
- Wide range of affinity values can be measured
- Reusable sensor chips
- Excellent reproducibility

Cons:
- High quality protein needed
- High quantity of protein needed
- Not very sensitive for small-molecule binding
- Expensive hardware

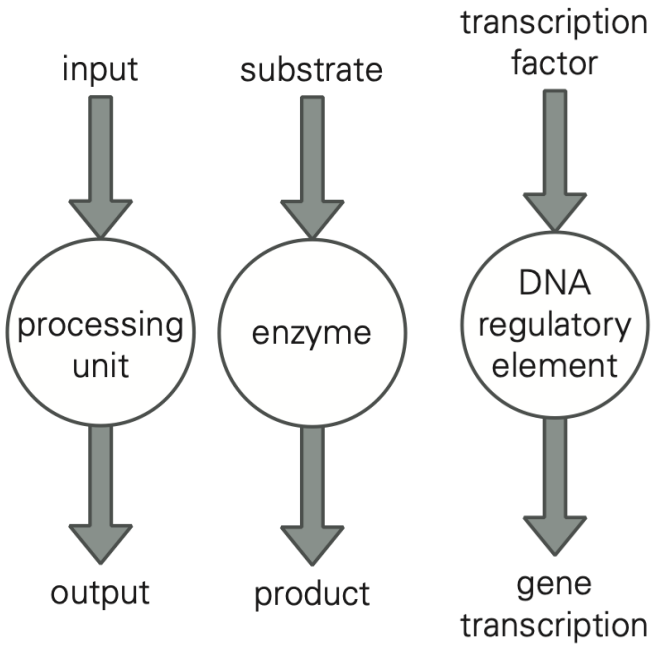
Summary of biophysical methods and their applications

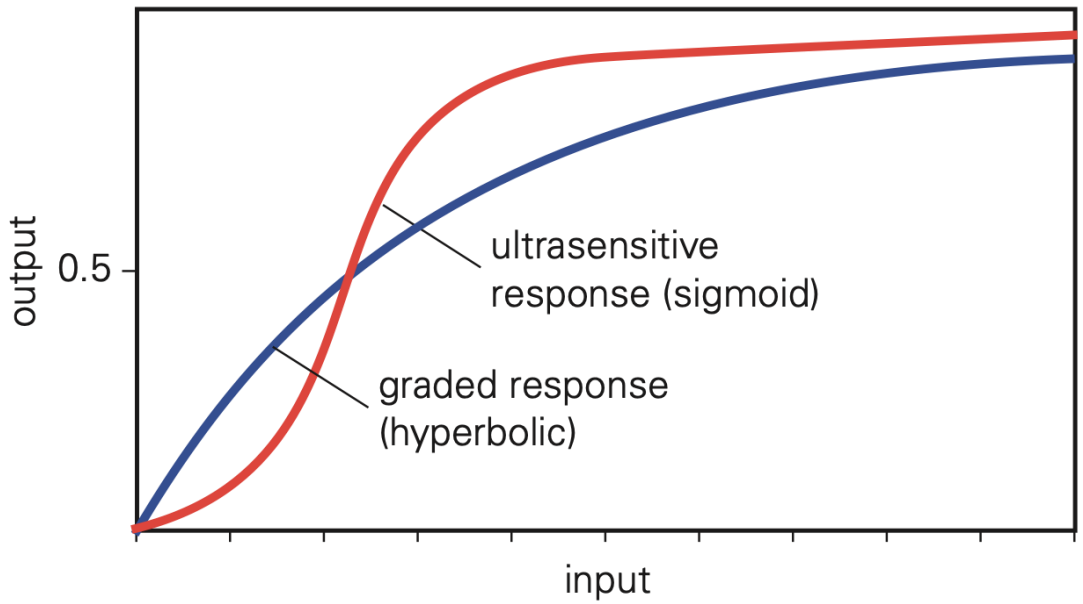


Cooperativity and Allostery

Response to molecular stimuli

- Biological functions are regulated by binding events (i.e., detection of input molecules produces output effect)
- However, the modalities of output effects need to be switchable - Either **ON** or **OFF**

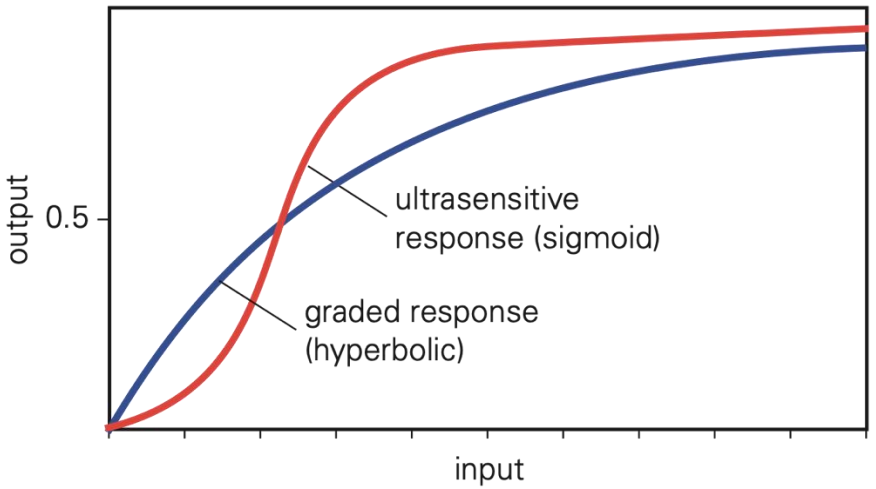


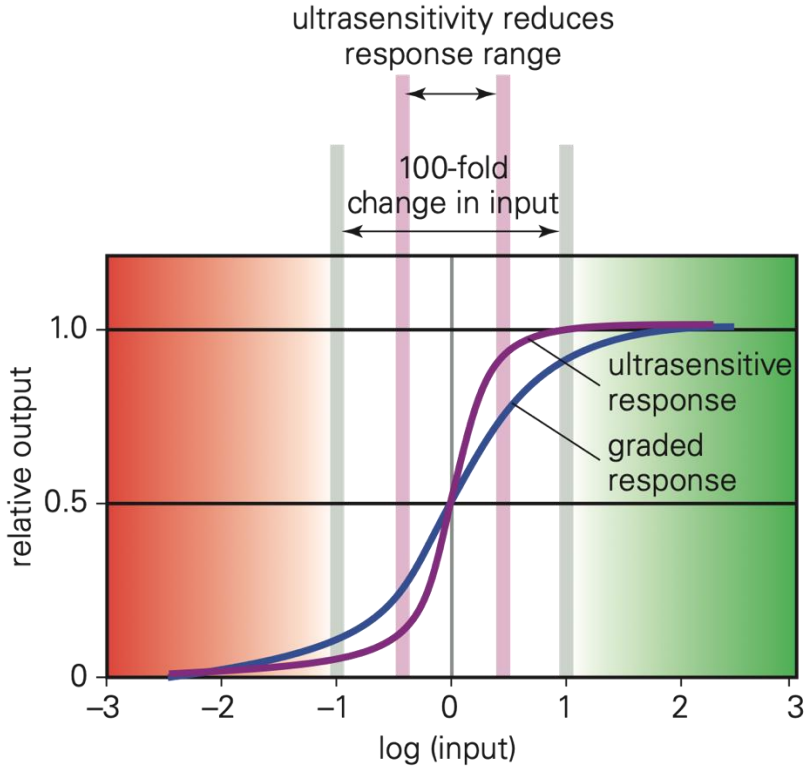


- This is usually achieved by changing the state and activity of proteins (via PTMs or binding), but it can also be achieved through **ultrasensitive binding**

Ultrasensitivity of binding

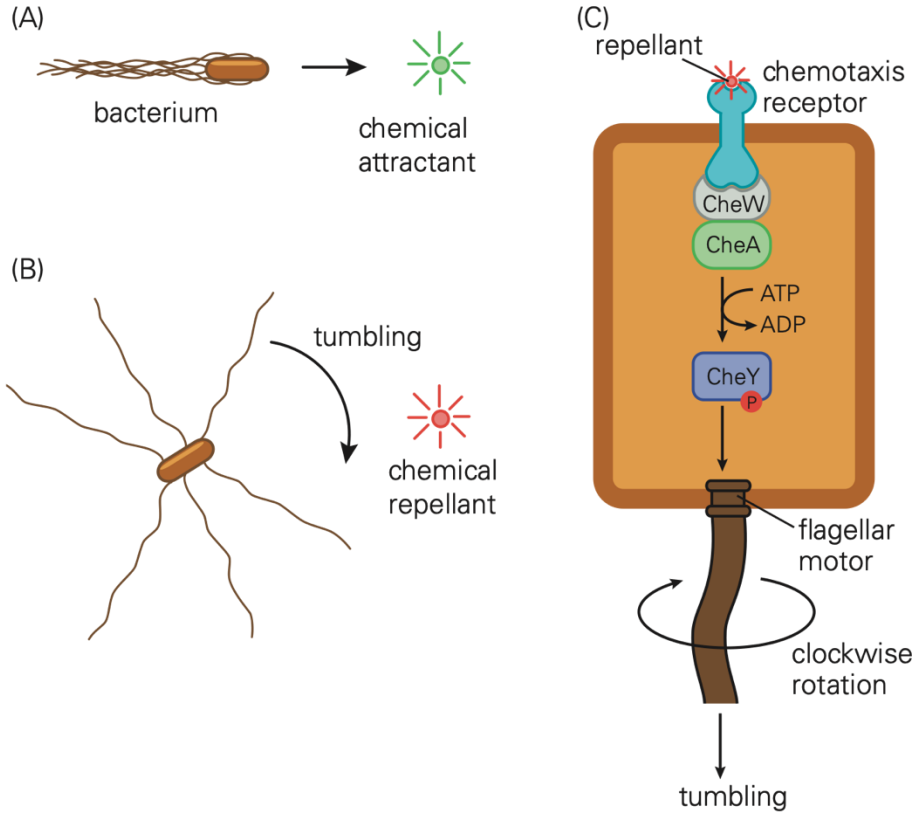
- Key systems in cells are **ultrasensitive**, rather than graded, in their response to input signals.
- These can switch from off to on over a less than 100-fold change in the strength of the input signal.





- Ultrasensitivity is achieved via **cooperativity,** ie ligand molecules appear to “cooperate” with each other so that the extent of binding increases more sharply as more ligand molecules are bound.

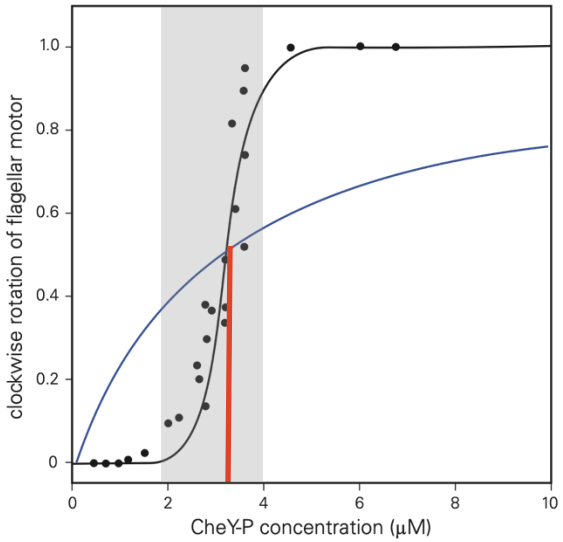
Example: Chemotaxis response in bacteria



- In bacteria, chemotaxis is the directed movement towards sources of food and away from toxins.
- Tumbling away from toxins depends on phosphorylation of CheY, thus on [CheY-P]

$$f = \frac{[\mathrm{L}]}{K_\mathrm{D} + [\mathrm{L}]} = \frac{[\text{CheY-P}]}{3.0 \times 10^{-6} + [\text{CheY-P}]}$$

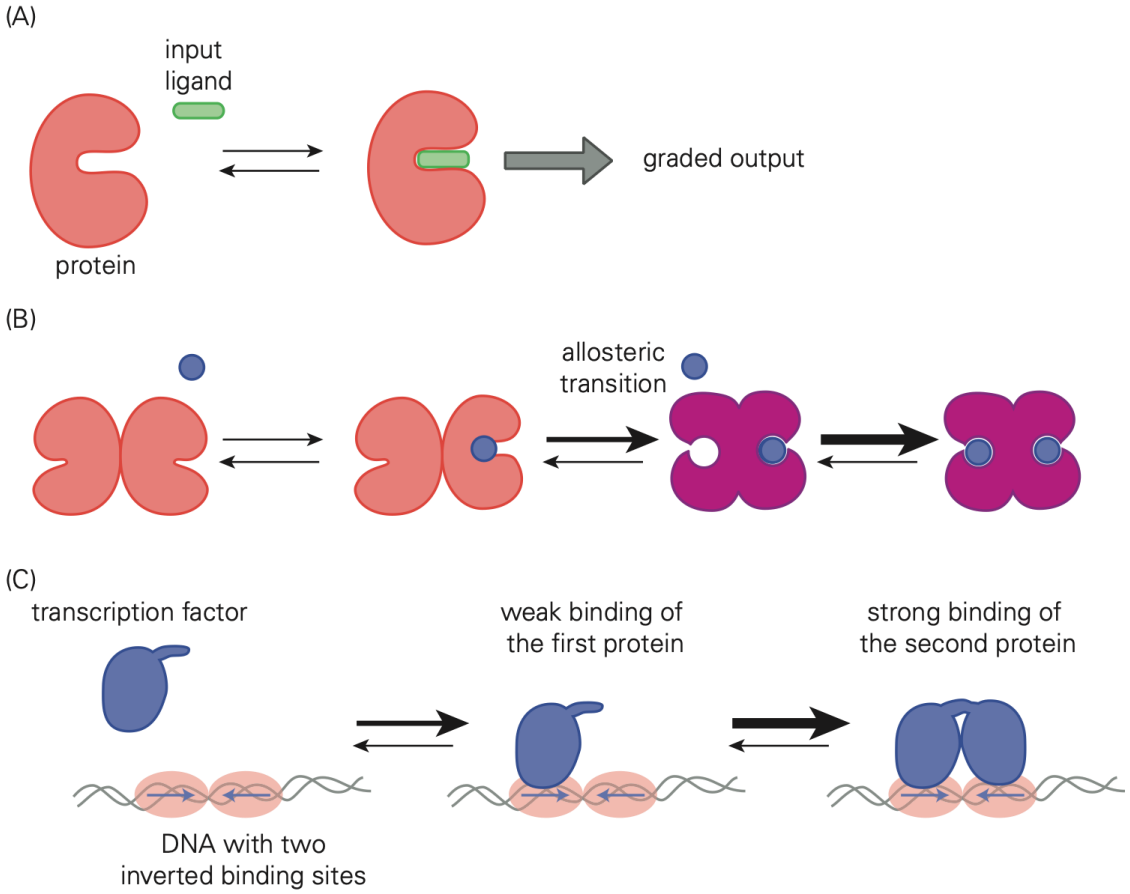
sigmoid curve



$K_D \sim 3\mu M$

- Increase from 2 to 4μM concentration of CheY-P results in switch to 100% switch to clockwise movement of the flagellar motor
- Bacteria uses this motion to make a sharp turn away from the toxin

Ultrasensitivity and Cooperative binding

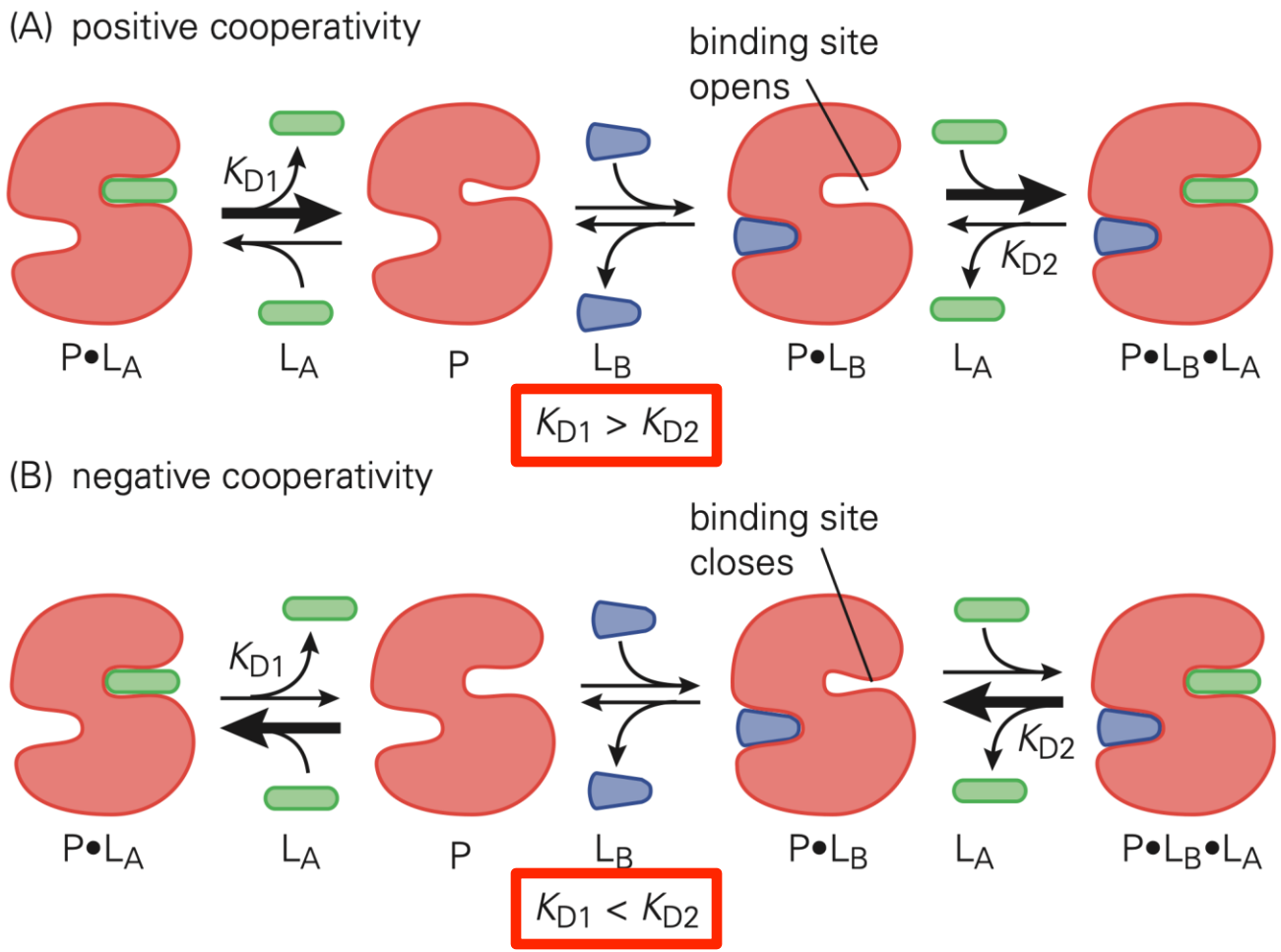


- For **cooperatively binding** an allosteric mechanism comes into play as a first binding event induces some effect that allows also a second binding event
- **Allostery** is thus not a direct effect between binding sites, but a mechanism that happens at a distance

- The possibility to have such a mechanism at distance is routed within the architecture of the protein itself, there exists a specific network that permits the cooperative behavior

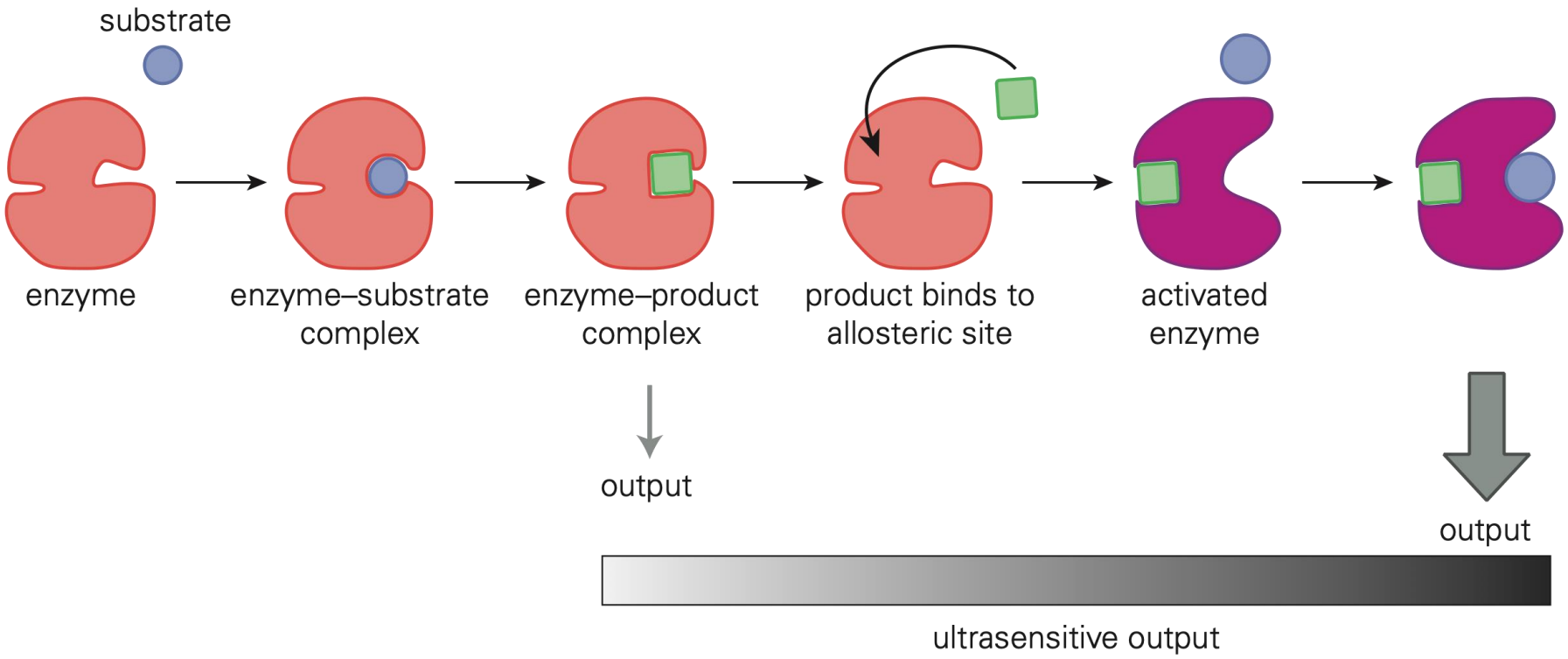
Cooperativity and Allostery

- In **positive cooperativity** the binding of the allosteric ligand B increases the affinity - on the opposite for **negative cooperatively** the affinity is reduced



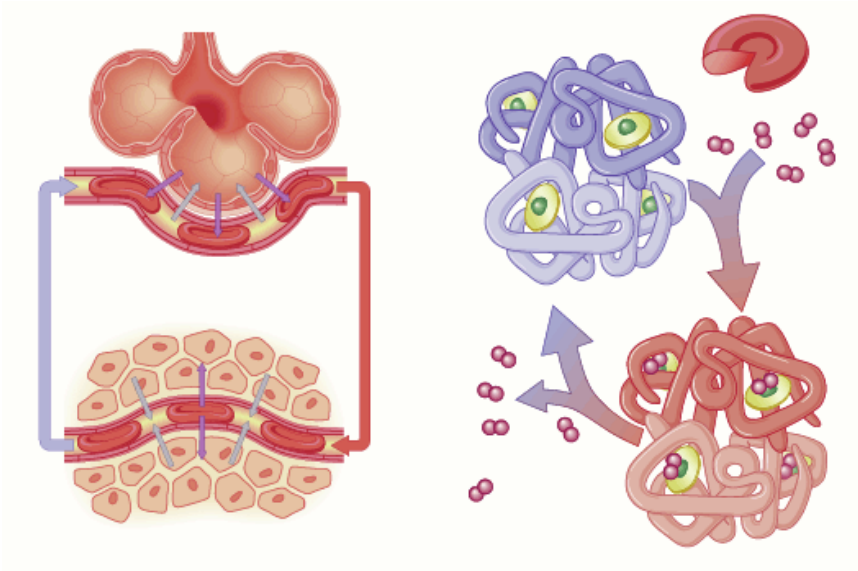
Allosteric feedback

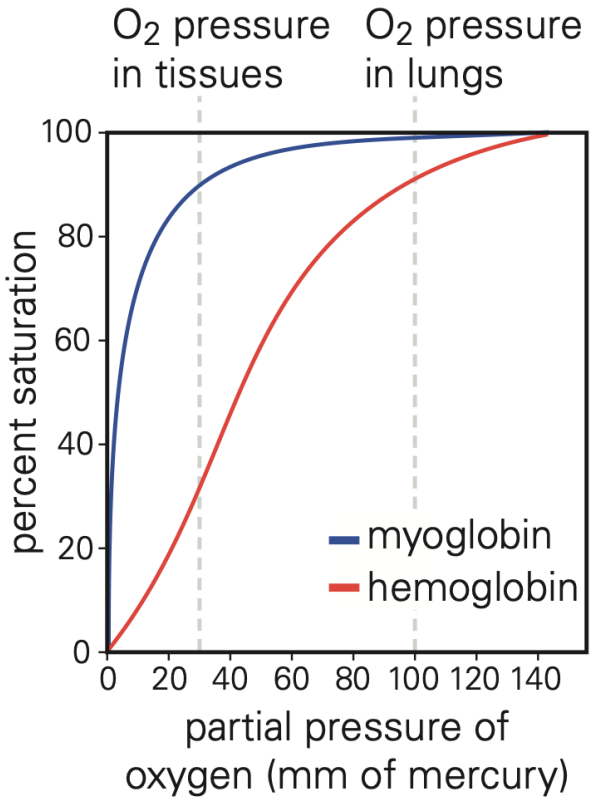
- Ultrasensitivity can also arise from **allosteric feedback.**
- An enzyme has low affinity for the substrate. The conversion of substrate to product activates the enzyme because the product molecule can bind to an allosteric site on the enzyme, causing a conformational change that opens up the active site.



Allostery in hemoglobin

- Myoglobin is found in muscle tissue and is a monomer - local storage of oxygen
- Hemoglobin in blood and is a tetramer - transport of oxygen from lung to tissues

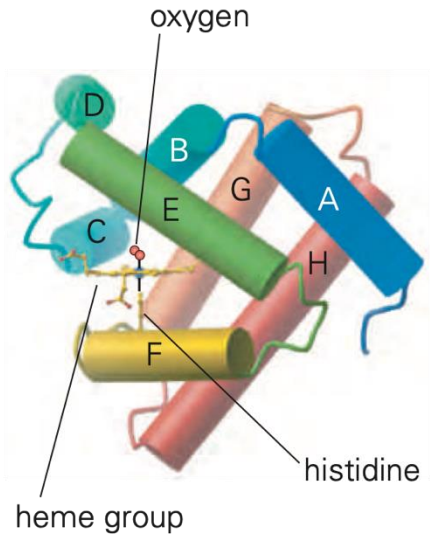


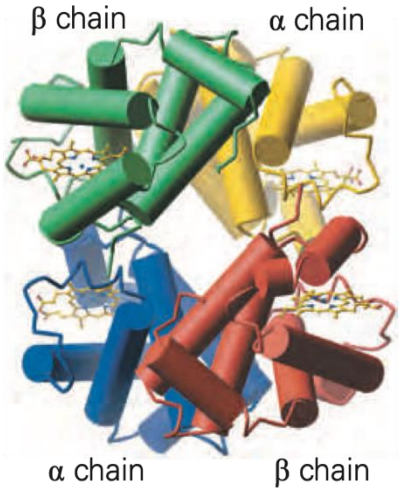


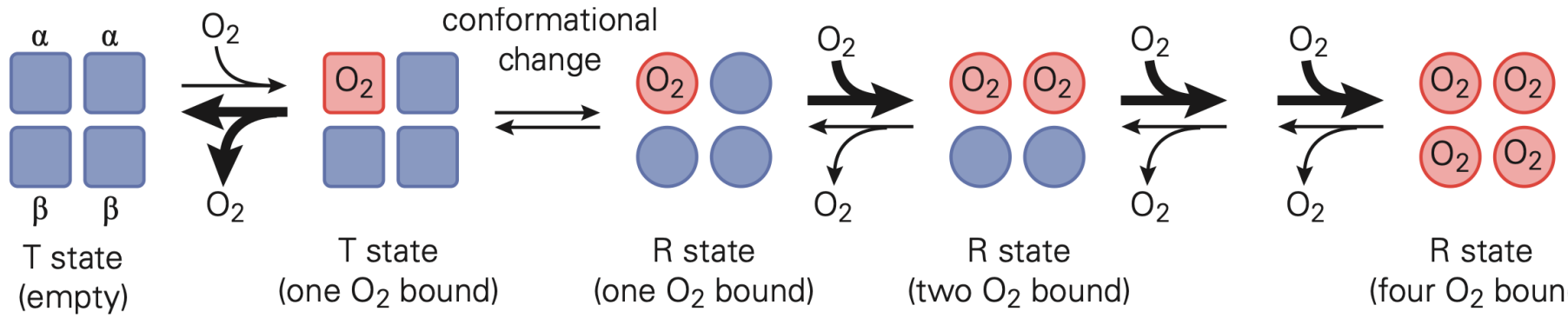
- Myoglobin has a graded binding while hemoglobin has cooperative binding to O_2
- Average O_2 concentration is 0.1 mM
- Difference in concentration of O_2 in lungs and tissues is only ~3 fold - that is why hemoglobin needs to have a ultrasensitive response
- Hemoglobin binds O_2 in the lungs (higher partial pressure) and releases it in the tissue (lower partial pressure)

Allostery in hemoglobin

- Myoglobin is found in muscle tissue and is a monomer - local storage of oxygen
- Hemoglobin in blood and is a tetramer - transport of oxygen from lung to tissues





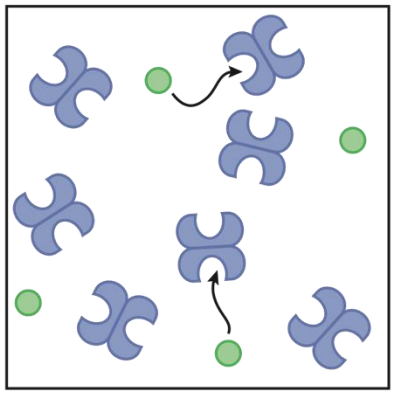


- Sigmoid binding curve arises from positively cooperativity
- Hemoglobin passes from the tense T state to the relaxed R state where affinity is increased

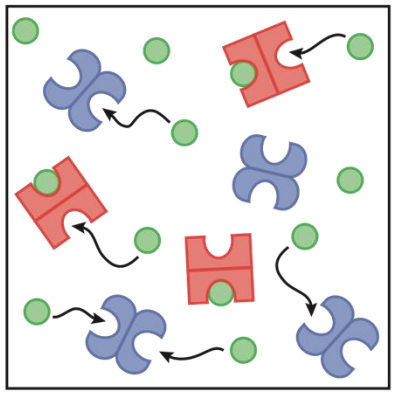
Sigmoid binding isotherm

- To simplify things let's imagine only 2 states, T and R
- As [L] increases, bound states of type R increases

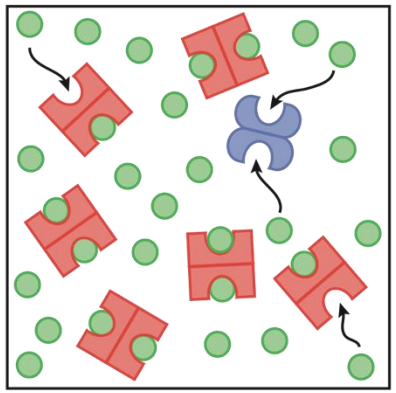
K_{D1}



low ligand concentration
T state

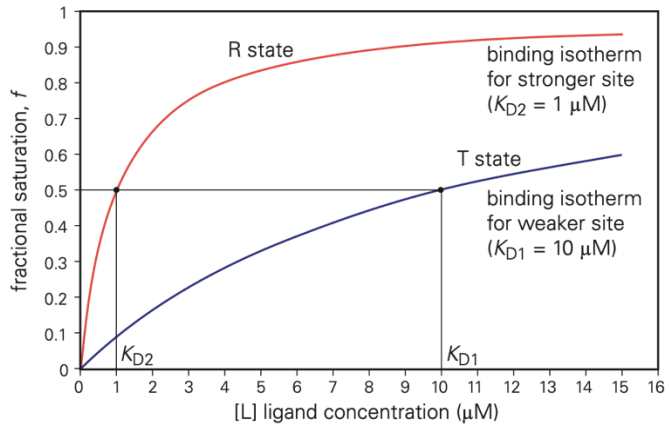


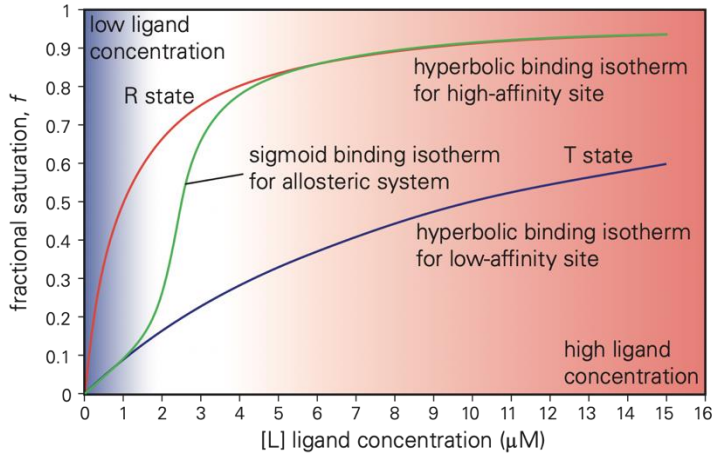
intermediate ligand concentration
mixture of T and R states



very high ligand concentration
R state

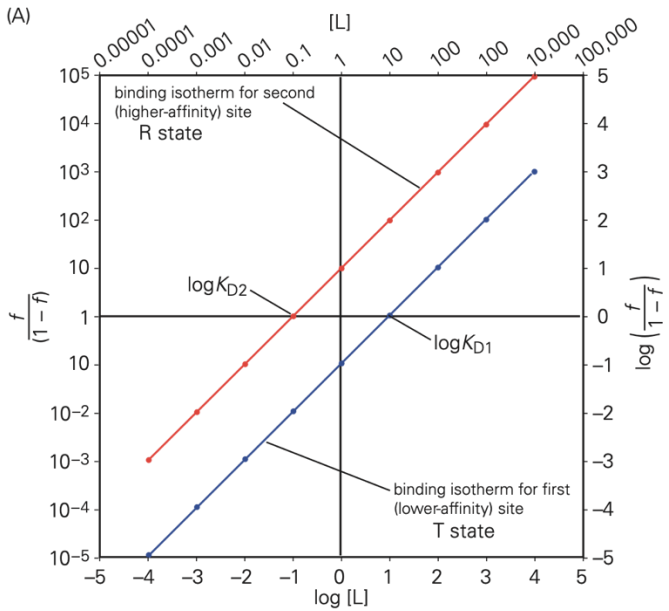
K_{D2}

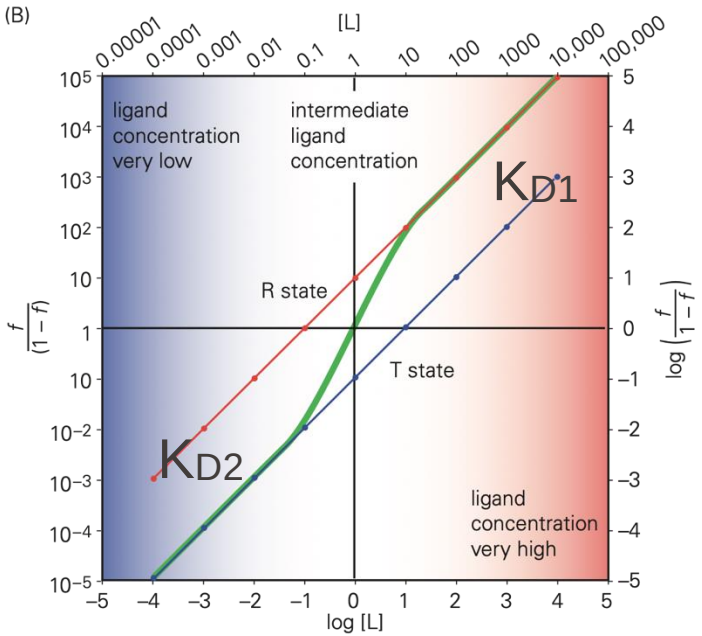




Degree of cooperativity - Hill Coefficient

- Log version of the isotherm (X axis) is often used to evaluate cooperative binding





$$\log\left(\frac{f}{1-f}\right) = \log[\mathrm{L}] - \log K_{\mathrm{D}}$$

- Slopes for both binding regimes is always 1 - true also for cooperativity
- At intermediate [L], slope is different from 1 and called **Hill coefficient:**

$$n_{\mathrm{H}} = \frac{2}{1+\sqrt{\frac{K_{\mathrm{D2}}}{K_{\mathrm{D1}}}}}$$

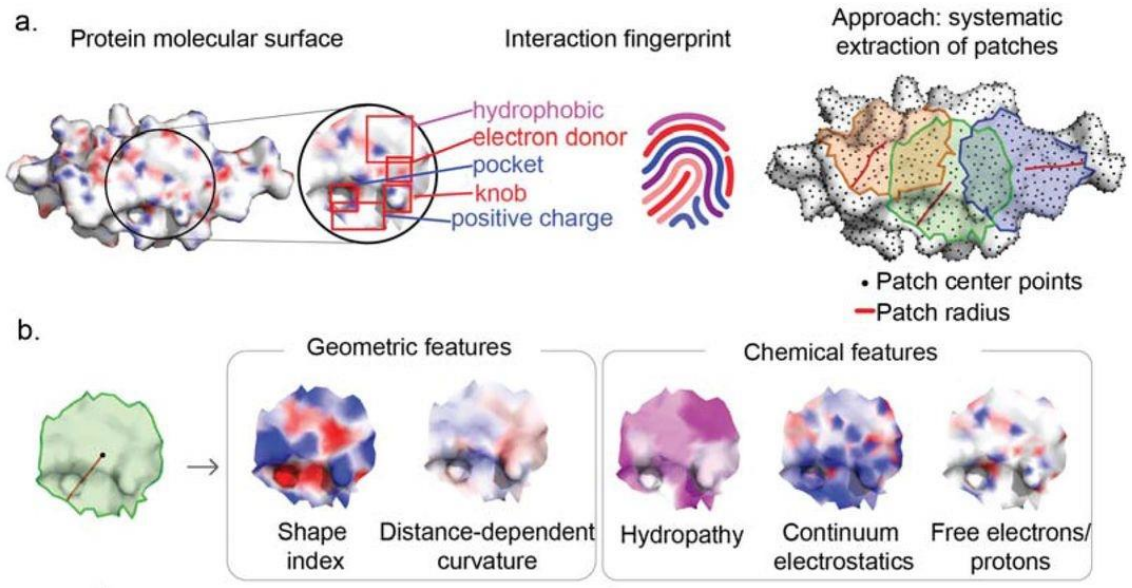
The value of the Hill coefficient is greater than 1 for positive cooperativity and less than 1 for negative cooperativity.

Summary

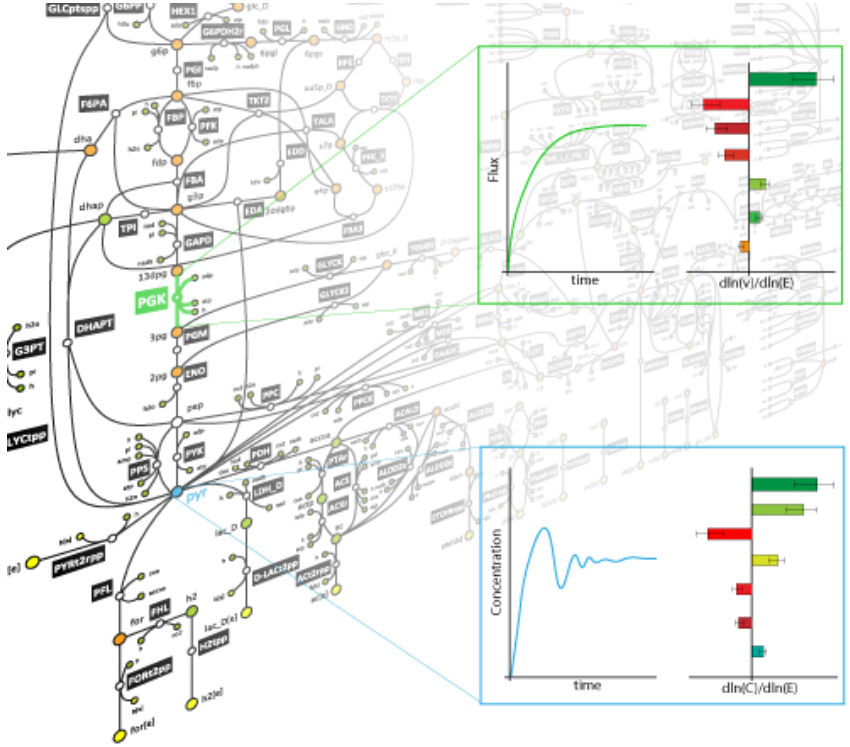
- Molecular binding in protein-protein complexes is the result of the balance between multiple contributions in order to optimize affinity and specificity at the same time (i.e., hydrophobic, H-bonds, salt bridges, interfacial water)
- ITC experiments allow to dissect the thermodynamic parameters of molecules binding
- FP and NMR are other useful techniques to quantify binding parameters
- SPR can provide information on kinetics (association and dissociation rates) of binding
- Ultrasensitive binding is often required in biological processes and it can be achieved by allosteric (cooperative) mechanisms
- Positively cooperativity is described by sigmoid binding isotherms (instead of hyperbolic) and is characterized by a Hill coefficient greater than 1

Macromolecular binding in bioengineering

Deciphering molecular rules of binding



Systems biology of cells, tissues, organs



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