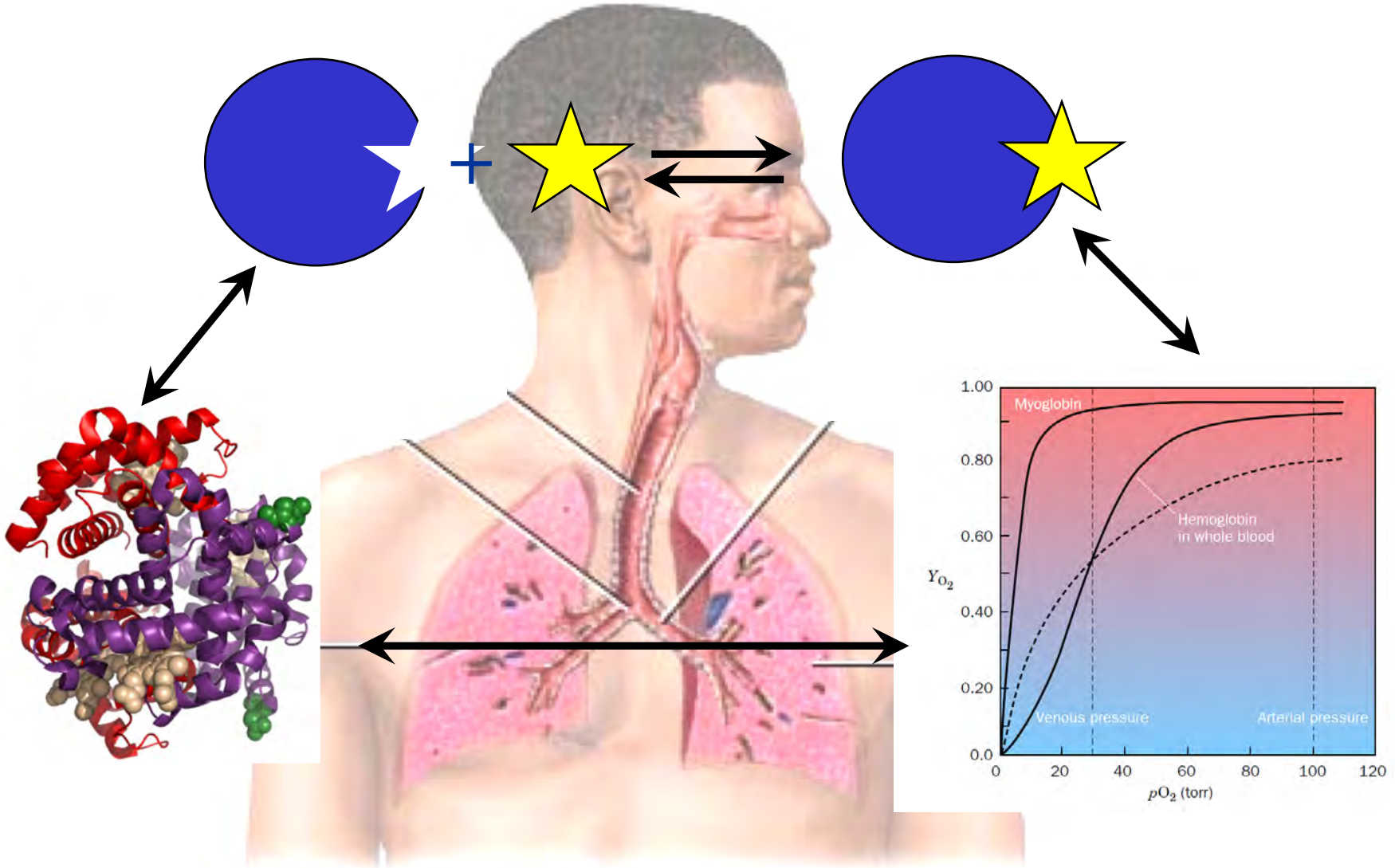
Protein Structure, Function and Disease

Ligand-Protein Interaction

(Partially adopted from Profs. John Baenziger and Patrick Giuère's lectures)

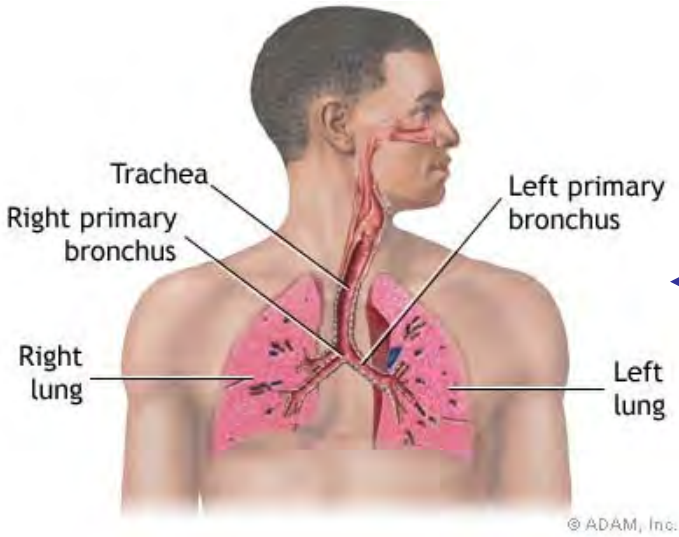
Jyh-Yeuan (Eric) Lee, Assistant Professor, BMI

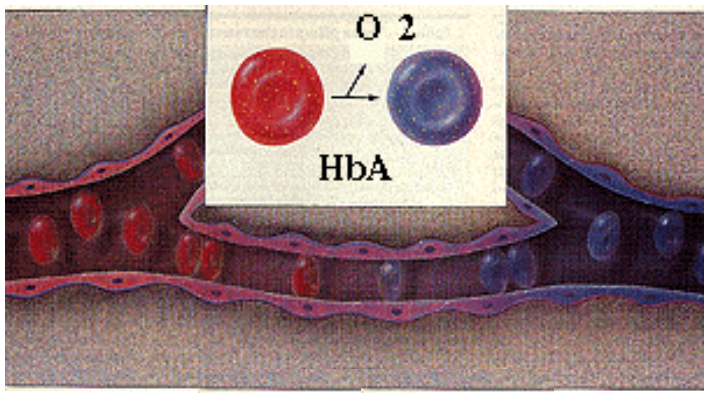


Protein-ligand interactions and the physiology of oxygen transport to peripheral tissues.

Oxygen transport in mammals

All multicellular organisms have developed systems for transporting oxygen to their cells to allow respiration to occur.





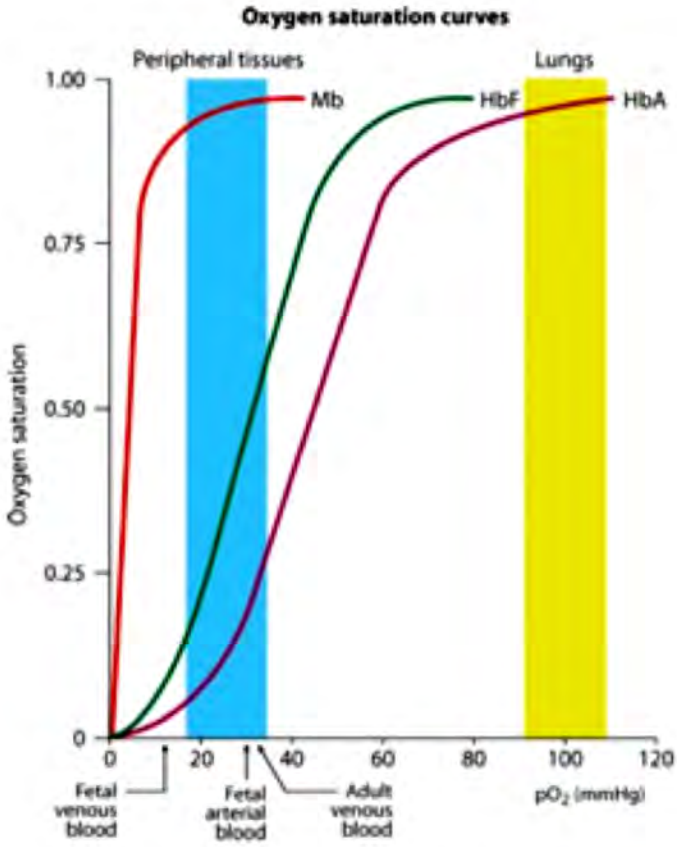
Intrinsic to these systems are the oxygen binding proteins, Hemoglobin (Hb) and Myoglobin (Mb). These are fine-tuned oxygen-binding *molecular machine*s that allow mammals to optimize oxygen delivery to tissues under widely varying conditions

Oxygen binding to heme proteins

All multicellular organisms have developed systems for transporting oxygen to their cells to allow respiration to occur.

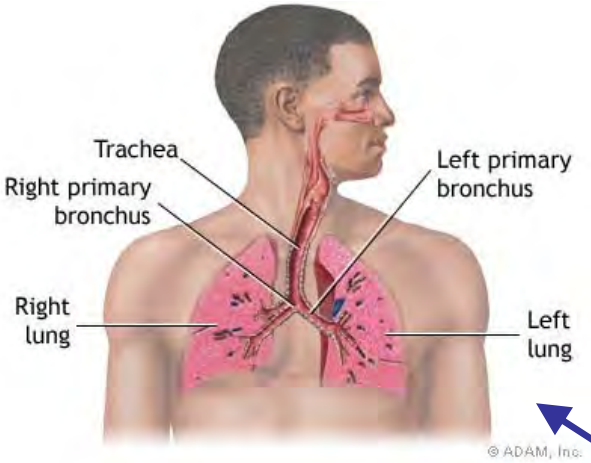
Oxygen binding to Mb is **hyperbolic**, while the binding to HbA and HbF is **sigmoidal**. Note that Mb is fully oxygen saturated in peripheral tissues (where it stores O_2), while HbA requires the much higher oxygen concentrations in the lungs to fully saturate. HbF saturates at lower oxygen concentrations than HbA, so that a fetus can acquire oxygen from the maternal circulation.

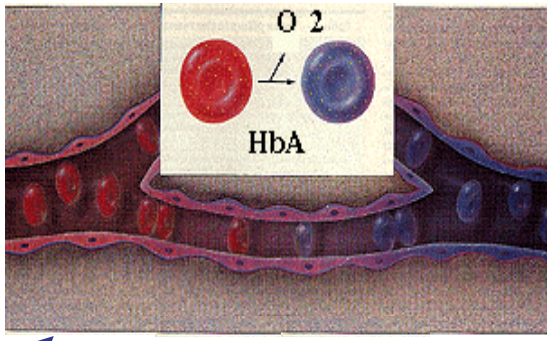
So, how has biology adapted the basic globin fold to create proteins with such different binding characteristics?

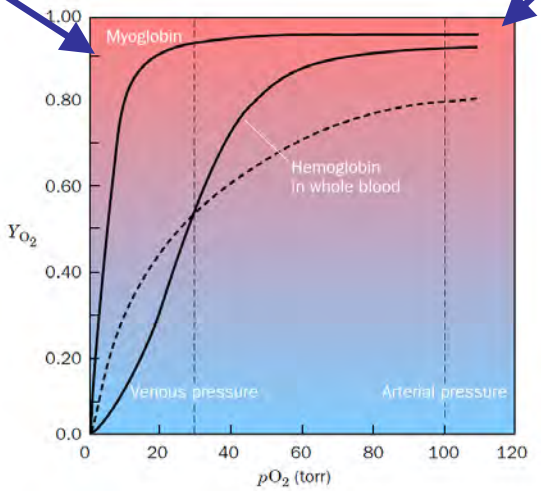


Oxygen binding to myoglobin (Mb) adult hemoglobin (HbA) and fetal hemoglobin (HbF).

Oxygen binding properties of Hb and Mb

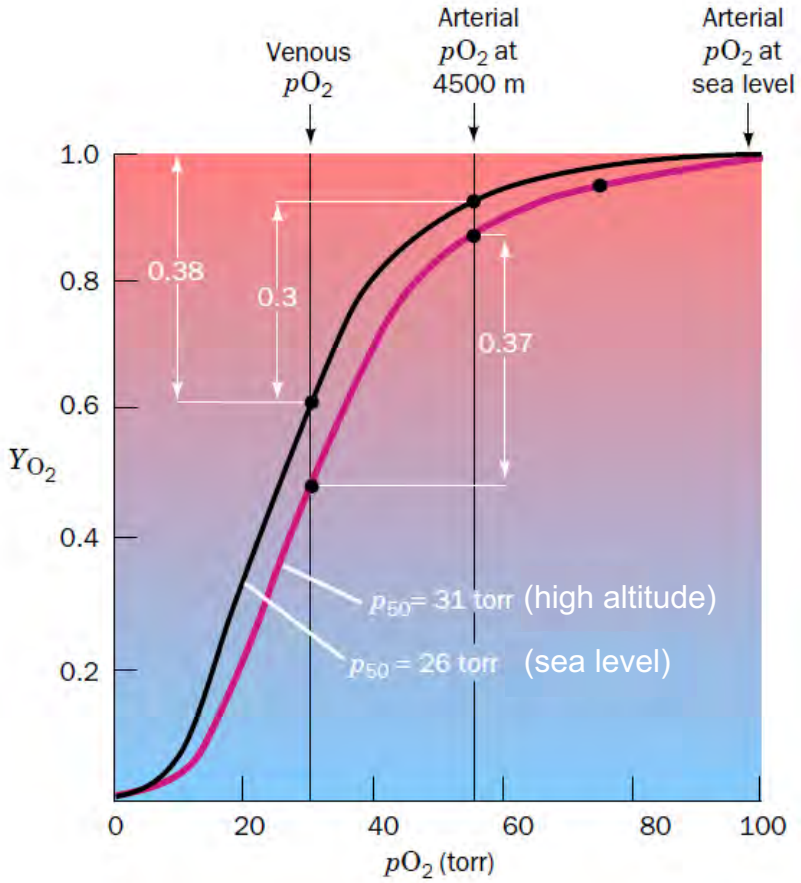






Hb in red blood cells binds oxygen in the lungs, transports it through the blood to peripheral tissues, and then releases the oxygen. Mb binds and stores oxygen in muscle. Hb and Mb have very different binding cures. But there is more…

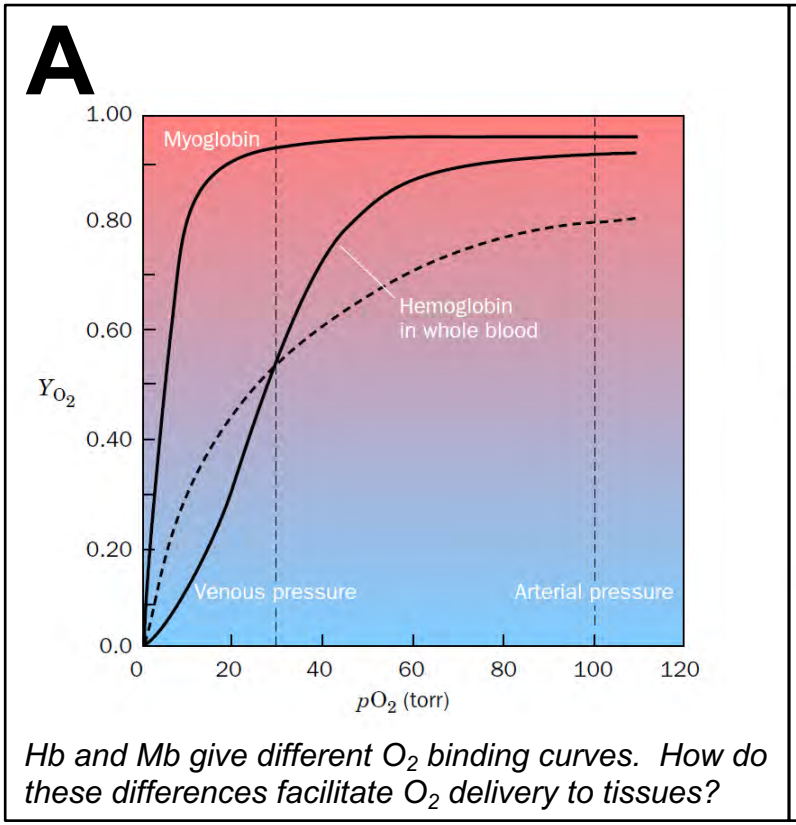
Hb adapts to maximize O_2 delivery at high altitude



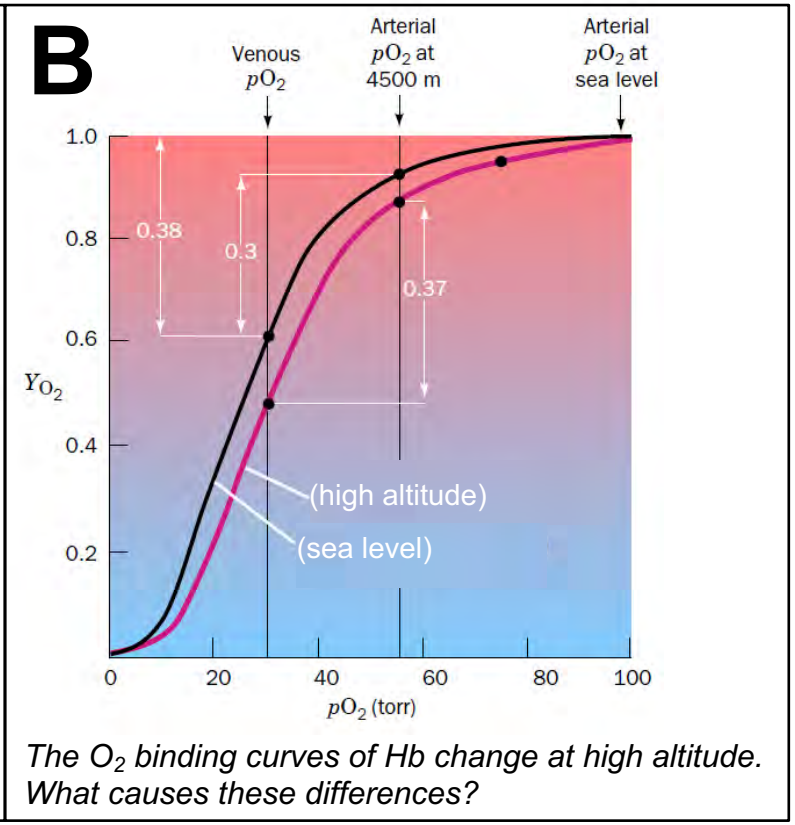
Breathing is difficult at high altitude because Hb is less efficient at delivering O_2 to tissue. While ~40% of binding capacity is delivered at sea level, only ~30% is delivered at 4500 m – i.e. O_2 delivery drops by 25%!

After even a day at high altitude, however, the O_2 binding properties of Hb change to increase the efficiency of O_2 delivery.

Our goal is to understand how Hb & Mb can exhibit such different binding characteristics



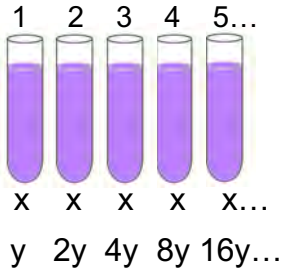
Hb and Mb give different O_2 binding curves. How do these differences facilitate O_2 delivery to tissues?



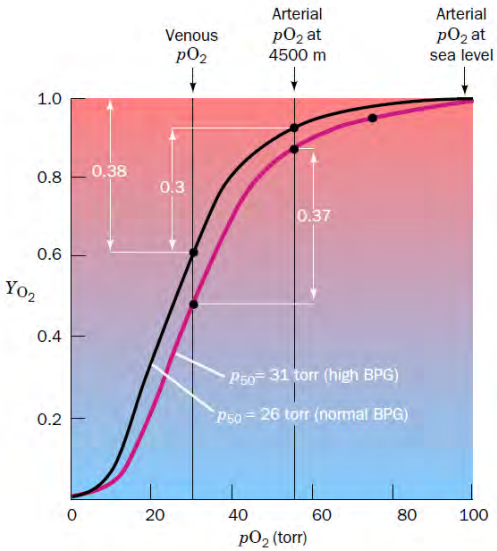
The O_2 binding curves of Hb change at high altitude. What causes these differences?

The different O_2 binding properties of Hb vs Mb (A) and the adaptability of Hb-O_2 interactions (B) are essential for O_2 delivery. <u>These properties are typical of the protein-ligand interactions that underlie biological processes.</u> We will develop an understanding of the different binding properties of Mb and Hb, and how Hb-O_2 interactions are modulated to maximize O_2 delivery.

How do we characterize protein-ligand interactions?



Binding experiment



Fit Binding Data

Interpret in terms of Biology

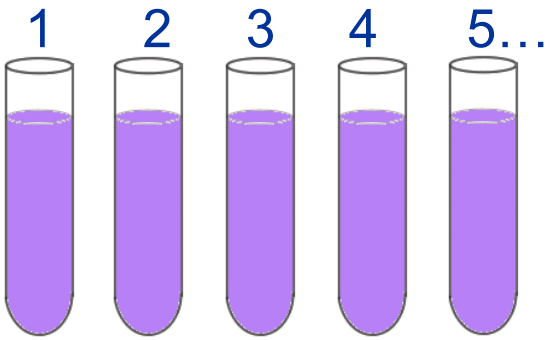
1) Perform a ligand binding experiment, such as shown on the left with oxygen binding to Hb.

2) Fit the binding data to obtain the binding parameters (number of sites, strength of binding (K_D) and cooperativity)

3) Use binding parameters to understand biology.

So let's start with 1) measuring and 2) interpreting binding data…

1.0 Protein-ligand binding experiment



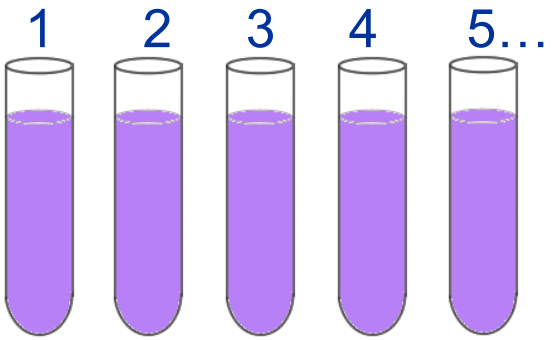
[P]: x x x x x…

[L] : y 2y 4y 8y 16y…

To each in a series of tubes:

1) Add a constant amount of protein
2) Add an increasing amount of substrate (sometimes radiolabeled)
3) Incubate 30 minutes to hours (depending on k_{on}*: rate*)
4) Separate L_{free} from L_{Bound} and measure the amount of each

1.0 Protein-ligand binding experiment



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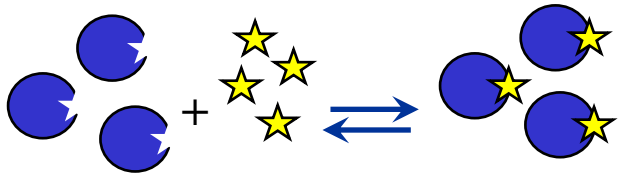
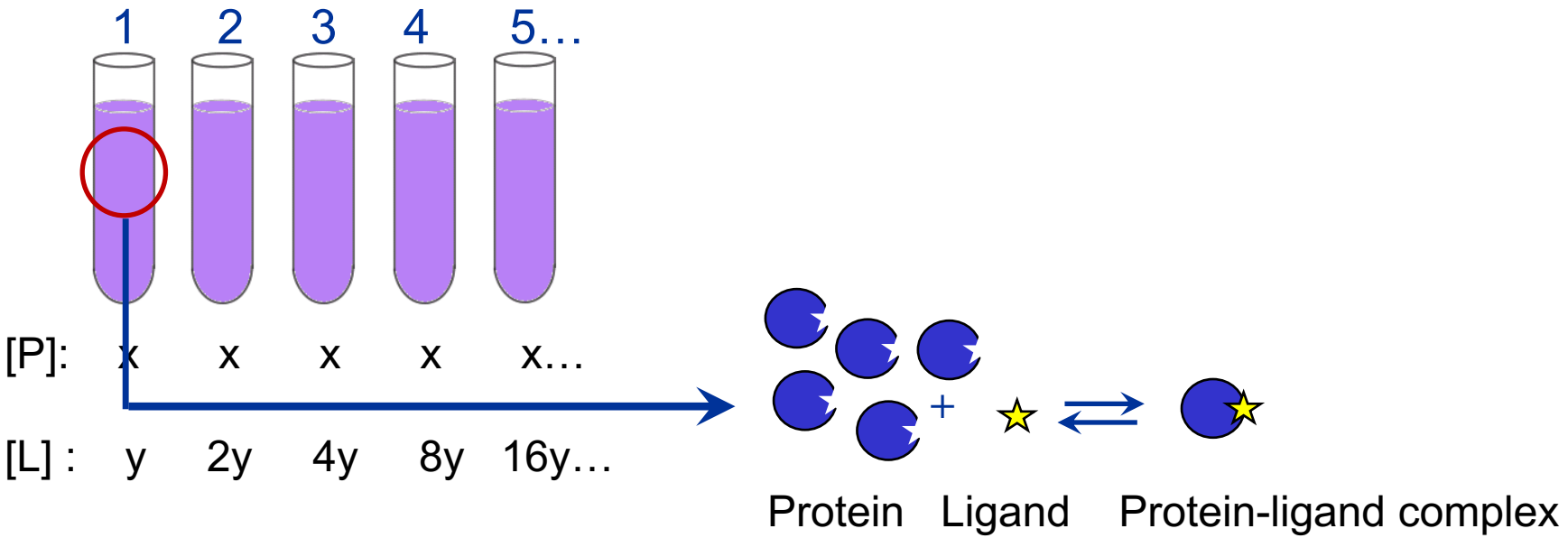


Protein Ligand Protein-ligand complex

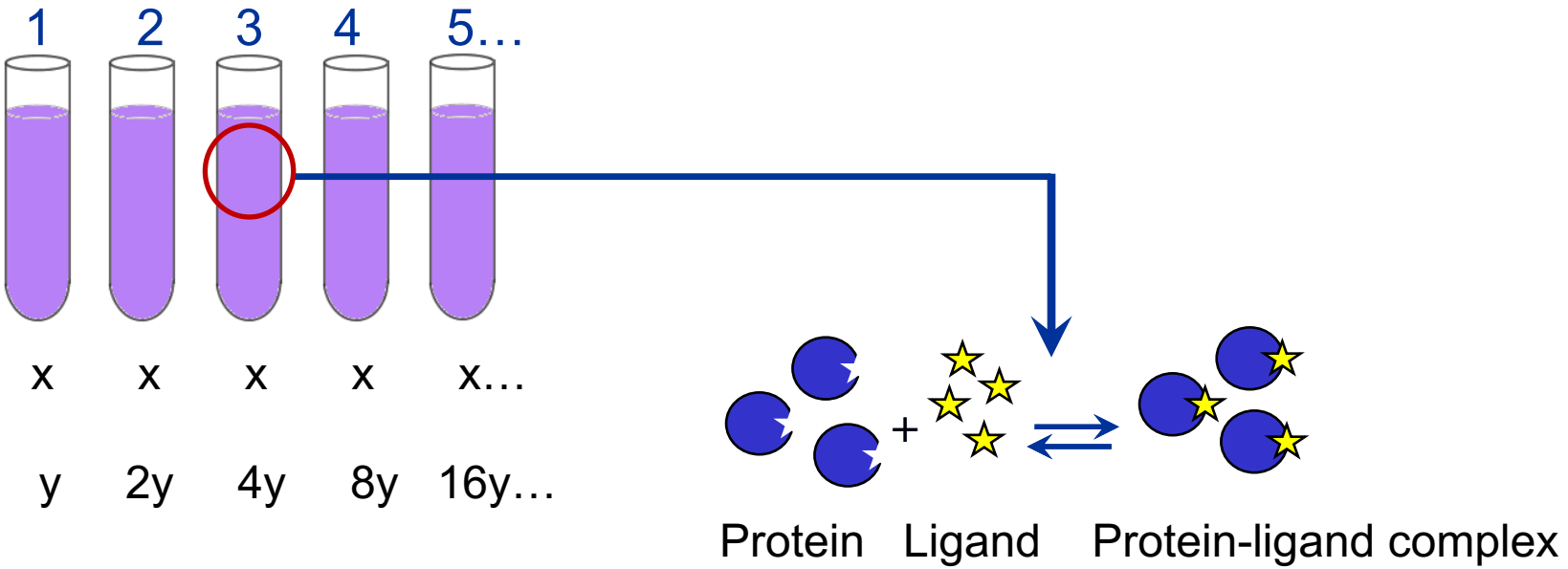
In each tube we have a mixture of protein bound to ligand and protein free in solution. As we increase the amount of ligand we drive the reaction towards the bound state. We ultimately want to define the K_D **(concentration where protein is 50% saturated)** because this tells us about affinity. At each [L], we therefore need to measure the amount of protein bound to ligands – so we must separate bound ligands (PL) from free ligands.

1.0 Protein-ligand binding experiment



In tube 1 at low concentrations of ligand, the equilibrium between P + L and PL strongly favors the ligand-free protein

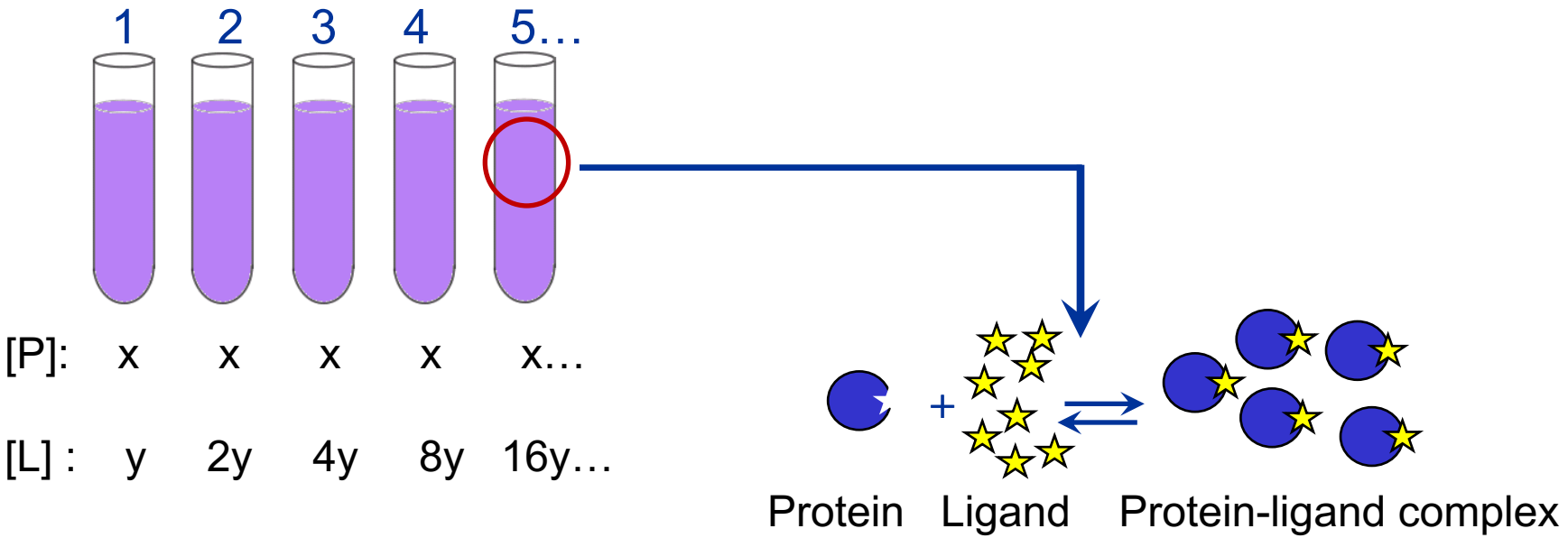
1.0 Protein-ligand binding experiment



In tube 3, the higher concentrations of ligand drive the equilibrium between P + L and PL more towards the ligand-bound protein, PL.

By measuring the binding at different concentrations, we are determining the concentration range in which binding occurs. The concentration range over which binding occurs tells us about the strength of binding, or affinity. Binding affinity is characterized by the K_D.

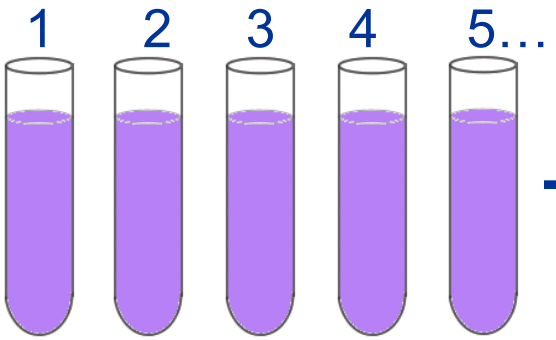
1.0 Protein-ligand binding experiment



In tube 5+, the high concentrations of ligand drive the equilibrium between P + L and PL in favor of the ligand-bound protein, PL.

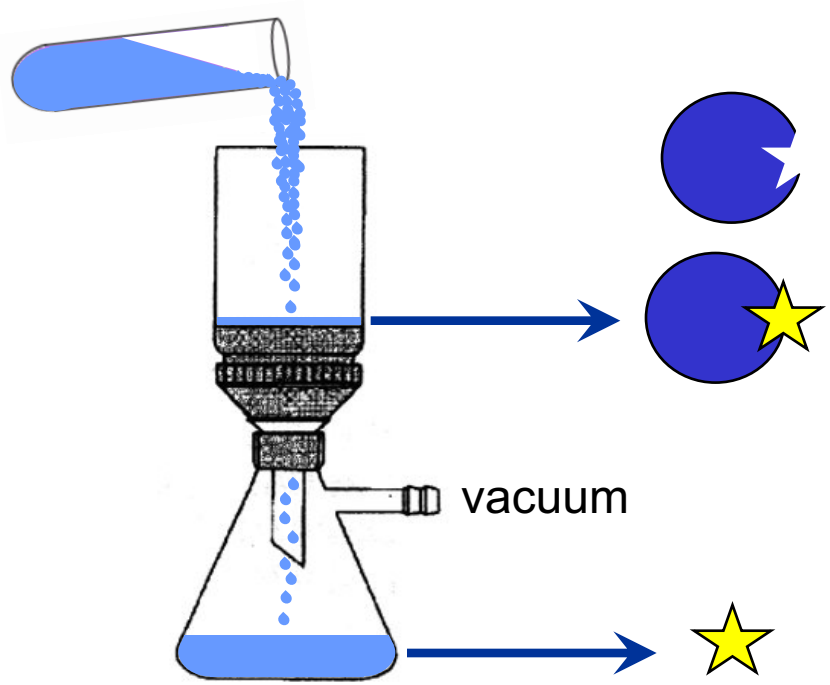
By measuring the binding at different concentrations, we are determining the concentration range in which binding occurs. The concentration range over which binding occurs tells us about the strength of binding, or affinity. Binding affinity is characterized by the K_D.

1.1 We need to separate the bound ligand from the free ligand



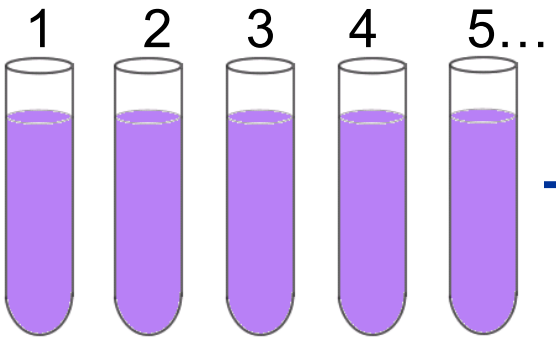
[P]: x x x x x…

[L] : y 2y 4y 8y 16y…



Filtration is one “established” method for separating bound ligand (PL) from free ligand, but there are many. We also need some way to measure the bond and free ligand – radio-labelled ligands are still common. We know the total [L] added to each tube, so if we can measure the $[L]_{free}$ and $[L]_{bound}$, we can calculate $[P]_{free}$ and [PL].

1.2 The binding experiment leads to a binding “isotherm”



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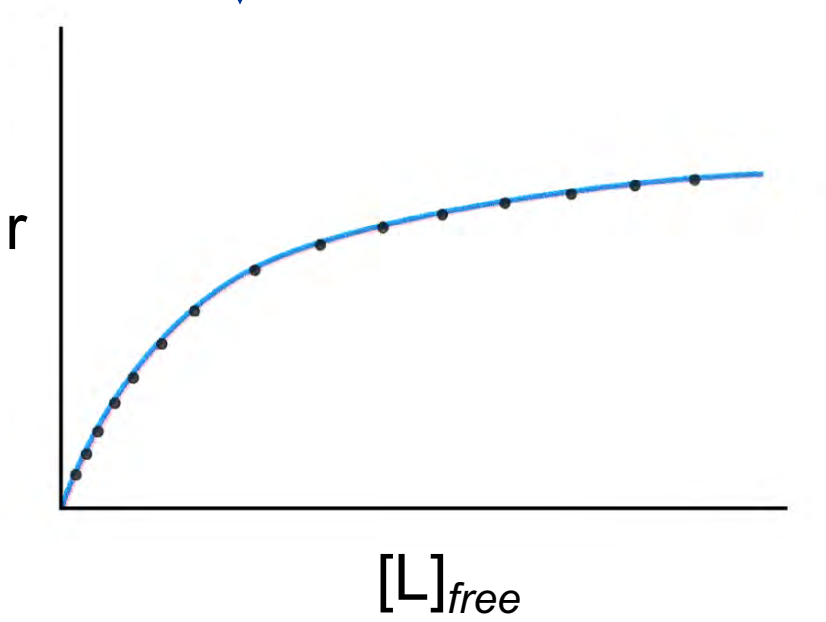


By measuring the $[L]_{free}$ and $[L]_{bound}$ at each $[L]_{total}$, we obtain a binding curve, although we usually convert $[L]_{bound}$ into the **molar binding, “r”**…

1.3 Molar binding, r

r is the number of moles of ligand bound in our test tube per mole of protein:

$$r = \frac{[L]_{bound}}{[P]_{total}} = \frac{[PL]}{[P]_{free} + [PL]}$$

r varies from 0 (none bound) to the a maximal value corresponding to the number of binding sites for the ligand on each molecule of protein, *n. (0 < r < n)*

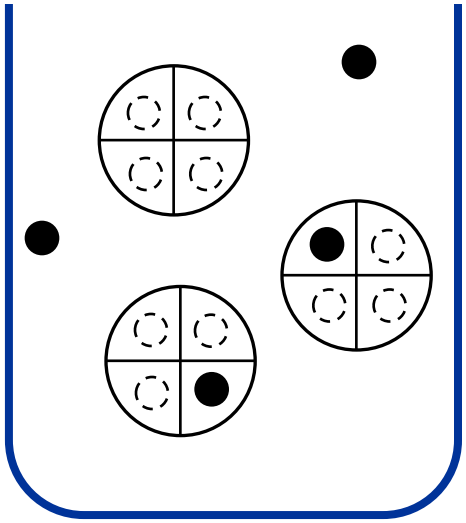
Note that later we will use the fractional binding, ***Y***, which tells us the percentage of bound sites. **Y** varies from 0 to 1 (i.e. 0 – 100%).

Y = r/n

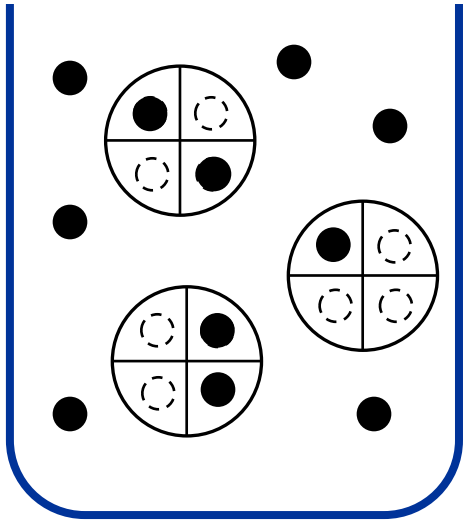
1.3 Molar binding, r

Consider three test tubes each with three protein molecules, but at three different concentrations of ligand increasing from left to right:

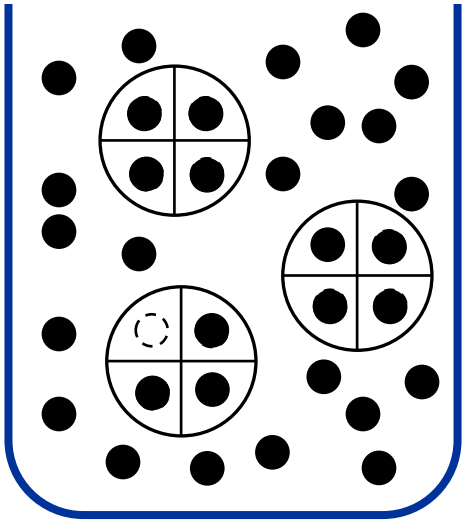
Tube A



Tube B



Tube C

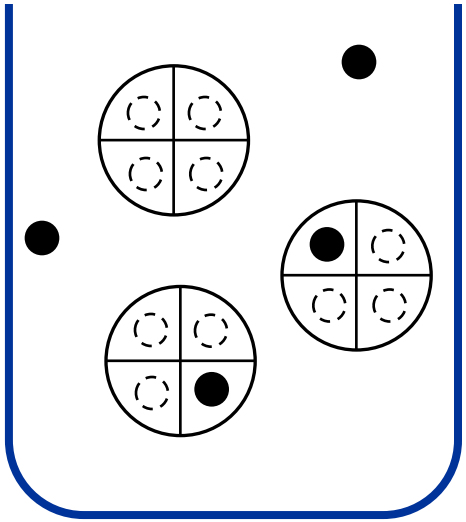


What is r:

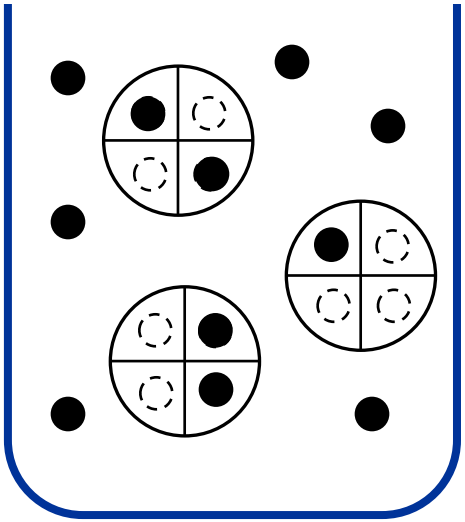
1.3 Molar binding, r

Consider three test tubes each with three protein molecules, but at three different concentrations of ligand increasing from left to right:

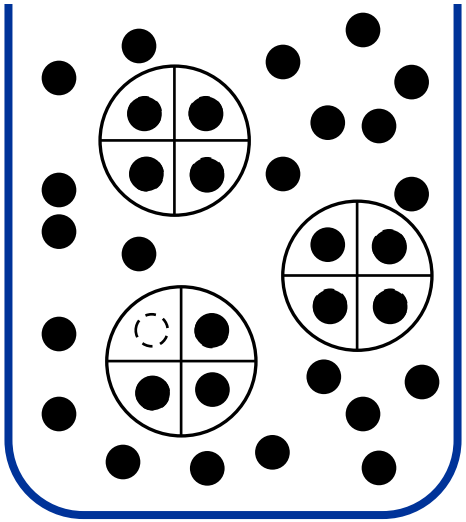
Tube A



Tube B



Tube C



What is r:

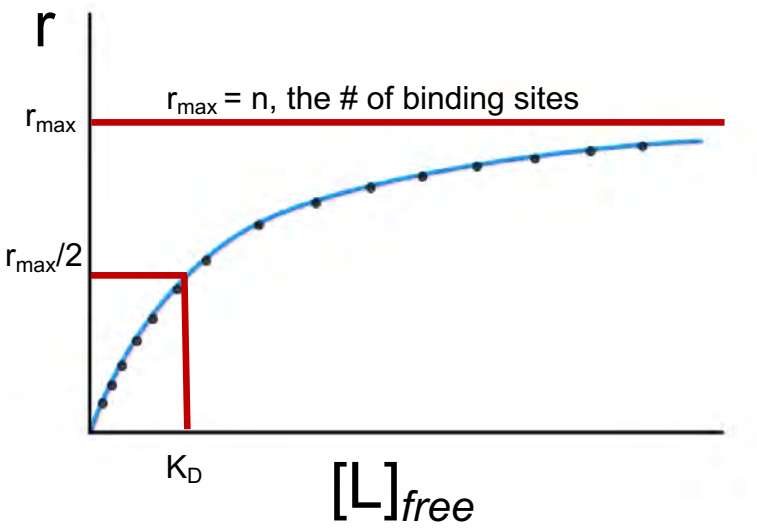
r = 2/3= 0.67

r = 5/3 = 1.67

r = 11/3 = 3.67

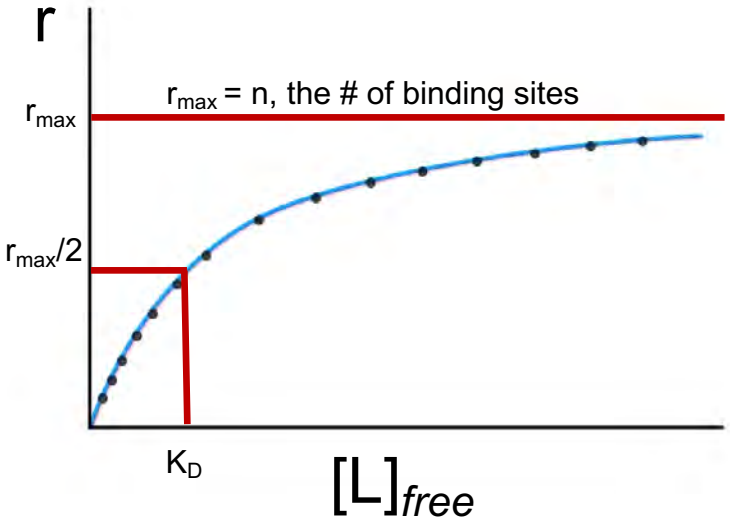
r_{max} = 12 ligands bound per 3 proteins = 12/3 = 4 (i.e. the number of binding sites on each protein)

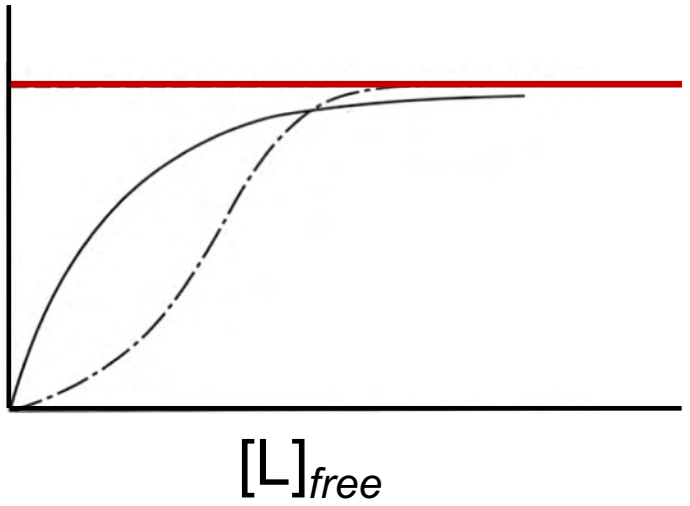
2.0 Interpreting binding data



Intuitively we can see that r approaches r_{max}, the # of binding sites on the protein, asymptotically. We can also define a parameter, K_D, which is the concentration of ligand that gives half maximal binding.

2.0 Interpreting binding data





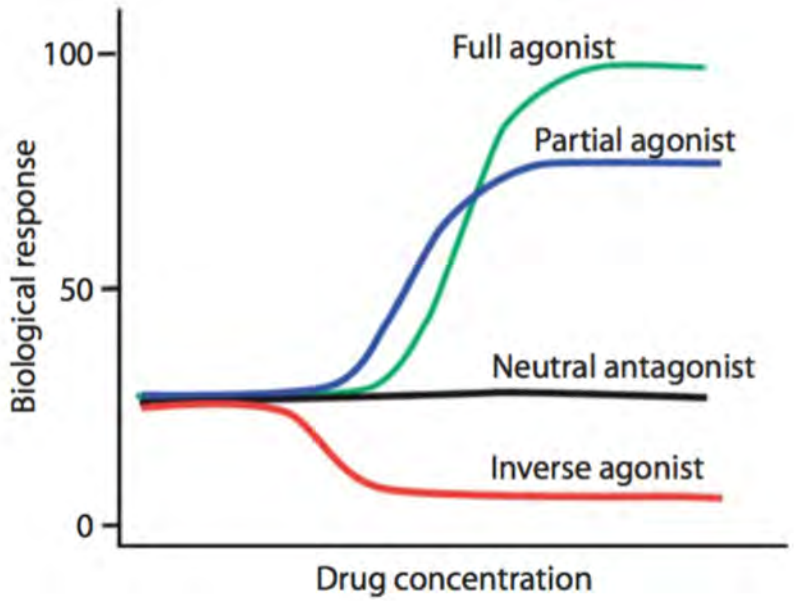
Intuitively we can see that r approaches r_{max}, the # of binding sites on the protein, asymptotically. We can also define a parameter, K_D, which is the concentration of ligand that gives half maximal binding.

BUT, not all curves are so easy to interpret, as shown on the right. To interpret binding data, we have to develop the underlying theory.

2.0 Interpreting binding data

Ligand Efficacy and potency

Efficacy relative to endogenous ligand



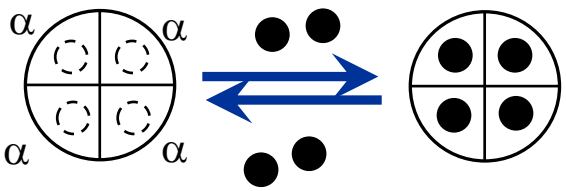
Potency

Potency compares the relative **effectiveness** of drugs to produce a desired effect
Related to efficiency: Ability to do produce something with less drug.

e.g. Drug A requires fewer milligrams than Drug B to achieve the same pharmacological response
--> Drug A has the higher potency, yet, both drugs have the same efficacy.

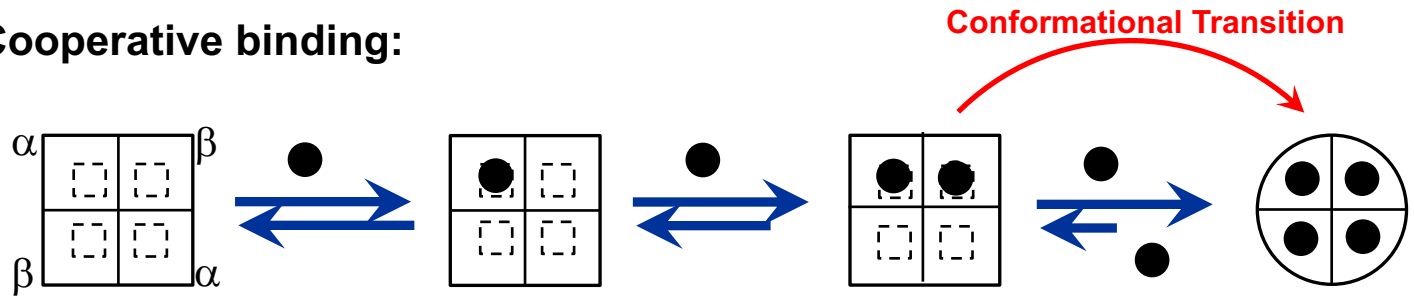
2.1 Independent versus cooperative binding

Case 1) Independent binding:



The binding of one molecule of ligand to one site on the protein has no effect on the binding of other molecules of the ligand to the same protein – the binding sites are independent!

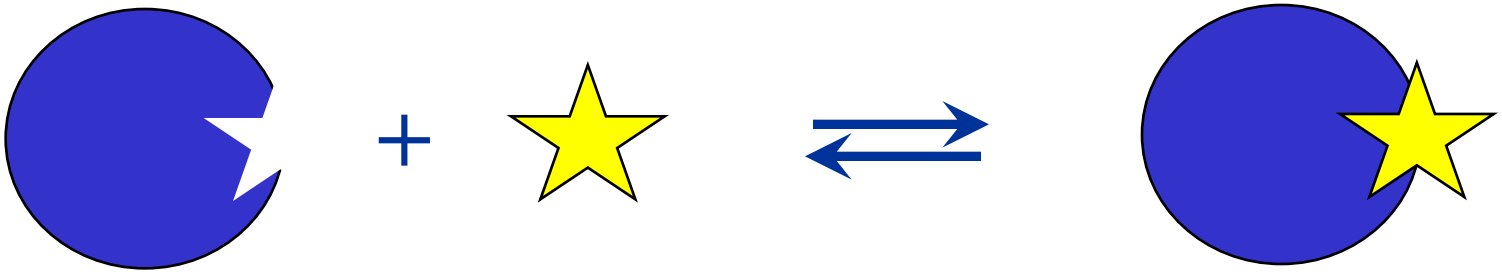
Case 2) Cooperative binding:



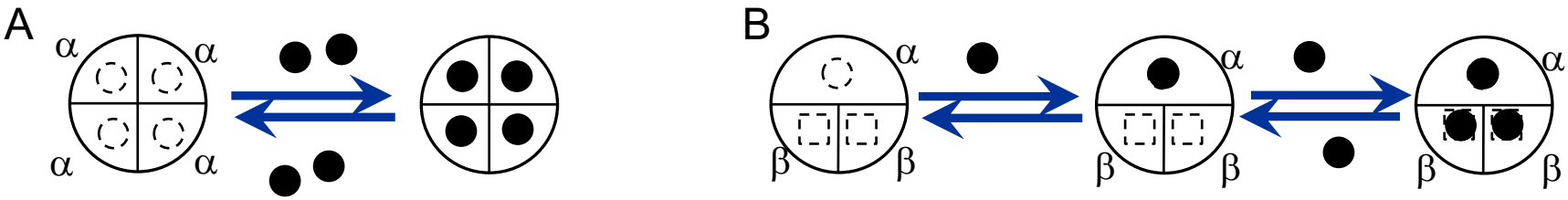
The binding of one molecule of ligand to one site on the protein changes the binding of other molecules to the same protein. In this example of positive cooperativity, the binding of one molecule increases the affinity (i.e. the strength) for binding other molecules.

2.2 Case I: Independent binding

Consider a macromolecule (Protein, P) with only a single binding site for a ligand (L):

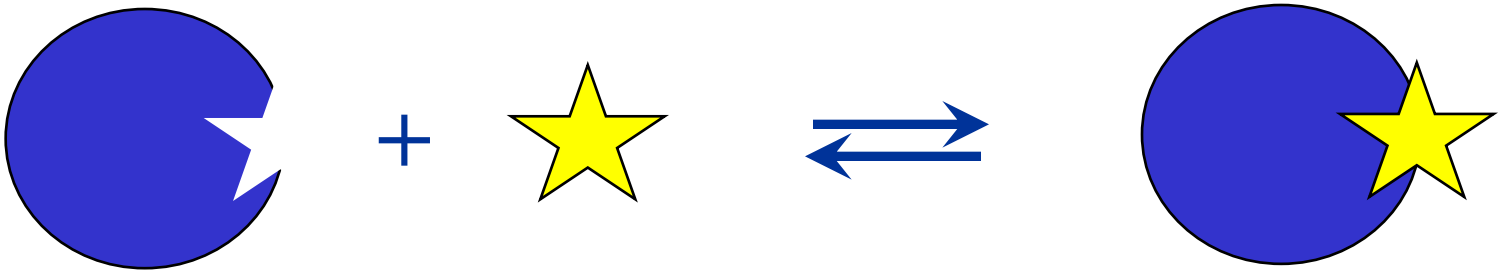


A general case is a protein with multiple identical (*A*) or multiple different (*B*) sites:



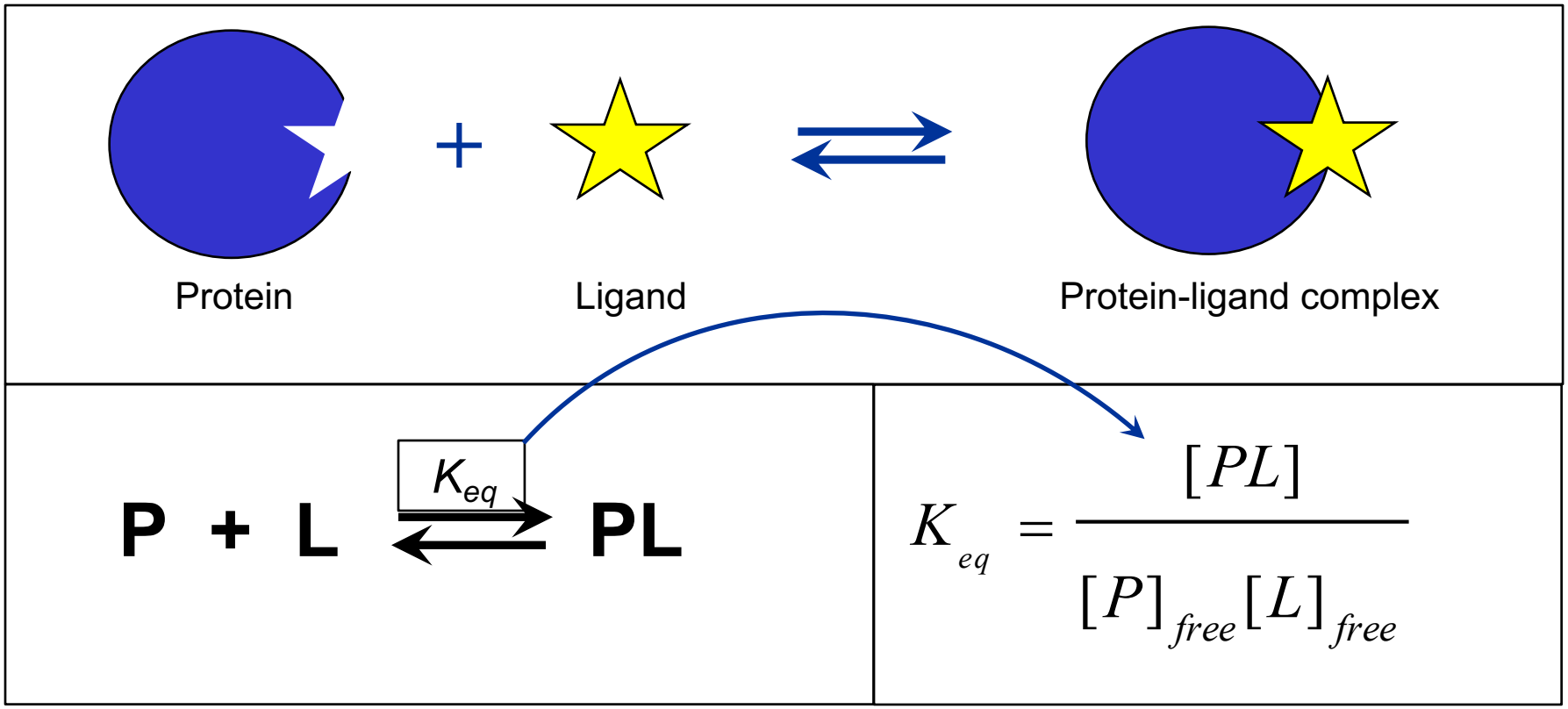
2.2 Case I: Independent binding with one site

Consider a macromolecule (Protein, P) with only a single binding site for a ligand (L):



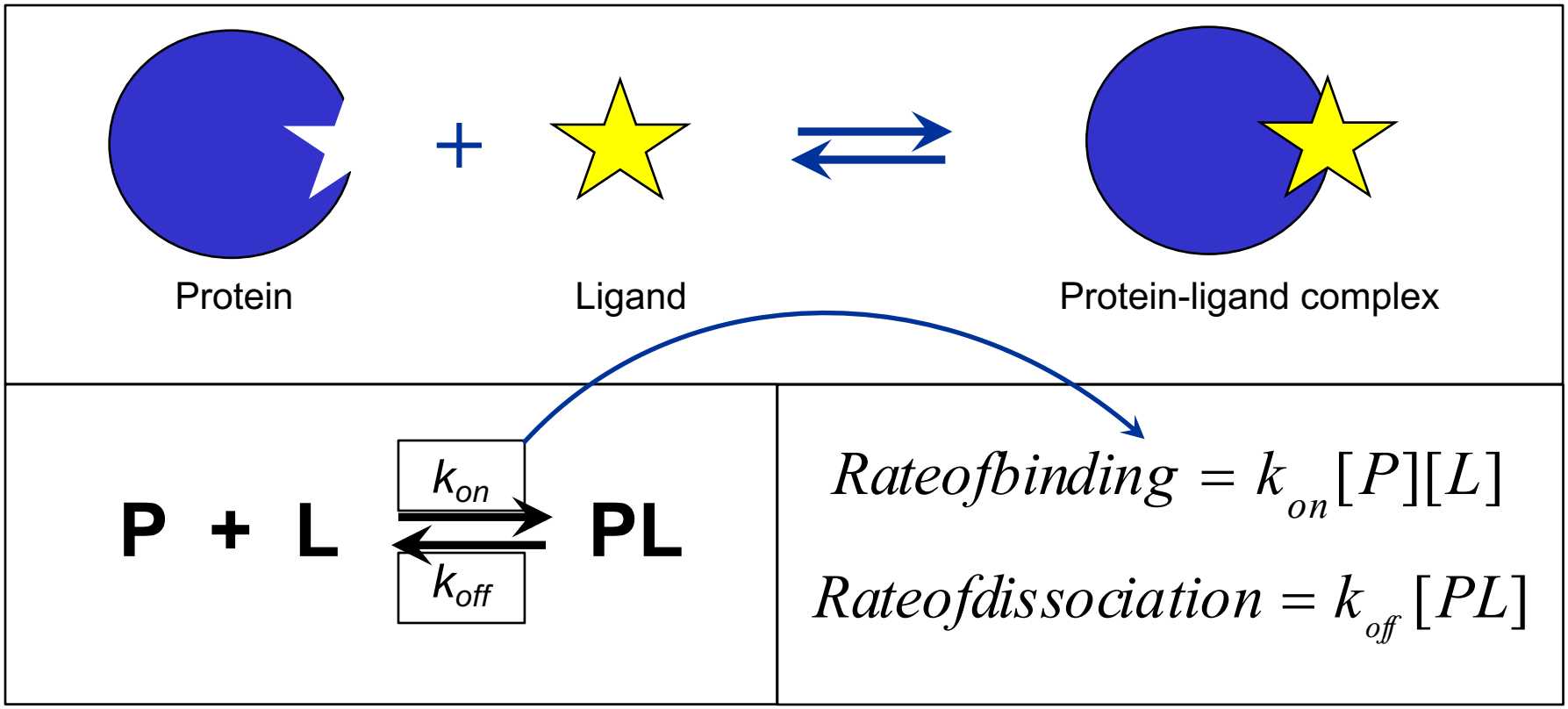
We will develop the basic concepts using the simplest case of a single ligand binding to one site on a protein.

2.2 The equilibrium constant



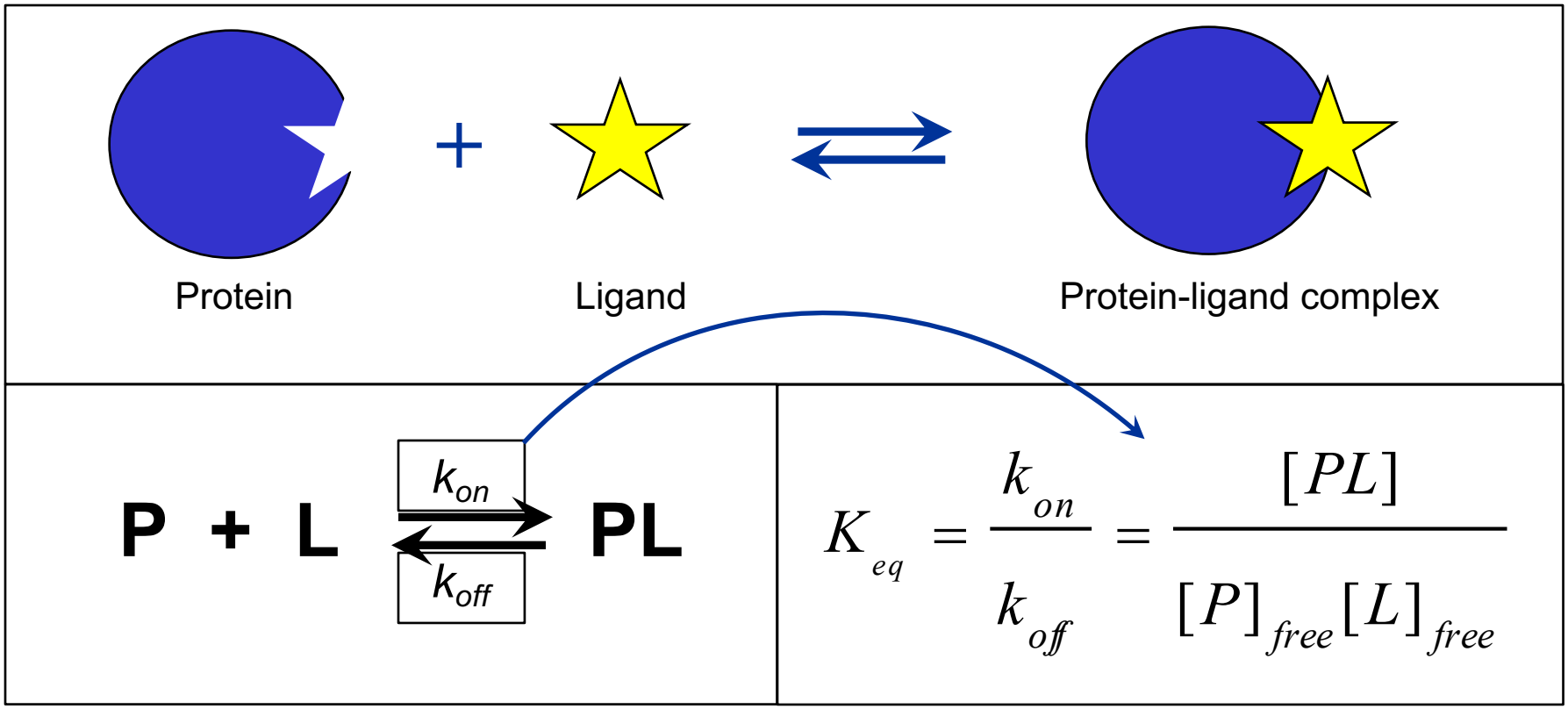
K_{eq} (units of M^{-1}): the equilibrium constant, which tells us the proportion of protein that is bound ligand *at equilibirum.*

2.2 The rate constants for binding



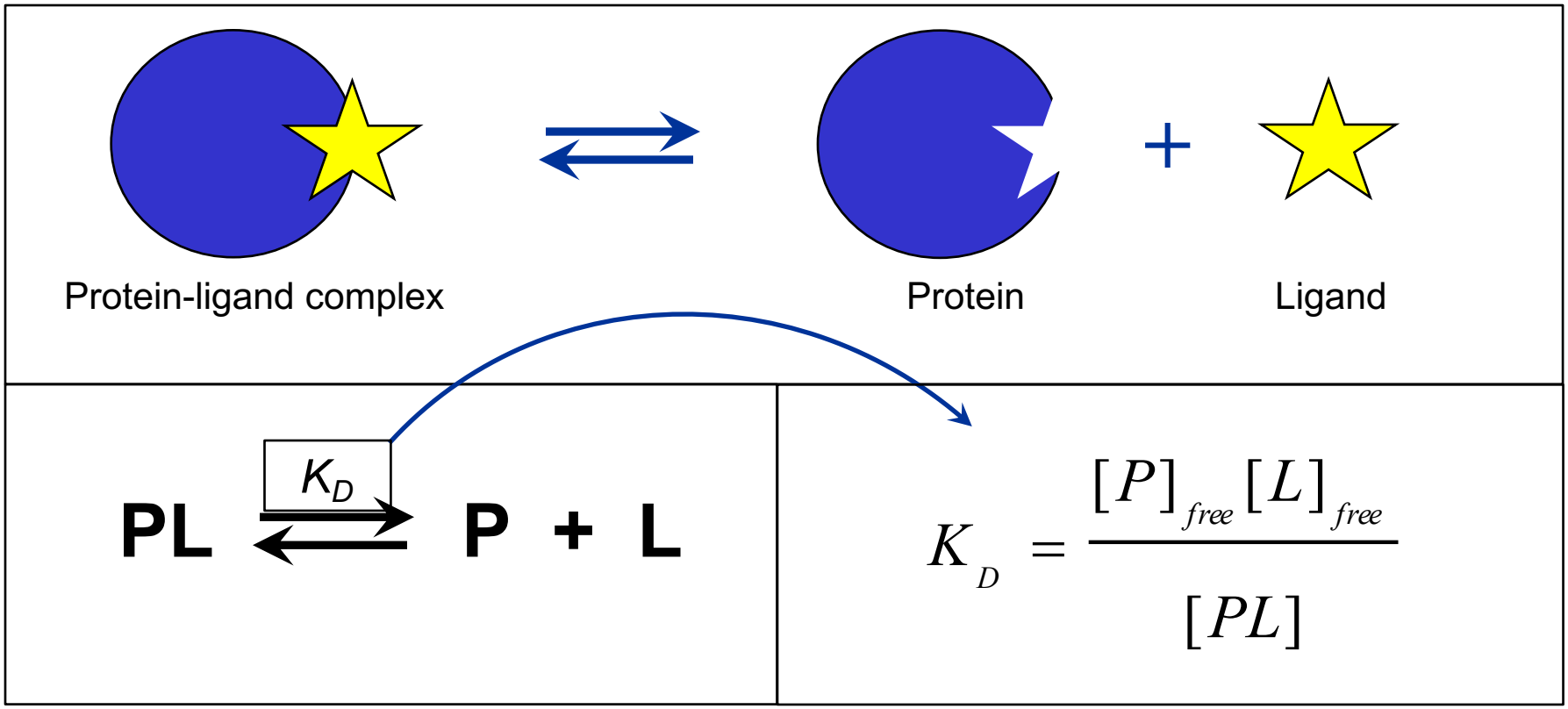
k_{on} and k_{off} are rate constants. k_{on} is the on-rate and it tells us how fast the protein binds the ligand ($M^{-1}s^{-1}$). k_{off} is the off-rate and tells us how fast the ligand dissociates from the protein (s^{-1}).

2.2 The equilibrium and rate constants are mathematically related



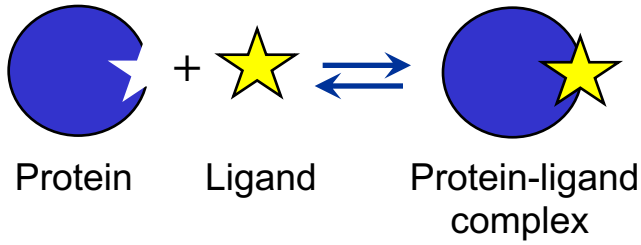
The equilibrium binding affinity depends on the relative values of k_{on} and k_{off}. If the ligand binds fast ($\uparrow k_{on}$) and dissociates slowly ($\downarrow k_{off}$), then it will spend most of its time bound and will have a large K_{eq}.

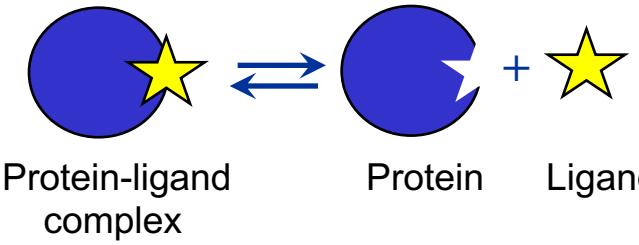
2.2 The dissociation constant, K_D



We typically characterize protein-ligand interactions in terms of the equilibrium constant for the reverse reaction, i.e. the dissociation of the ligand from the protein. This equilibrium constant is called a dissociation constant or K_D and is an intuitive measure of affinity – the strength of binding.

2.2 K_D is the reciprocal of K_{eq}





$$K_{eq} = \frac{[PL]}{[P]_{free}[L]_{free}}$$

$$K_D = \frac{1}{K_{eq}} = \frac{[P]_{free}[L]_{free}}{[PL]}$$

The association equilibrium constant, K_{eq}, has units "M^{-1}" (i.e. 1/molar or liter/mol). **The dissociation constant, K_D, has units of "M" (i.e. molar or mols/liter). Note that $K_{eq} = 1/K_D$.**

Deriving the binding equation for Case I

Now we must define *r* in terms of the things we can measure ($[P]_{total}$ and $[PL]=[L]_{bound}$) and the things that we want to define (K_D):

$$r = \frac{[PL]}{[P]_{free} + [PL]} \quad (1)$$

$$K_D = \frac{[P]_{free}[L]_{free}}{[PL]} \quad (2)$$

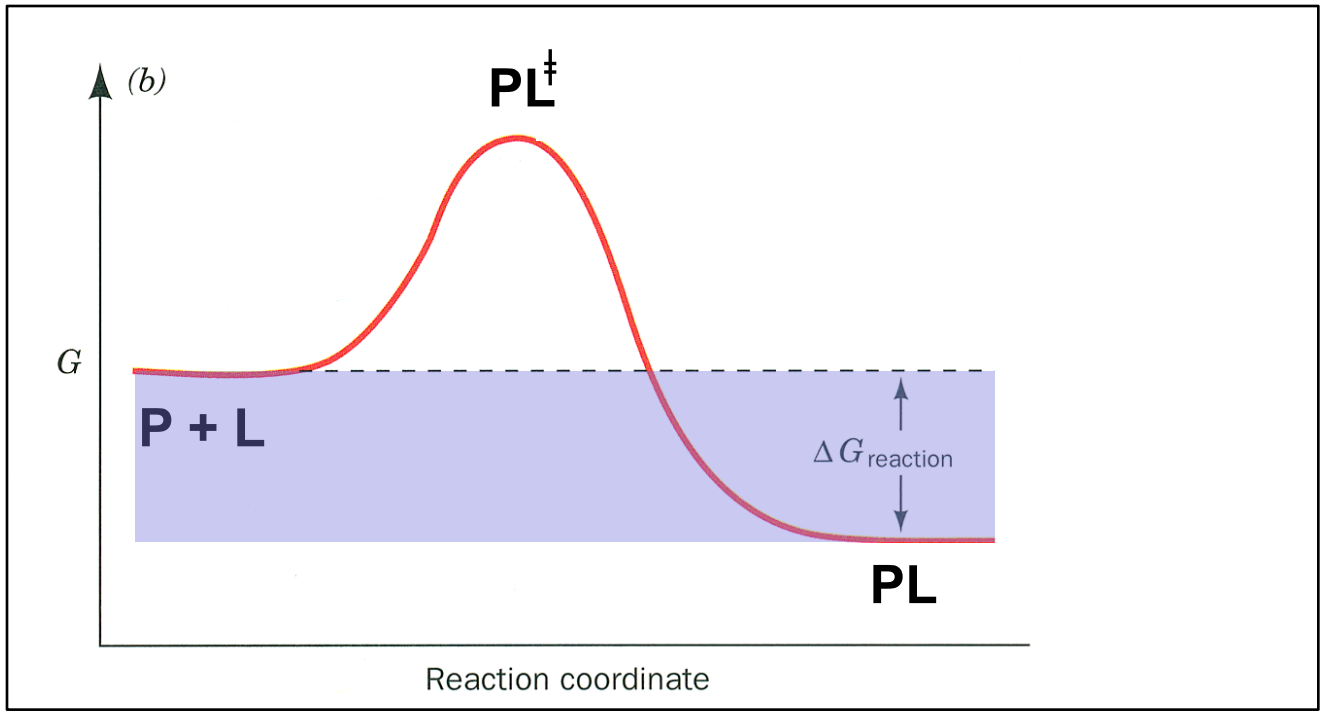
$$r = \frac{[L]_{free}}{K_D + [L]_{free}}$$

$$[PL] = \frac{[P]_{free}[L]_{free}}{K_D}$$

Rearrange equation “2” and solve for [PL], sub [PL] into “1”and rearrange to get our binding equation.

2.2 The reaction coordinate diagram and binding constants

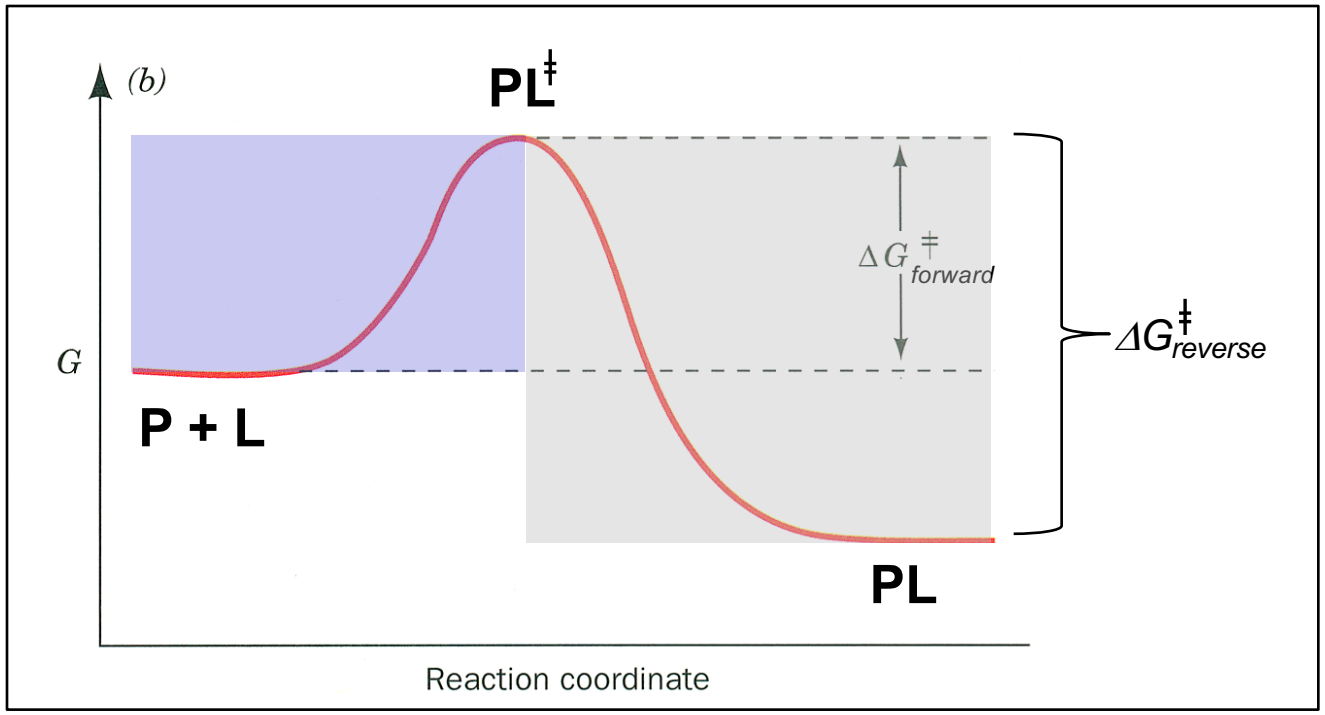
$$P + L \underset{}{\overset{K_{eq}}{\rightleftharpoons}} PL$$



K_{eq}, K_D are related to ΔG_{rxn} - the energy difference between reactants and products (P+L vs PL).

2.2 The reaction coordinate diagram and binding constants

$$P + L \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} PL$$



k_{on} is related to the "activation energy", $\Delta G^{\ddagger}_{forward}$

k_{off} is related to the reverse activation energy, $\Delta G^{\ddagger}_{reverse}$

- i.e. the energy between the reactants and the activation energy barrier ($PL^{\ddagger}$)

2.2 The significance of K_D

$$P + L \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} PL$$

$$K_{eq} = \frac{[PL]}{[P]_{free}[L]_{free}} \qquad K_D = \frac{1}{K_{eq}} = \frac{[P]_{free}[L]_{free}}{[PL]}$$

K_D provides a direct estimate of both binding affinity and biological significance (i.e. the K_D is similar to the concentration of ligands that occurs *in vivo*, then the binding is relevant).

2.2 The significance of K_D

$$P + L \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} PL \qquad K_D = \frac{[P]_{free}[L]_{free}}{[PL]}$$

Let $[L]_{free} = K_D$ $\longrightarrow$ $K_D = \dfrac{[P]_{free} K_D}{[PL]}$

$$\therefore \quad \frac{K_D}{K_D} = 1 = \frac{[P]_{free}}{[PL]}$$

$$\therefore \quad [PL] = [P]_{free}$$

Calculation box 1

When the concentration of free ligands equals K_D, 50% of the protein molecules have ligands bound (50% saturation).

2.2 The significance of K_D

$$P + L \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} PL \qquad K_D = \frac{[P]_{free}[L]_{free}}{[PL]}$$

Let $[L]_{free} = 10K_D \longrightarrow$ $K_D = \dfrac{[P]_{free}10K_D}{[PL]}$

$$\therefore \frac{K_D}{10K_D} = \frac{1}{10} = \frac{[P]_{free}}{[PL]}$$

$$\therefore [PL] = 10[P]_{free}$$

Calculation box 2

When the concentration of free ligands equals 10x K_D, 10 out of 11 of the protein molecules have ligands bound (~90% saturation).

2.2 We can intuitively estimate % saturation from K_D

What will be the percentage of P bound to L when:

$[L]_{free} = K_D$ ⟶ *50% of P will be bound to L*

$[L]_{free} = 10K_D$ ⟶ *~90% of P will be bound to L*

$[L]_{free} = 100K_D$ ⟶ *~99% of P will be bound to L*

$[L]_{free} = 0.1K_D$ ⟶ *~10% of P will be bound to L*

$[L]_{free} = 0.01K_D$ ⟶ *~1% of P will be bound to L*

2.2 K_D as a measure of affinity

Protein A and B have K_Ds for binding the hormone epinephrine of 100 nM and 100 μM, respectively. Which has the greater affinity for epinephrine?

Ans: Protein A will be at 50% saturation at 100 nM concentrations, well below the concentration required for 50% saturation of Protein B (100 μM). In fact, Protein A will be at 90% and 99% saturation at 1 μM and 10 μM epineprhine. In contrast, Protein B at the same concentrations will be at only 1% and 10% saturation at 1 μM and 10 μM epinephrine, respectively.

Clearly, Protein A thus has a much higher affinity for epinephrine

K_D is a direct measure of binding affinity! The larger the K_D the lower the affinity – i.e. the weaker the binding.

2.2 K_D tells us about biological relevance

A receptor on the surface of a blood cancer cell binds a drug with a K_D of 100 nM. When 90% of the receptors are activated, cell death is triggered and the cancer cells die.

1) What concentration must the drug achieve in blood to be fully effective?

Ans: To activate 90% of the receptors, we must have 90% saturation, which means we need to be at 10x K_D or 1 μM.

2) If the drug is cleared from circulation quickly so that it never achieves a concentration above 500 nM, will it be effective?

Ans: If the drug in the blood never achieves concentrations above 500 nM, then we will never achieve the required 1 μM concentrations, so the drug will not be effective.

2.2 K_D tells us about biological relevance

In your fourth year project, you identify a new protein receptor located on the surface of cells that binds hormone X. Activation of this protein with hormone X triggers cell proliferation. You propose that this protein is a receptor for hormone X – which implies new mechanisms to activate cell signaling pathways possibly leading to cancer. You next measure that the protein binds hormone X with a K_D of 300 μM. Maximal endogenous levels of hormone X are ≤ 500 nM.

1) At endogenous levels, will hormone X activate this receptor?

Ans: at a hormone concentration equal to K_D, 50% of the receptors binding sites will have hormone bounds, so 50% of the receptors will be activated. At [hormone] = 30 μM, you will get10% activation; at 3 μM - 1% activation; 300 nM – 0.1% activation. At maximal endogenous hormone levels, there will be almost not activation of this receptor, so this does not appear to be a natural receptor for the hormone – better luck next time…

2.3 Case I: The general case with multiple identical sites

$$r = \frac{[L]_{free}}{K_D + [L]_{free}}$$

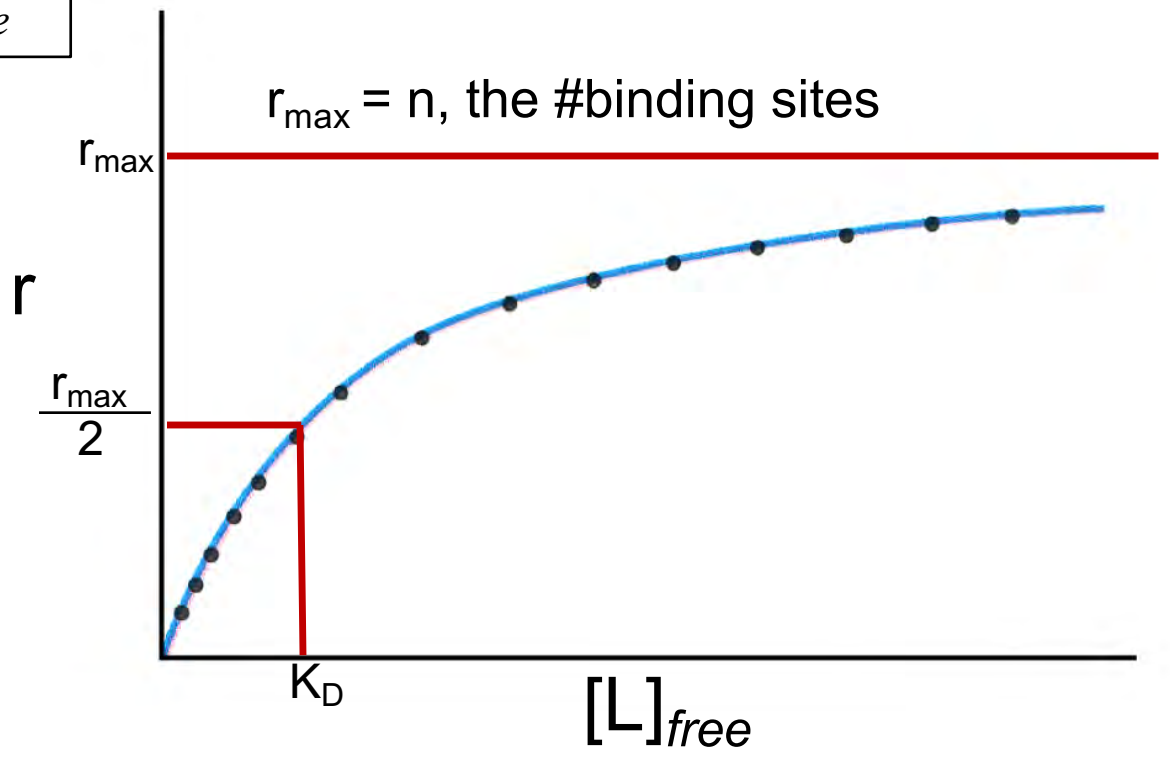
(one binding site)

$$r = \frac{n[L]_{free}}{K_D + [L]_{free}}$$

(*n* binding sites)

2.3 Case I: The general case with multiple identical sites

$$r = \frac{n[L]_{free}}{K_D + [L]_{free}}$$

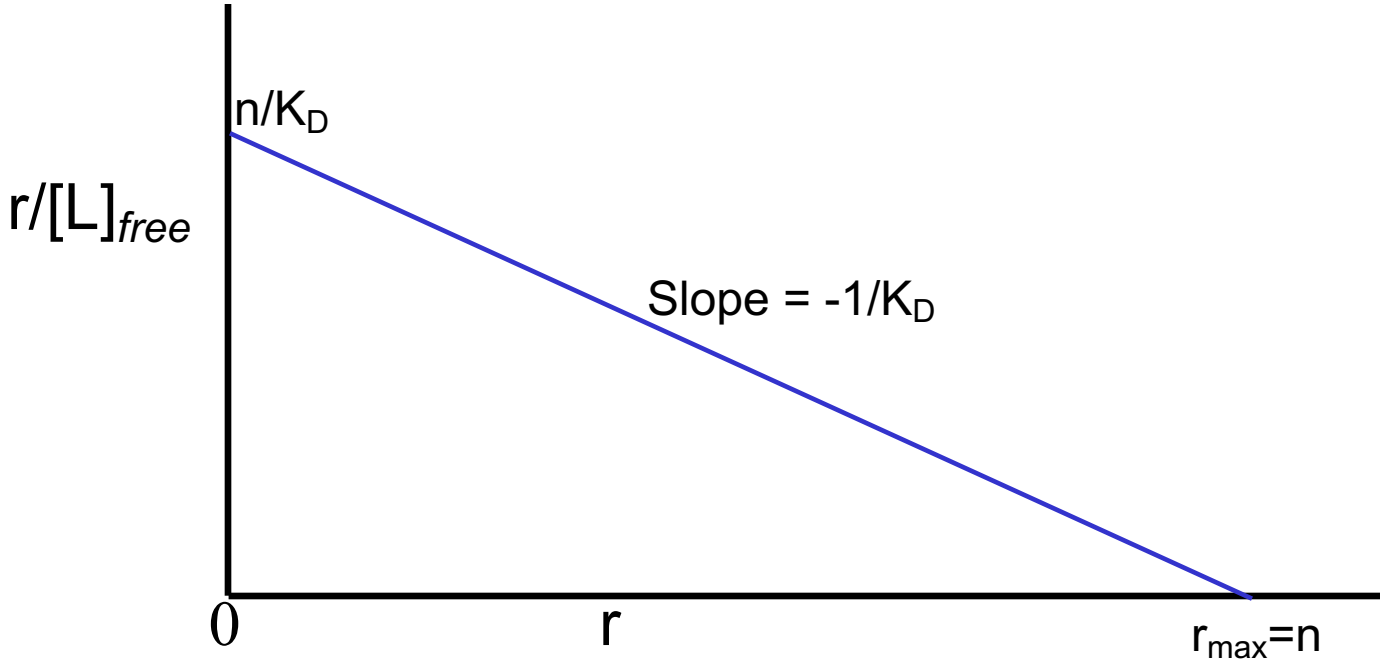


Plots of *r* vs $[L]_{free}$ have a hyperbolic shape and plateau at r_{max}, the number of binding sites.
The ligand concentration that gives $r = r_{max}/2$ is: $[L]_{free} = K_D$.

The binding equation can be rearranged to plot the data in different ways:

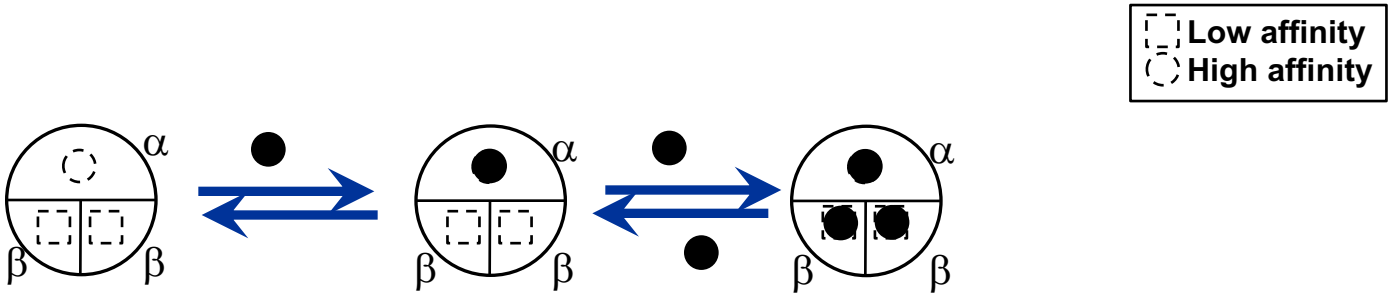
2.3 Scatchard Plot

$$\frac{r}{[L]_{free}} = \frac{n}{K_D} - \frac{r}{K_D}$$



The Scatchard plot allow us to calculate r_{max}. A linear curve is indicative binding to one or multiple sites all with the same affinity. Deviations from linearity suggest either more than one type (affinity) of site or cooperative binding

2.4 Case I: multiple non-identical sites

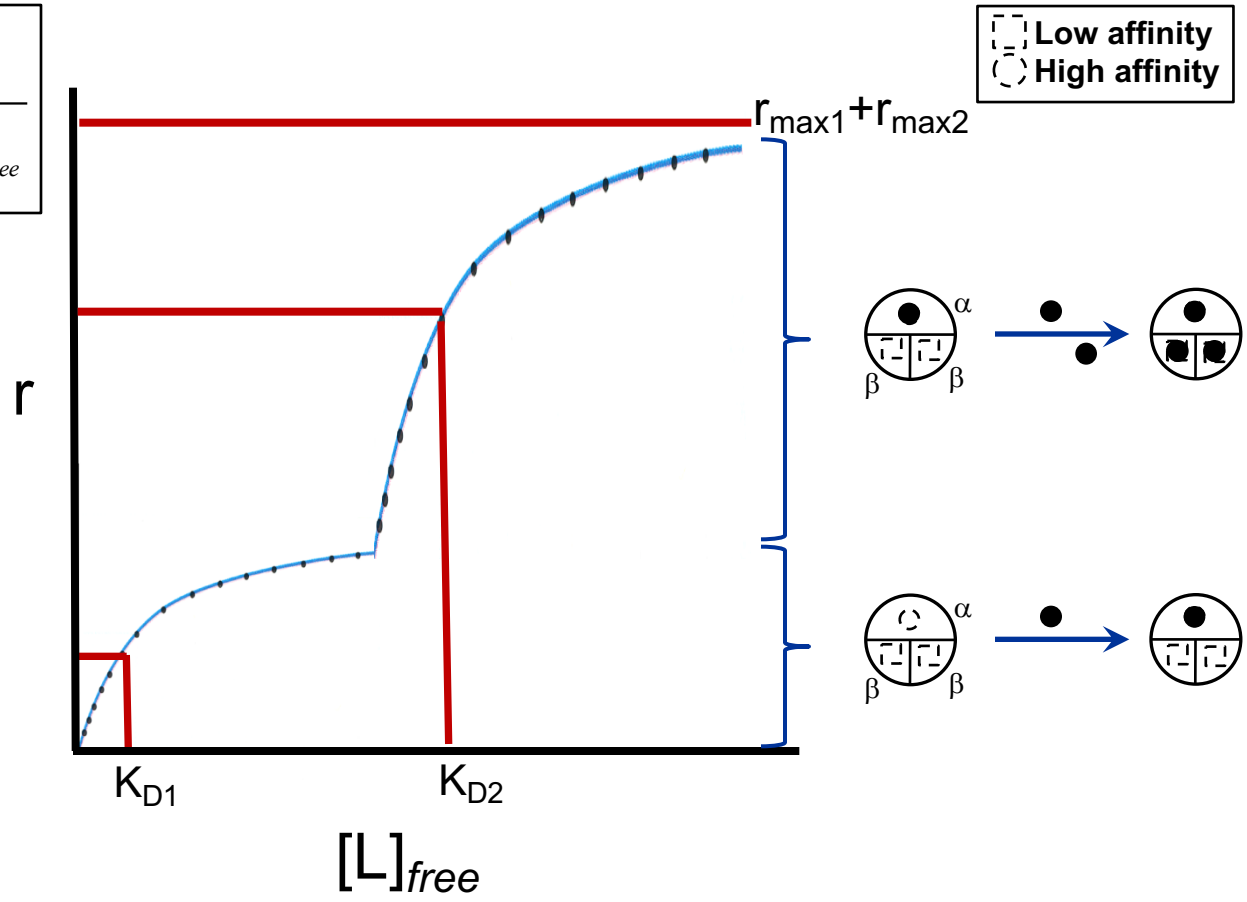


$$r = \frac{[L]_{free}}{K_{D1} + [L]_{free}} + \frac{2[L]_{free}}{K_{D2} + [L]_{free}}$$

In this hypothetical example, the protein consists of one α and two β subunits. The binding site on the α subunit has a relatively high affinity (low K_{D1}) compared to the two binding sites on the two β subunits (high K_{D2}). Binding occurs independently binding to α does not affect binding to β. If $K_{D1} << K_{D2}$, then the site on α saturates before binding occurs to the sites on β.

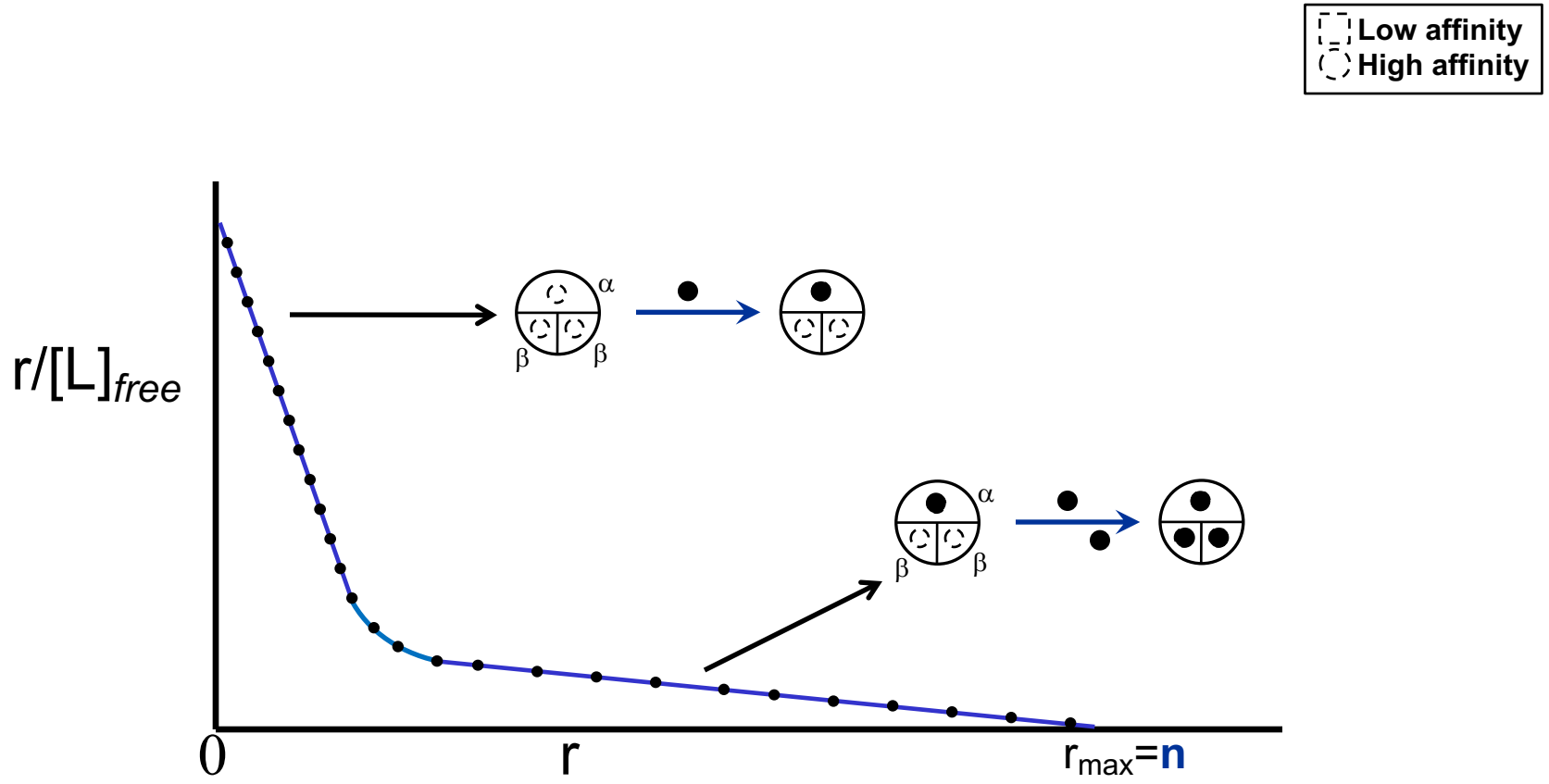
2.4 Case I: multiple non-identical sites

$$r = \frac{[L]_{free}}{K_{D1} + [L]_{free}} + \frac{2[L]_{free}}{K_{D2} + [L]_{free}}$$



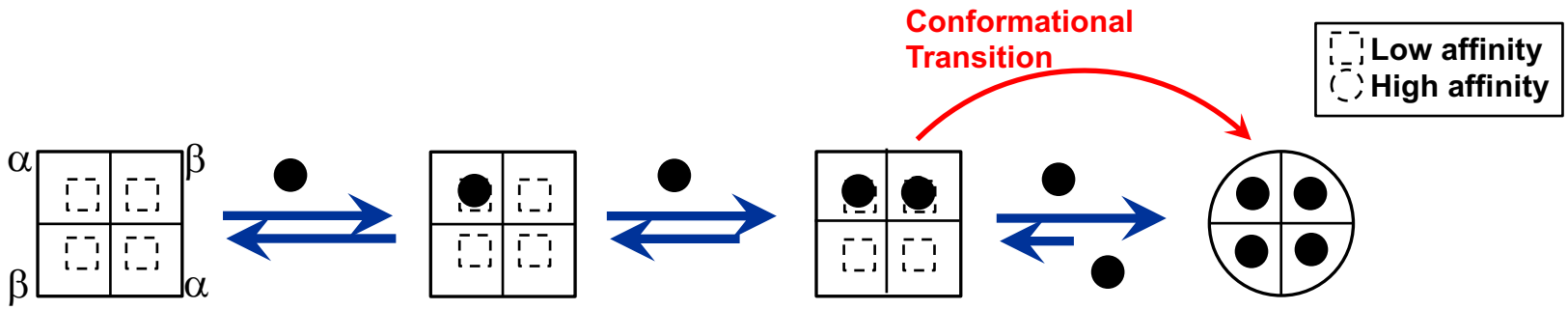
To clearly distinguish binding event #1 from #2, K_{D1} must be << than K_{D2} (~1000 fold). To see data points over a >1000-fold concentration range, you usually plot r versus $\log[L]_{free}$. *In the semilog plot, all curves become sigmoidal.*

2.4 Case I: multiple non-identical sites – Scatchard plot



Two distinct binding events are easily seen in a Scatchard plot, as the line deviates from linearity giving a two slope curve

2.5 Case II: cooperative binding (allosteric)



Cooperative binding means that the binding of the first molecule of ligand influences (changes the affinity) of binding for other ligands (and so on):

Positive – binding of ligand X increases binding of X or Y ($\downarrow K_D$)

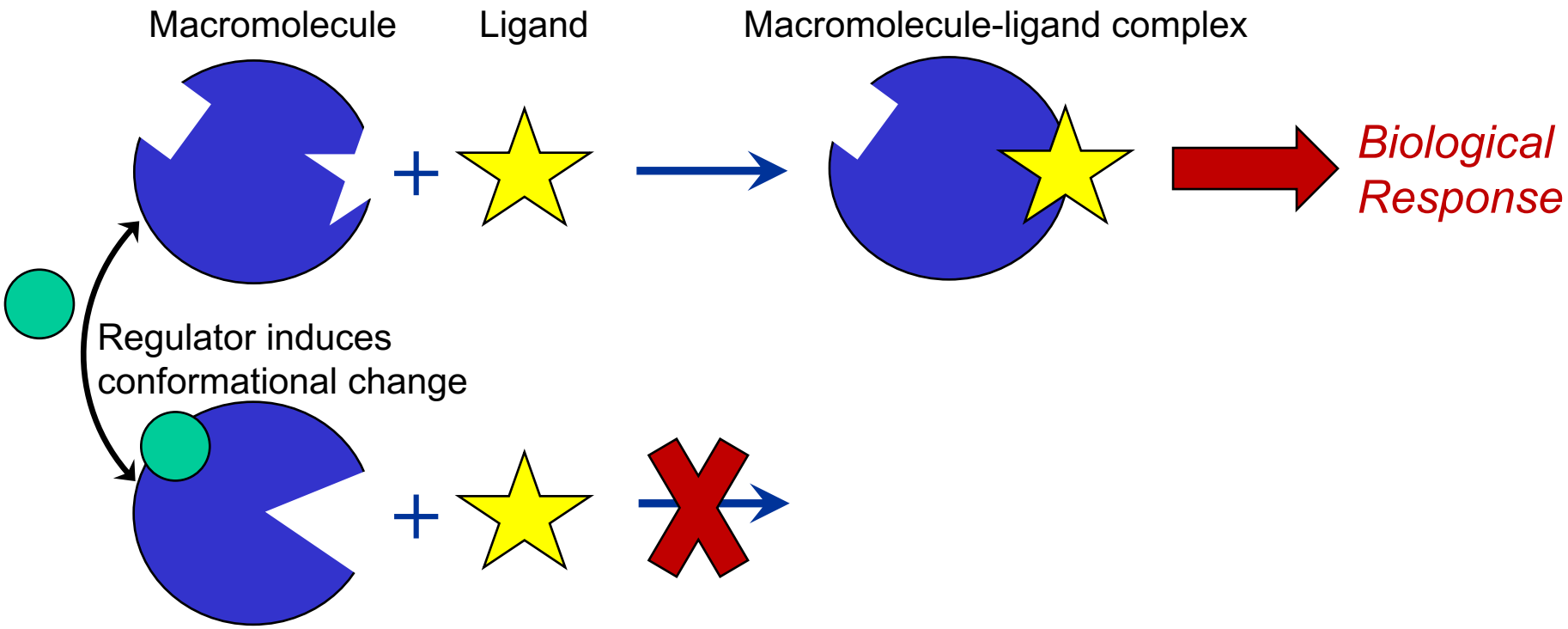
Negative - binding of ligand X decreases binding of X or Y ($\uparrow K_D$)

Homotropic - binding of ligand X influences binding of other molecules of X

Heterotropic – binding of ligand X influences binding of ligand Y

The diagram is an example of positive homotropic cooperativity. Heterotropic cooperativity is often called "allostery"

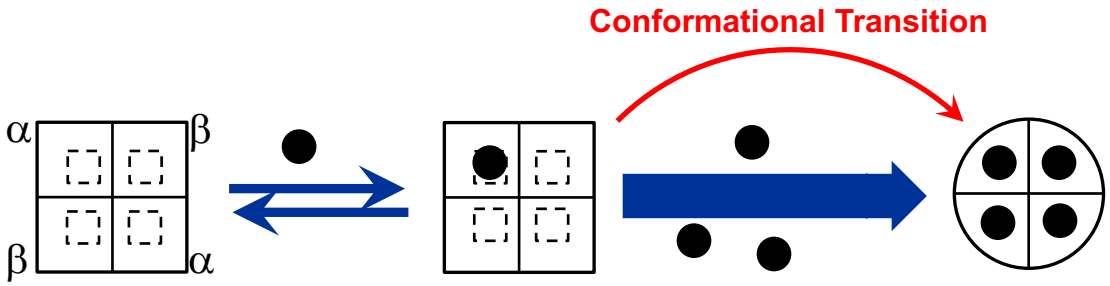
2.5 Case II: cooperative binding (allosteric)



The binding of the green round molecule stabilizes a different conformation or shape of the protein so that the protein's interactions with the yellow ligand is altered.

This is an example of classic protein allostery, where binding to one site influences binding of a different molecule at another site. The case shown is for negative cooperativity (allostery) because the binding of the green ligand diminishes the binding of the yellow ligand.

2.5 If we assume infinite cooperativity…



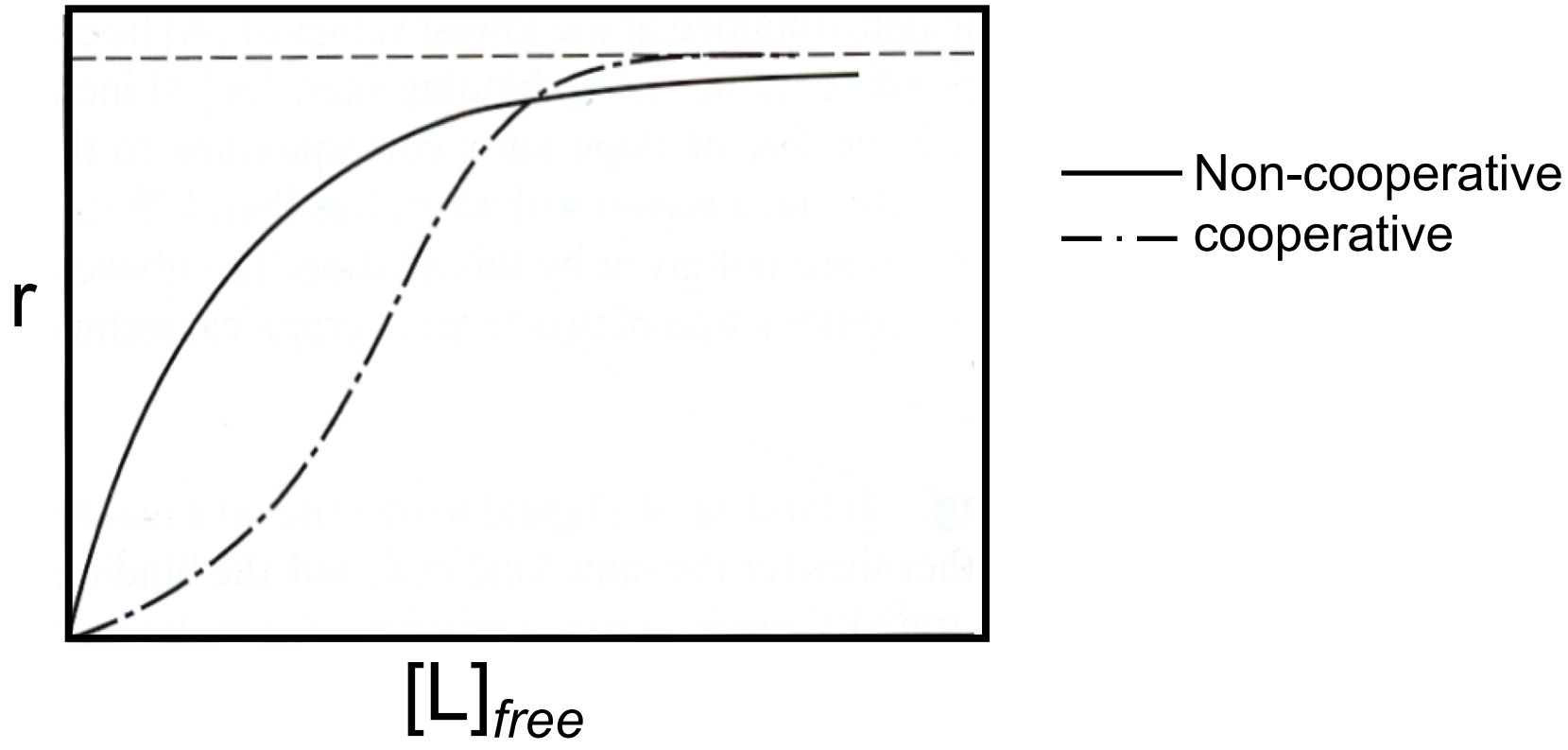
…where cooperativity is so strong that binding to one site leads to change in structure at all other sites so that they immediately saturate with ligand.

$$P + nL \rightleftharpoons PL_n$$

$$r = \frac{n[L]_{free}^{n}}{K_D^{n} + [L]_{free}^{n}}$$

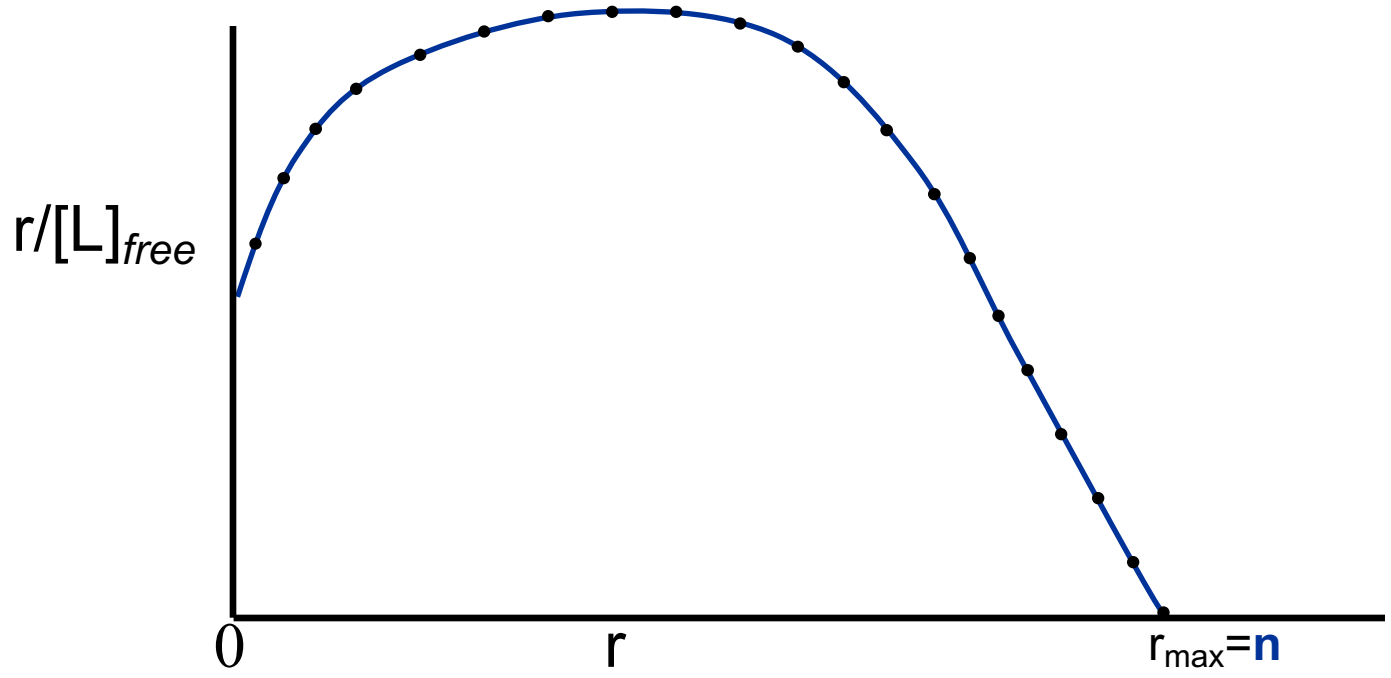
where K_D is the [L] that leads to 50% saturation.

2.5 Cooperative versus noncooperative binding



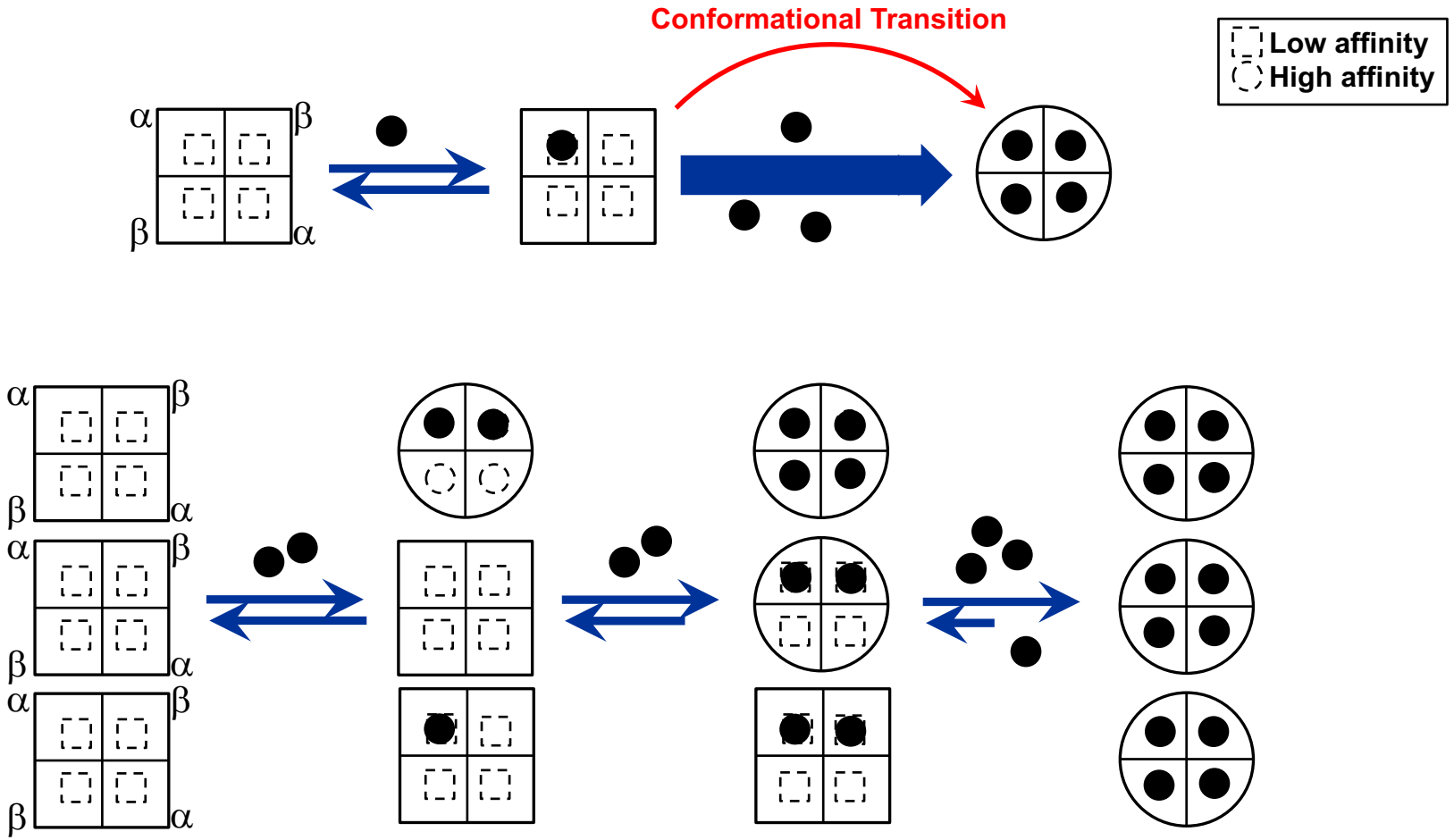
Sigmoidal shape results because binding of first ligand is weak. After the first ligand is bound, the binding of other ligands becomes stronger so these sites fill quickly (NB both types of binding give a sigmoidal curve in plots of r vs log[L]).

2.5 Case II: cooperative binding Scatchard plot



A Scatchard plot for cooperative binding gives a distorted shape, which is difficult to analyze.

2.5 Infinite versus “partial” cooperative binding



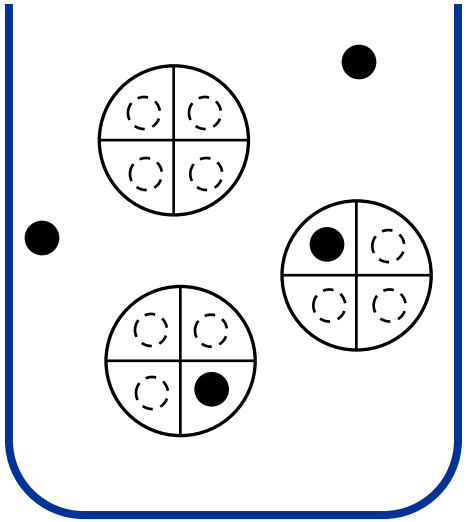
The infinite case of cooperative binding never occurs in real life. In reality, binding at one site increases the binding affinity for the second, binding at the second site increases binding affinity for the third, and so on. Increased binding eventually overcomes the energy barrier between the low and high affinity states, so you get a conformational transition.

2.5 Fractional saturation

The fractional saturation tells us the percentage of sites that are filled.

$$Y = r/n$$

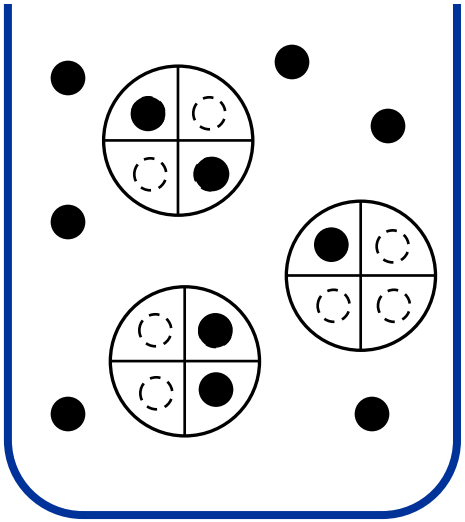
Tube A



r = 2/3= 0.67

Y = 2/12= 0.17

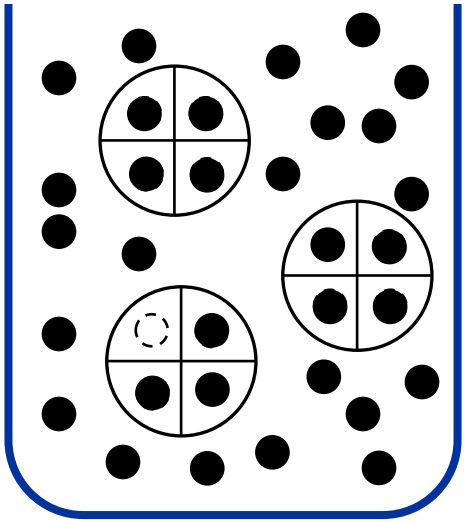
Tube B



r = 5/3 = 1.67

Y = 5/12 = 0.42

Tube C



r = 11/3 = 3.67

Y = 11/12 = 0.92

$0 < r < r_{max}$
$(0 < r < 4)$

$0 < Y < 1$

2.5 “Degree” of cooperativity

Traditionally, we define the Hill equation, which is the ratio of sites filled vs sites not filled:

$$\frac{r}{n-r} = \frac{Y}{1-Y} = \frac{\text{Fraction of sites filled}}{\text{Fraction of sites not filled}}$$

Sub our binding curve into the above equation, solve and take the log to give*:

$$r = \frac{n[L]_{free}^{n}}{K_D{}^{n} + [L]_{free}^{n}}$$

Hill equation:

(See Lecture 13 or Slide 56)

$$\log\frac{Y}{1-Y} = n\log[L]_{free} - \log K_D{}^{n}$$

Hill equation

$$\text{(1)}\quad \frac{Y}{1-Y} = \frac{r}{n-r} \qquad r = \frac{n[L]_{free}^{n_H}}{K_D^{n_H} + [L]_{free}^{n_H}}\quad \text{(2)}$$

Sub equation 2 into equation 1 to give (for simplicity the subscript for n_H is omitted):

$$\frac{Y}{1-Y} = \frac{\dfrac{n[L]_{free}^{n}}{K_D^{n} + [L]_{free}^{n}}}{n - \dfrac{n[L]_{free}^{n}}{K_D^{n} + [L]_{free}^{n}}} = \frac{\dfrac{n[L]_{free}^{n}}{K_D^{n} + [L]_{free}^{n}}}{\dfrac{n K_D^{n} + n[L]_{free}^{n} - n[L]_{free}^{n}}{K_D^{n} + [L]_{free}^{n}}} = \frac{[L]_{free}^{n}}{K_D^{n}}$$

Take the log of both sides:

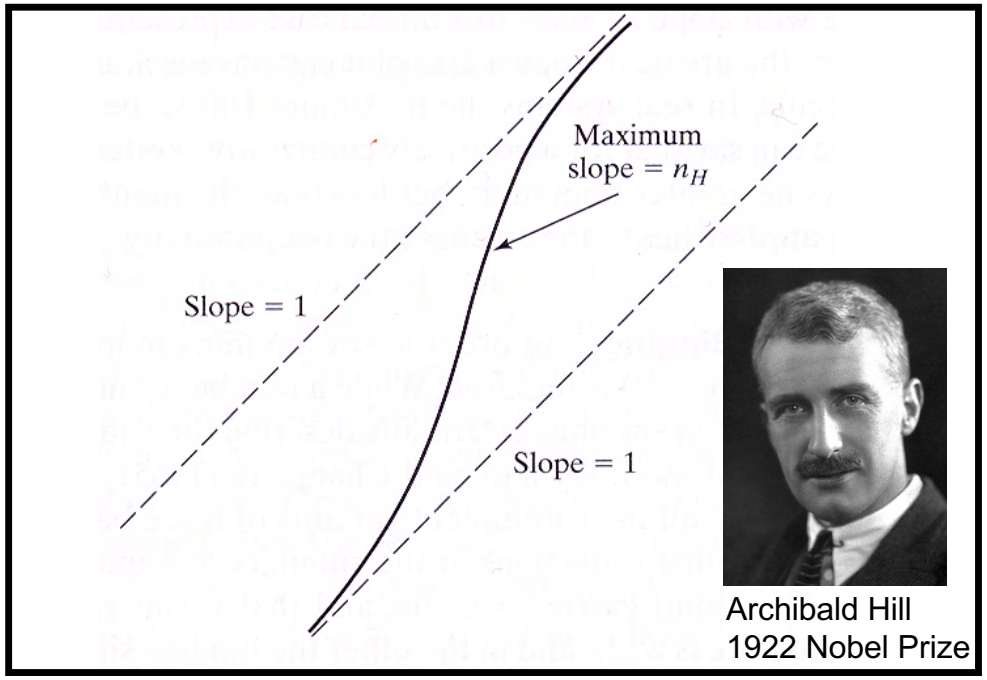
$$\log \frac{Y}{1-Y} = n \log[L]_{free} - \log K_D^{n}$$

The Hill plot defines the Hill coefficient

At low $[L]_{free}$, the binding is weak and the binding curve has a slope=1. As $[L]_{free}$ increases and L binds, the curve shifts over to another curve with a slope of 1 which is characteristic of high affinity binding. The max slope of the intervening curve is defined as n_H, the Hill coefficient. The Hill coefficient tells us the degree of cooperativity

$$\log \frac{Y}{1-Y} = n \log[L]_{free} - \log K_D^n$$

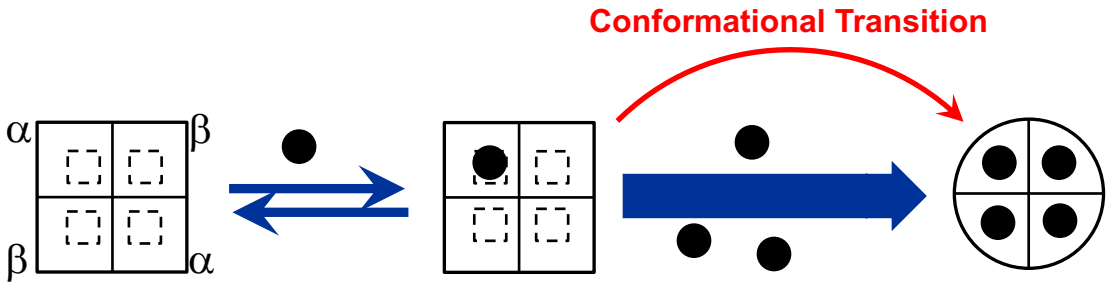
$$\log \frac{Y}{1-Y}$$



Archibald Hill
1922 Nobel Prize

$$\log [L]_{free}$$

The degree of cooperativity



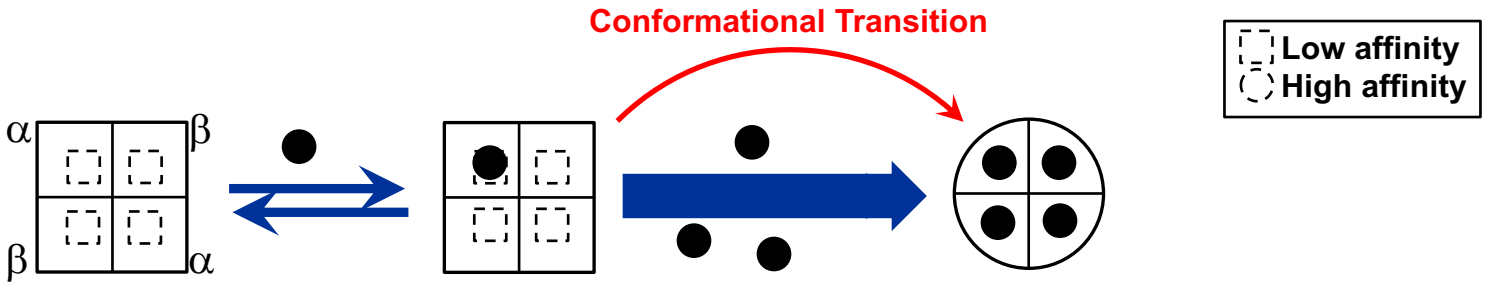
In this extreme case of infinite cooperativity, $n_H = n$. In most cases:

$$1 \leq n_H \leq n.$$

The closer the value of n_H to n *(the number of binding sites),* the greater the degree of cooperativity! Cooperative binding is defined by the following curve:

$$r = \frac{n[L]_{free}^{n_H}}{K_D^{n_H} + [L]_{free}^{n_H}}$$

The binding affinity K_D



Note that the formula for cooperative binding is often written as follows:

$$r = \frac{n[L]_{free}^{n_H}}{K_D^{n_H} + [L]_{free}^{n_H}}$$

instead of as:

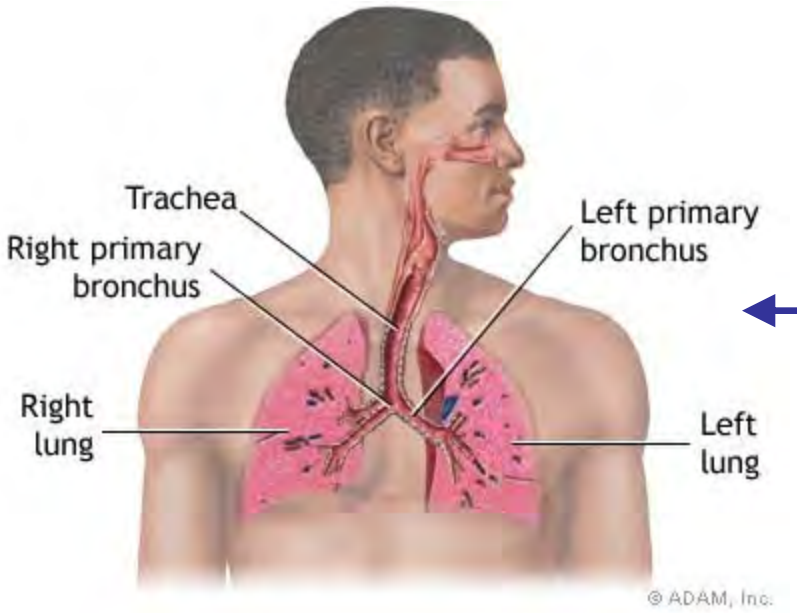
$$r = \frac{n[L]_{free}^{n_H}}{K_D^{n_H} + [L]_{free}^{n_H}}$$

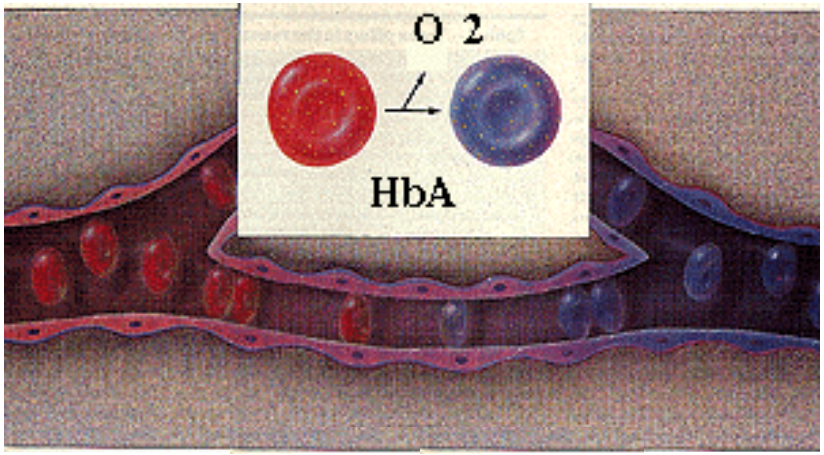
On the right, in this formulation, when $[L]_{free} = K_D$, then $r = n/2$ – i.e. you get 50% saturation.

So this fits with our operational definition that K_D is the concentration of ligand that gives 50% saturation. This interpretation of K_D is standard in the literature.

The more common derivation on the left requires that $[L]_{free} = K_D$ to get 50% saturation.

Oxygen transport in mammals





All multicellular organisms have developed systems for transporting oxygen to their cells for respiration. Intrinsic to these systems are the oxygen binding proteins, Hemoglobin (Hb) and Myoglobin (Mb). *These are fine-tuned oxygen-binding molecular machines* that allow mammals to optimize oxygen delivery to tissues under widely varying conditions.

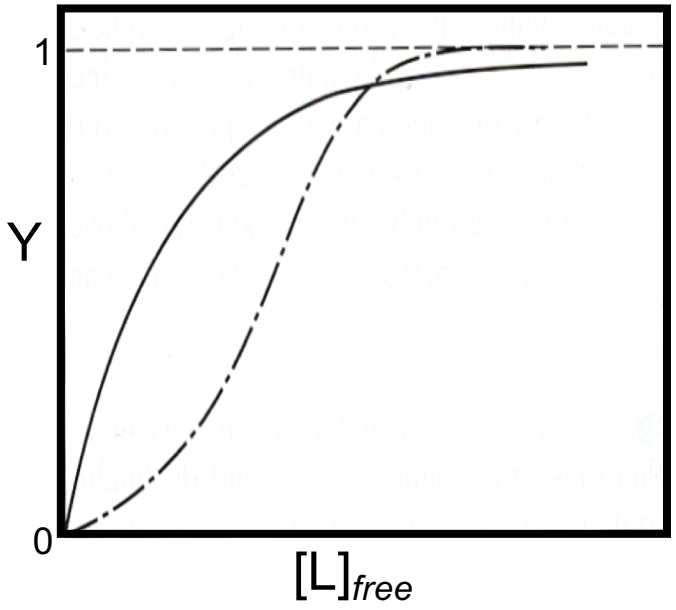
Mb and Hb classic examples of independent vs cooperative binding

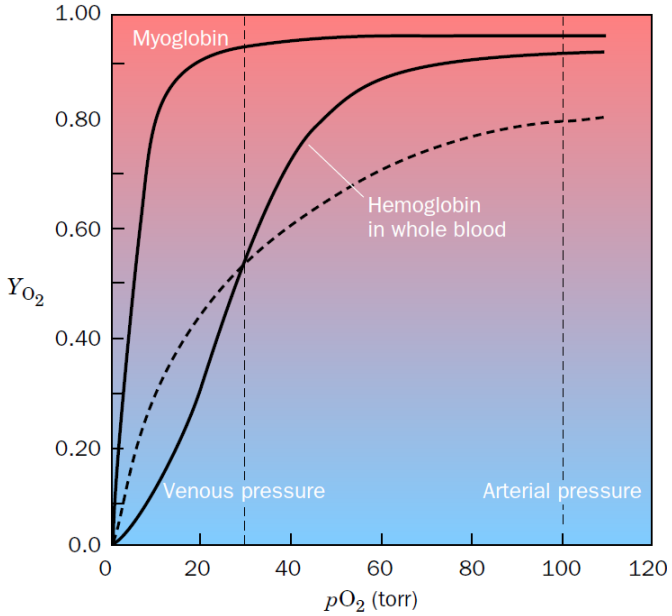
$$r = \frac{n[L]_{free}}{K_D + [L]_{free}}$$

——— Non-cooperative

$$r = \frac{n[L]^{n_H}_{free}}{K_D^{n_H} + [L]^{n_H}_{free}}$$

— · — cooperative

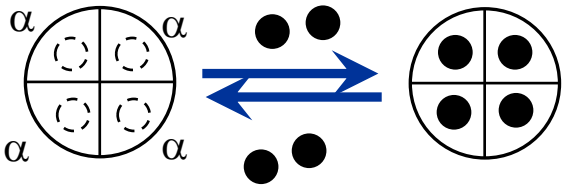




Our next step is to use the developed equations for independent and cooperative binding to understand the O_2 binding properties of Hb and Mb.

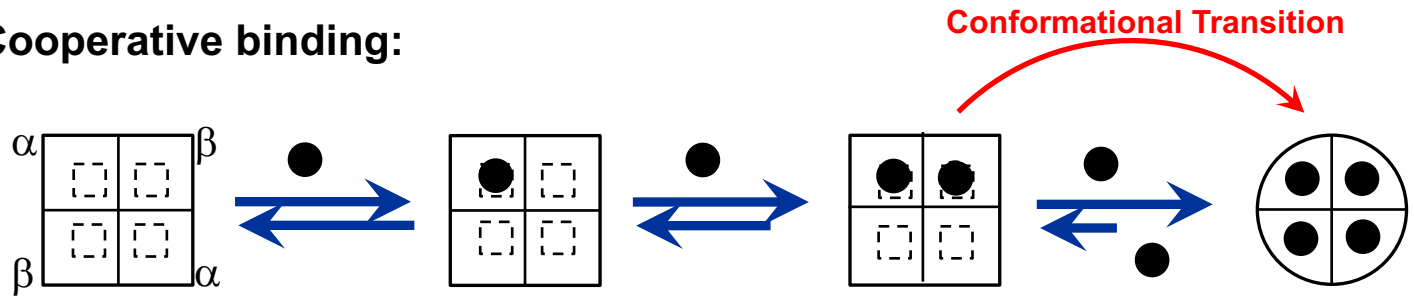
Mb and Hb classic examples of independent vs cooperative binding

Case 1) Independent binding:



The binding of one molecule of ligand to one site on the protein has no effect on the binding of other molecules of the ligand to the same protein – the binding sites are independent!

Case 2) Cooperative binding:



The binding of one molecule of ligand to one site on the protein changes the binding of other molecules to the same protein. In this example of positive cooperativity, the binding of one molecule increases the affinity (i.e. the strength) for binding other molecules.

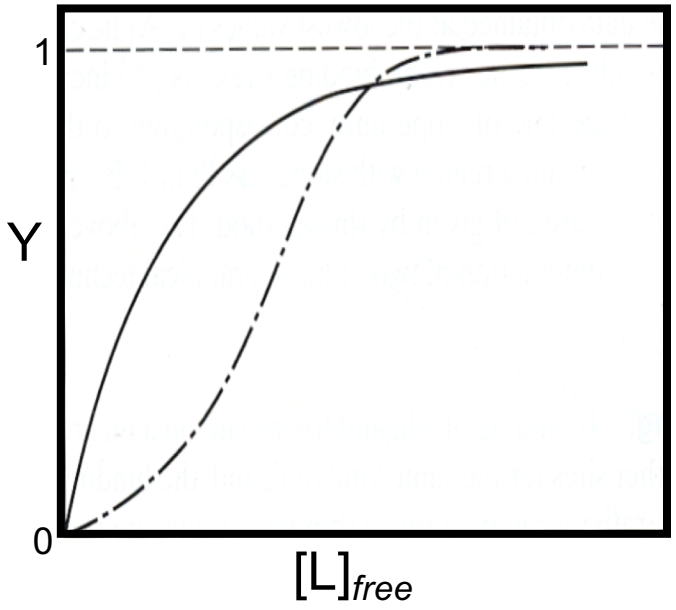
Mb and Hb classic examples of independent vs cooperative binding

$$r = \frac{n[L]_{free}}{K_D + [L]_{free}}$$

—— Non-cooperative

$$r = \frac{n[L]_{free}^{n_H}}{K_D^{n_H} + [L]_{free}^{n_H}}$$

—·—· cooperative



- Independent binding gives a rectangular hyperbola binding curve
- Cooperative binding gives a sigmoidal binding curve.
  - *Y* is the fractional (i.e. %) saturation
  - K_D is the [L] that gives 50% saturation of binding
  - n_H is the Hill coefficient and tells us the degree of cooperativity

Mb and Hb classic examples of independent vs cooperative binding

$$r = \frac{n[L]_{free}}{K_D + [L]_{free}}$$

—— Non-cooperative

$$r = \frac{n[L]_{free}^{n_H}}{K_D^{n_H} + [L]_{free}^{n_H}}$$

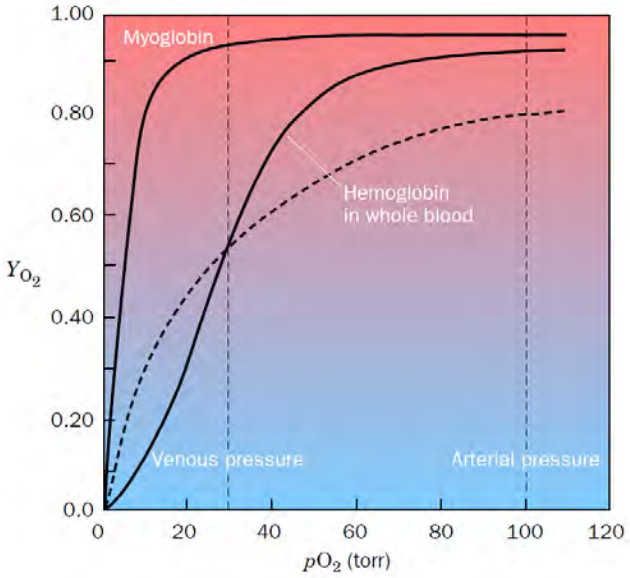
—·—· cooperative

Objectives today:

1) Use these binding equations to assess the O_2 delivery capabilities of Hb and Mb.

2) Examine how the Hb structure has evolved to facilitate cooperative binding.

Effective versus the "real" K_D



The K_D is the concentration of ligand that leads to 50% saturation. For O_2 binding, we traditionally measure the [O2] using the partial pressure, so historically *p50* is used instead of K_D.

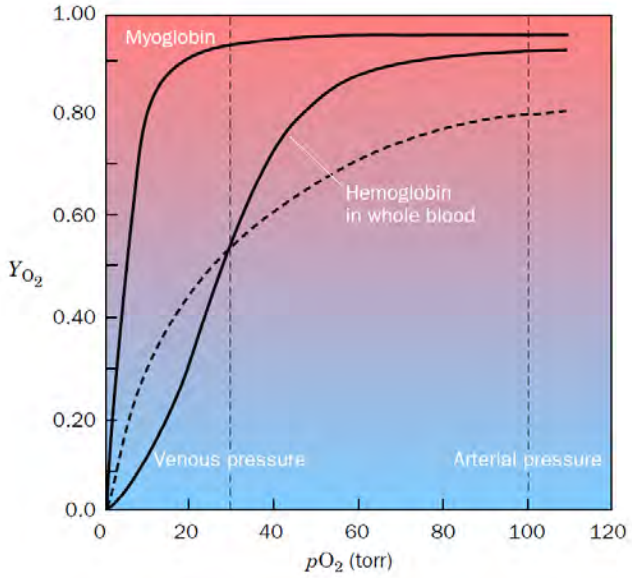
p50 is the partial pressure of O_2 that leads to 50% saturation.

Note that K_D/*p50* is a "constant" that reflects the structure of the binding site and its affinity for the ligand.

When we experimentally measure a K_D/p50, however, the measured value sometimes reflects complex processes – particularly when we are measuring the K_D/p50 for an allosteric protein, such as Hb.

With Hb, the measured K_D/p50 typically reflects an average of the K_D values for two different state, R and T. The measured K_D is not a constant, so we typically refer to the measured value as an "effective K_D or p50 value. The *"effective" moniker signifies that the measured value does not directly reflect the fundamental binding affinity of Hb.*

Effective versus the "real" K_D



p50 for Mb = 2.8 torr
effective p50 for Hb = 26 torr

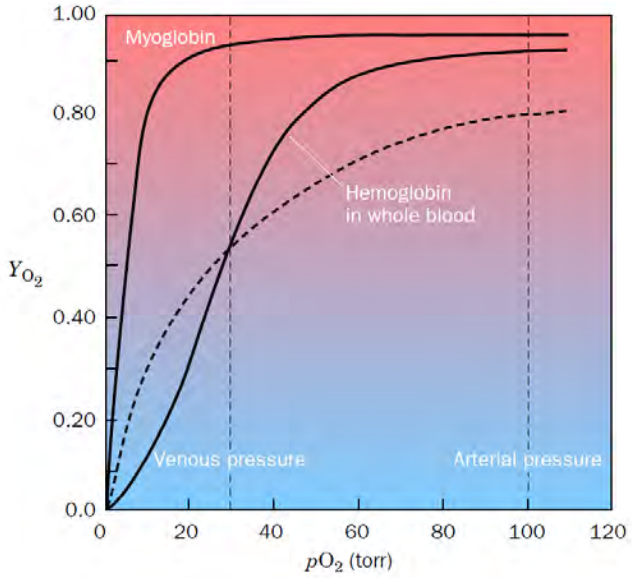
Venous pO_2 = 30 torr
Arterial pO_2 = 100 torr

$p50 = K_D$

O_2 binding to Mb follows a rectangular hyperbola and is non-cooperative. O_2 binding to Hb follows a sigmoidal curve and is cooperative! From a Hill plot, we can show that n_H = 2.8-3.0 (n=4 sites), so Hb is strongly cooperative!

Question 1: Is cooperativity important for oxygen delivery? To answer this question, lets first compare the relative abilities of Mb and Hb to deliver O_2.

How much O_2 can Mb deliver to peripheral tissues?



p50 for Mb = 2.8 torr (*n*=1)
effective p50 for Hb = 26 torr (*n*=4)

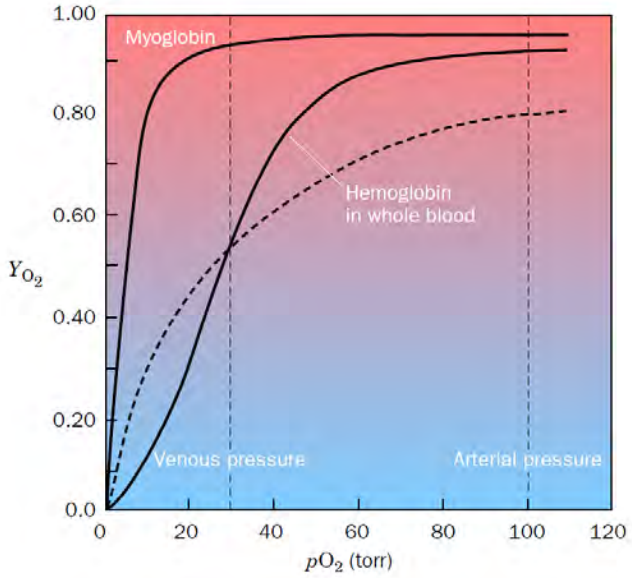
Venous pO_2 = 30 torr
Arterial pO_2 = 100 torr

$$r = Y_{O_2} = \frac{n[L]_{free}}{K_D + [L]_{free}} = \frac{pO_2}{p50 + pO_2}$$

At pO_2 =100 torr Y_{O2} = 0.95
At pO_2 = 30 torr Y_{O2} = 0.93

$\therefore$ **2%** of binding capacity is delivered (arterial - venous)

How much O_2 can Hb deliver to peripheral tissues?



p50 for Mb = 2.8 torr (*n*=1)
effective p50 for Hb = 26 torr (*n*=4)

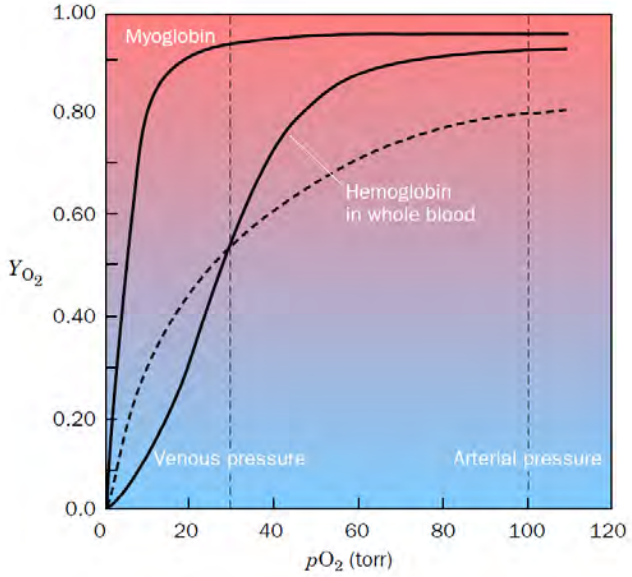
Venous pO_2 = 30 torr
Arterial pO_2 = 100 torr

$$Y_{O_2} = \frac{[L]_{free}^{n_H}}{K_D^{n_H} + [L]_{free}^{n_H}} = \frac{(pO_2)^{n_H}}{(p50)^{n_H} + (pO_2)^{n_H}}$$

At pO_2 =100 torr Y_{O2} = 0.93
At pO_2 = 30 torr Y_{O2} = 0.55

$\therefore$ **38%** of binding capacity is delivered (arterial - venous)

How much O_2 could Mb deliver if it had the same *p50* as Hb? (dashed line)



p50 for Mb = 2.8 torr (*n*=1)
effective p50 for Hb = 26 torr (*n*=4)

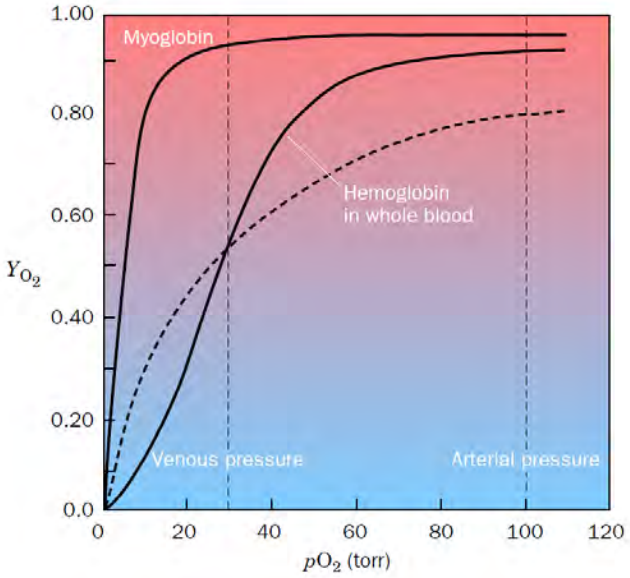
Venous pO_2 = 30 torr
Arterial pO_2 = 100 torr

$$Y_{O_2} = \frac{pO_2}{p50 + pO_2}$$

At pO_2 =100 torr Y_{O2} = 0.79
At pO_2 = 30 torr Y_{O2} = 0.54

$\therefore$ **25%** of binding capacity would be delivered

How much O_2 could Mb deliver if it had the same *p50* as Hb?



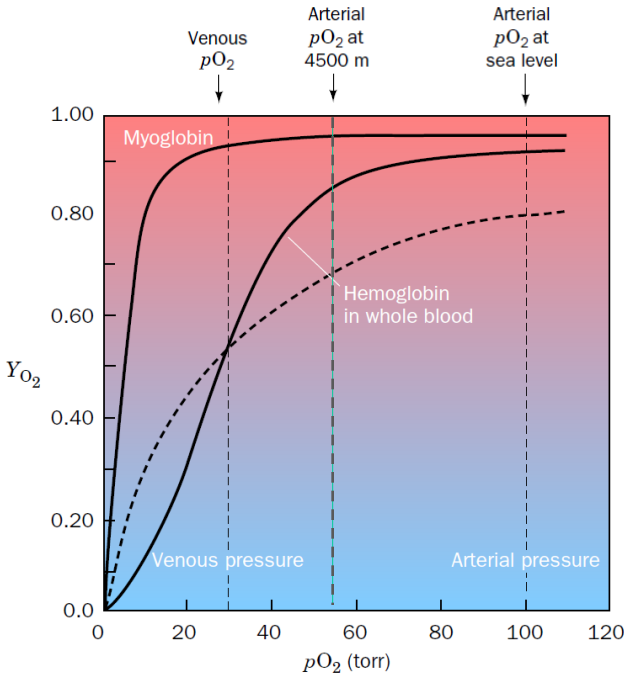
p50 for Mb = 2.8 torr (*n*=1)
effective p50 for Hb = 26 torr (*n*=4)

Venous pO_2 = 30 torr
Arterial pO_2 = 100 torr

Even with identical p50 values, Hb is much better adapted for delivering O_2 to peripheral tissues than Mb. This is because cooperative binding curves have a steeper response to changes in [ligand], and thus go **from low to high saturation over a smaller concentration range**.

The greater O_2 delivery capacity under normal physiological conditions is due to the cooperativity of binding! But there are additional advantages to cooperative binding…

How much O_2 is delivered at high altitude (4500 m)



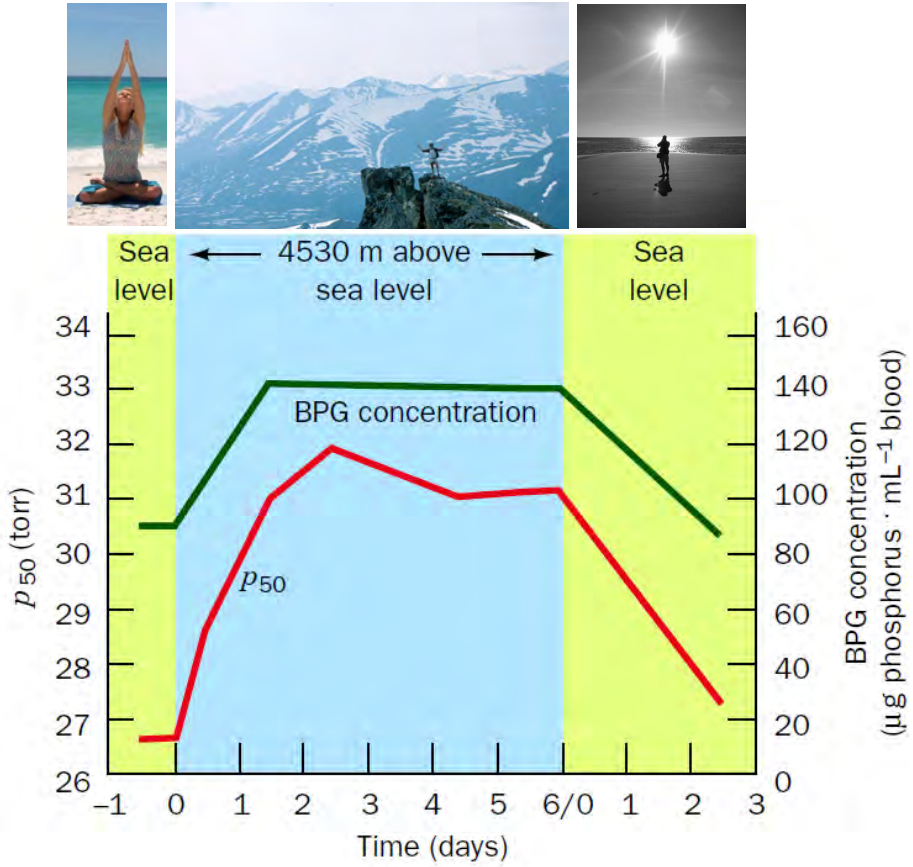
pO_2 = 100 torr at sea level
pO_2 = 56 torr at 4500 m

At pO_2 = 56 torr Y_{O2} = 0.85
= 30 torr Y_{O2} = 0.55

$\therefore$ **30%** of binding capacity is delivered

30% of O_2 capacity is delivered to tissues at 4500 m, relative to 38% at sea level. This means that 20-25% less O_2 is delivered at high altitude.

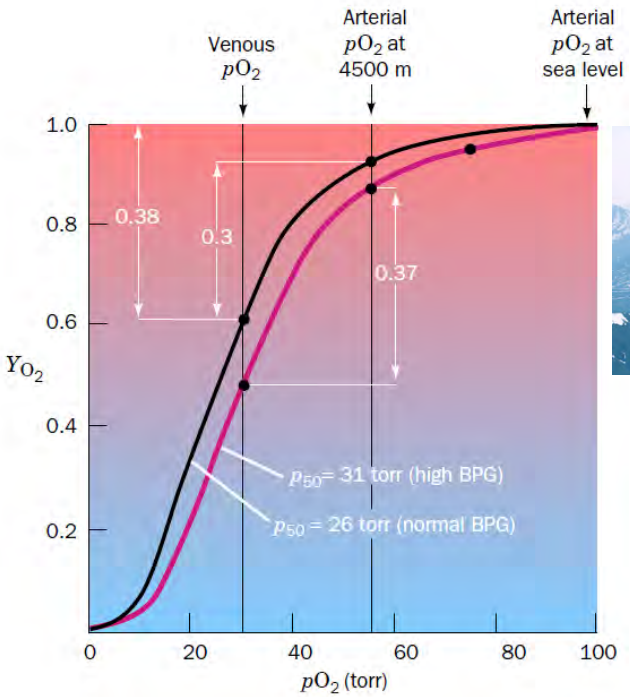
At high altitude, the body adapts to enhance O_2 delivery

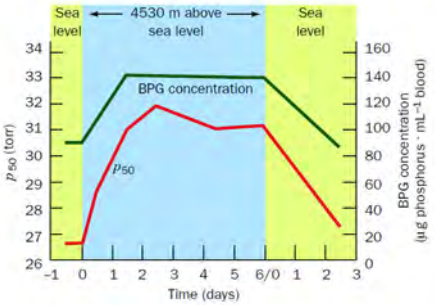


Spending time at high altitude leads to an increase in the production of Hb and red blood cells.

There is also an increase in the concentration of D-2,3 –bisphosphoglycerate (BPG) . The binding of BPG to Hb alters its binding properties so that it more effectively delivers O_2. BPG is a heterotropic allosteric modulator of Hb.

BPG binds to Hb shifting the p50 from 26 to 31 torr.





With a *p50* = 31 torr, how effective is Hb at delivering O_2?

At pO_2 = 56 torr Y_{O2} = 0.84
= 30 torr Y_{O2} = 0.48

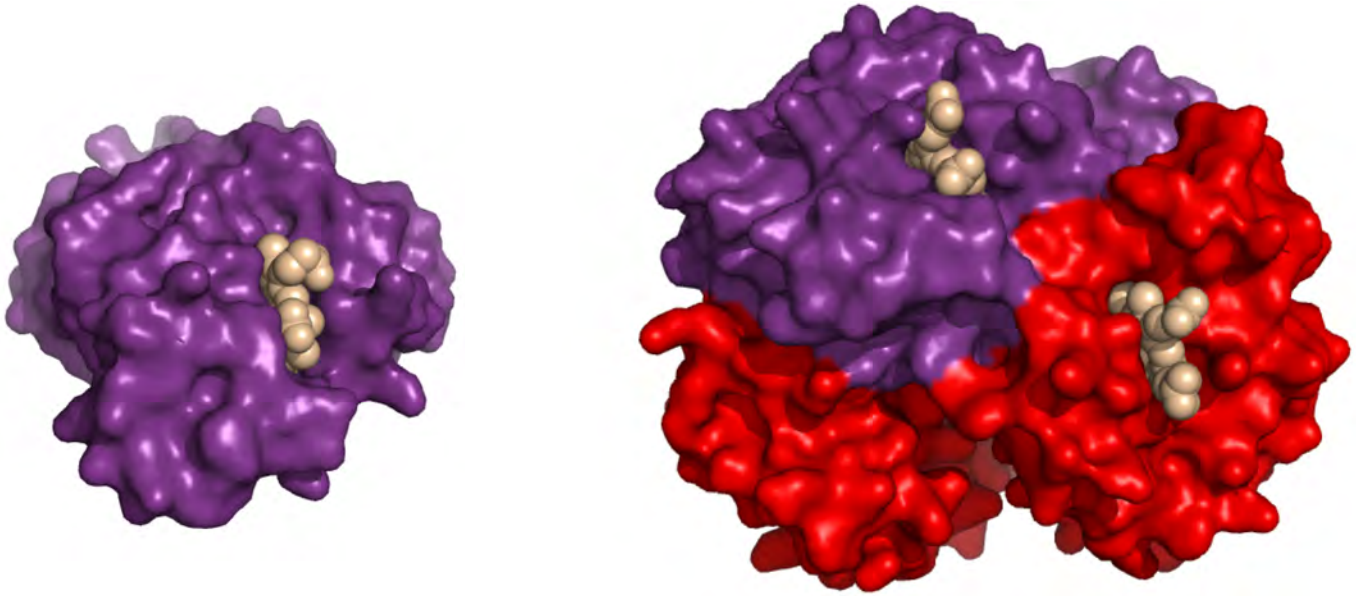
∴36% of binding capacity is delivered (38% at sea level)

The binding of BPG to Hb shifts the O_2 binding curve to the right so that Hb now delivers 36% of its binding capacity to peripheral tissues. *Is BPG a positive or negative allosteric modulator?*

What is the structural basis for cooperative binding to Hb?

Hb is a finely tuned O_2 delivery machine. Hb is efficient ad delivering O_2, yet has the capacity to adapt to a changing environment. Cooperative O_2 binding is key to both properties.

So how has Hb evolved to exhibit cooperative binding?



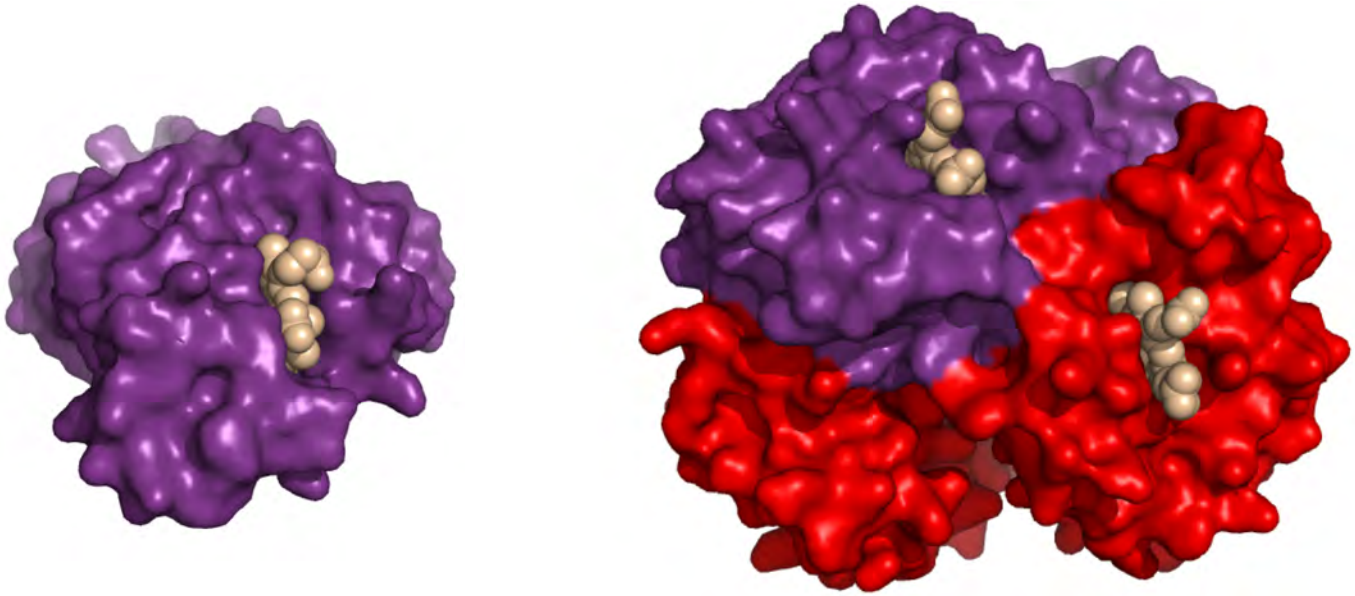
Myoglobin Hemoglobin

1) What are the structural and thermodynamic bases for cooperative O_2 binding?

2) What is the structural and thermodynamic bases for allosteric regulation by BPG?

Tetrameric structure is key to cooperative binding

Hb binds O_2 cooperatively while Mb does not. Cooperative binding means that the binding of one molecule of O_2 influences the binding of other molecules of O_2.



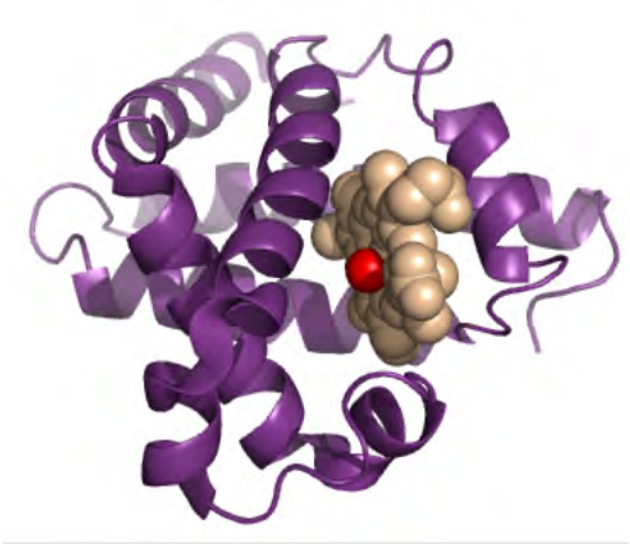
Myoglobin

Hemoglobin

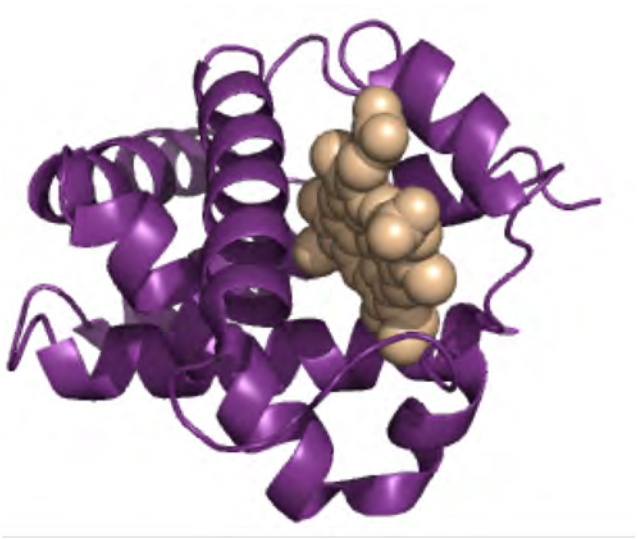
The evolution of tetrameric Hb from a monomeric myoglobin-like ancestor was an early step in the evolution of cooperative O_2 binding – it is the movement of subunits relative to each other that leads to cooperativity.

Why do the α and β subunits of Hb form a tetramer?

Mb and both the Hb α and β subunits have remarkably similar tertiary structures, each with a number of α-helices (helices A, B, C, …H) wrapped around a heme group in what is referred to as the globin fold.



Myoglobin

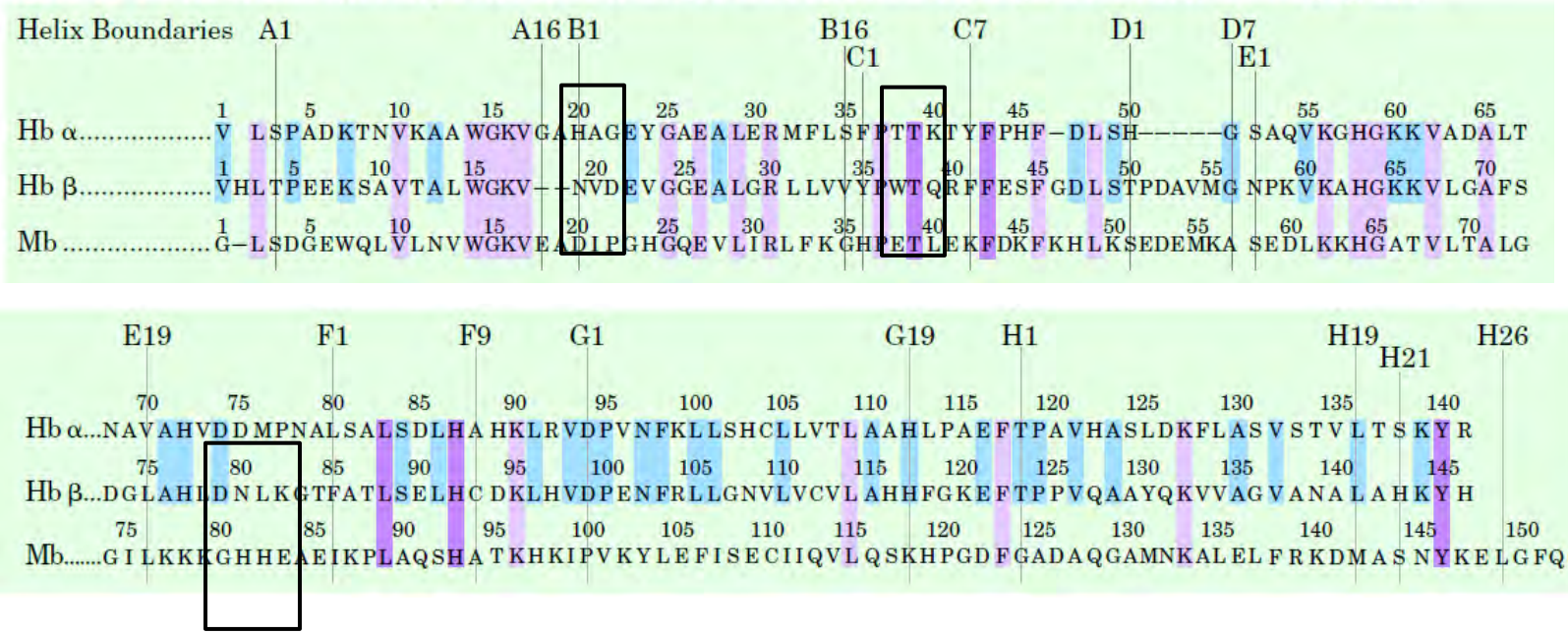


Hemoglobin

Why does Hb tetramerize while Mb does not?

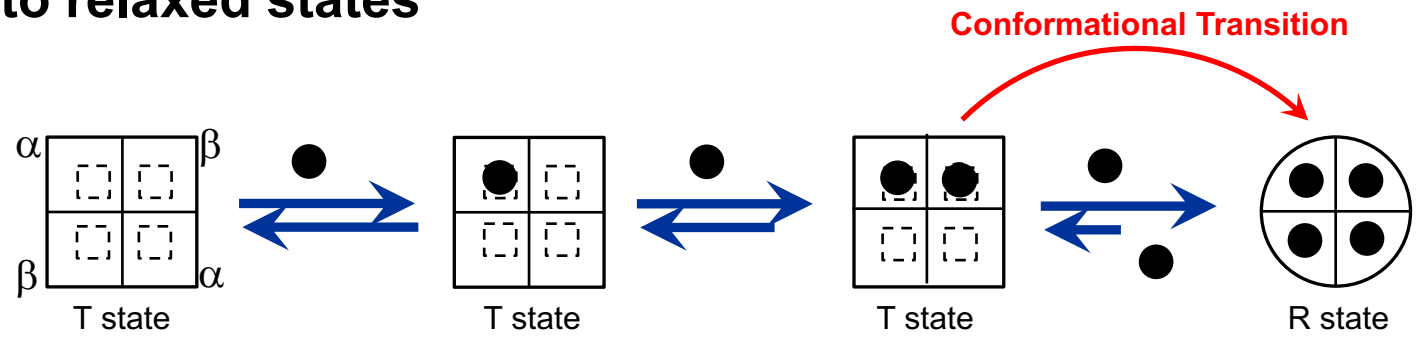
Sequence differences along the surfaces of the α and β subunits drive tetramer formation

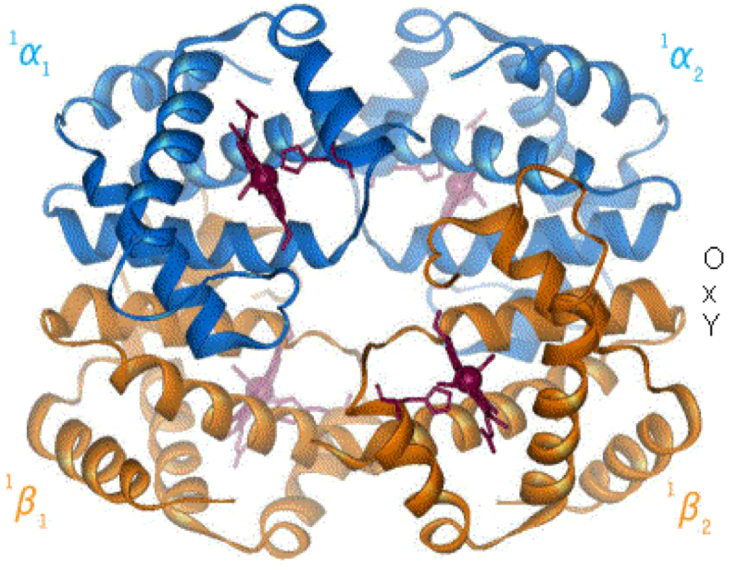
Table 10-1 **The Amino Acid Sequences of the α and β Chains of Human Hemoglobin and of Human Myoglobin**[a,b]



The sequence of Mb and the two Hb subunits share only 18% identity (in blue). There are several amino acid substitutions on the surface of each subunit that drive tetramerization.

Cooperative binding results from a conformational change from tense to relaxed states

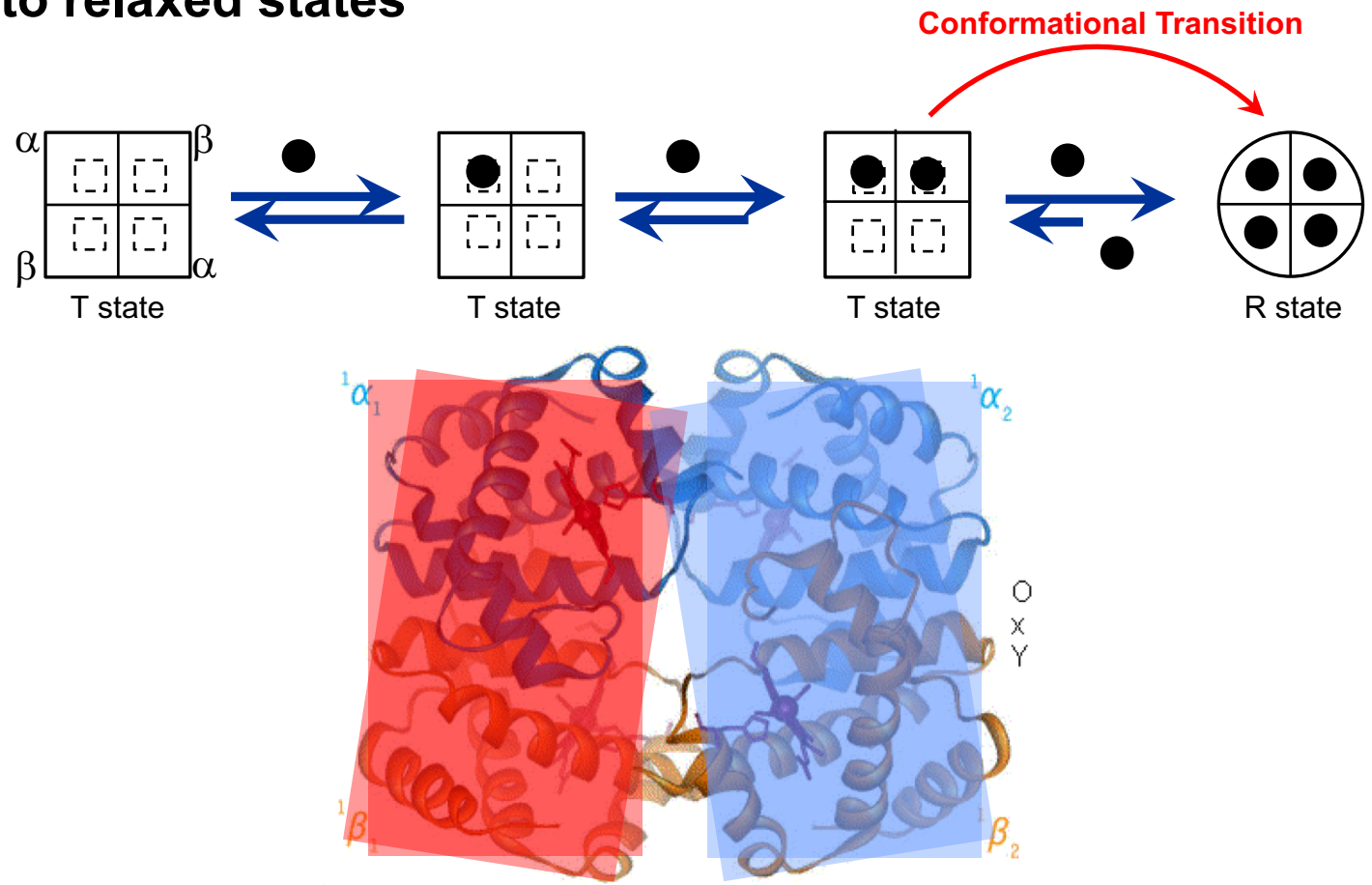




Hb is a dimer of αβ dimers ($\alpha_1\beta_1$ and $\alpha_2\beta_2$). The α_1/β_1 and α_2/β_2 interfaces are tighter than the interface between the $\alpha_1\beta_1$ and $\alpha_2\beta_2$ dimers. O_2 binding causes the $\alpha_1\beta_1$ dimer to move relative to the $\alpha_2\beta_2$ dimer. This change in dimer packing leads to a conformation transition that underlies cooperativity.

The high affinity O_2 bound state is called the relaxed (R) state. The low affinity unbound state is called the tense (T) state.

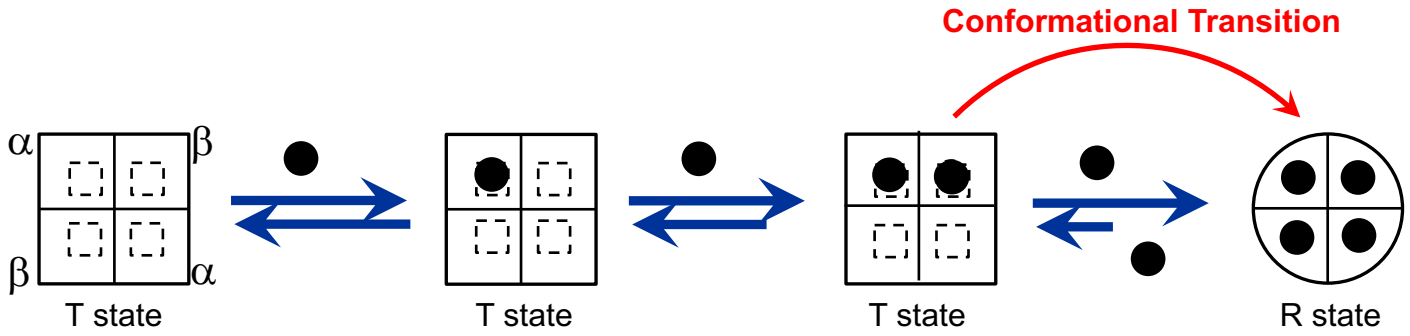
Cooperative binding results from a conformational change from tense to relaxed states

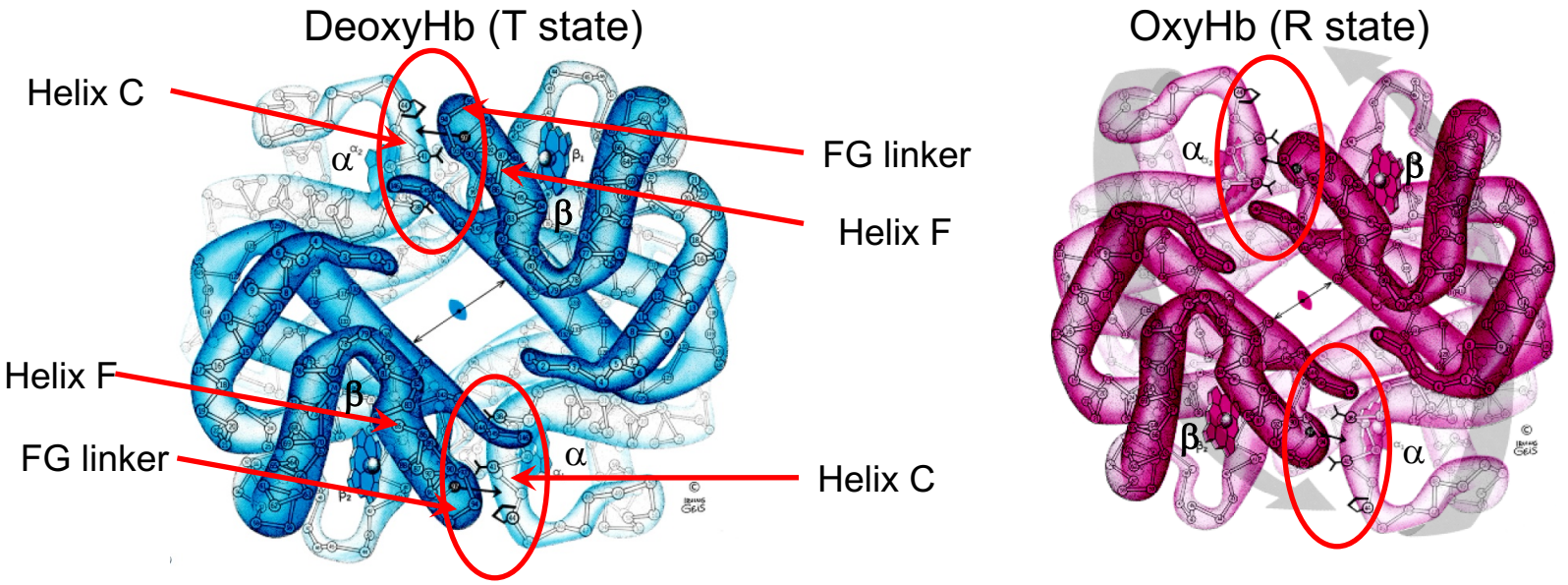


Hb is a dimer of αβ dimers ($\alpha_1\beta_1$ and $\alpha_2\beta_2$). The α_1/β_1 and α_2/β_2 interfaces are tighter than the interface between the $\alpha_1\beta_1$ and $\alpha_2\beta_2$ dimers. O_2 binding causes the $\alpha_1\beta_1$ dimer to move relative to the $\alpha_2\beta_2$ dimer. This change in dimer packing leads to a conformation transition that underlies cooperativity.

The high affinity O_2 bound state is called the relaxed (R) state. The low affinity unbound state is called the tense (T) state.

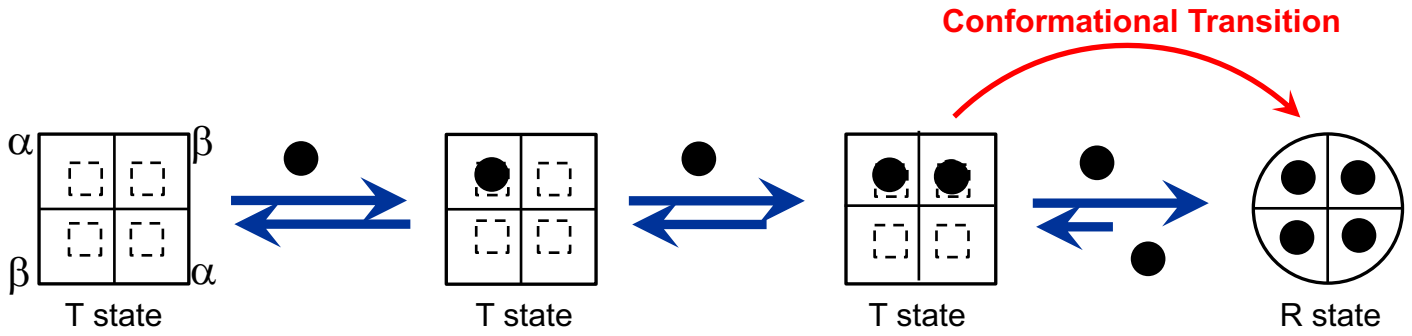
R and T states are governed by ridges into grooves packing



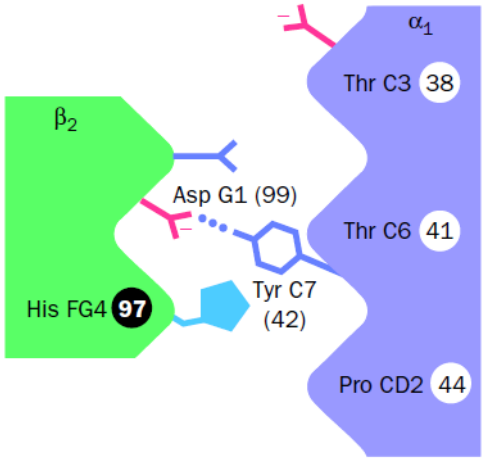


The linker between helix F and G (FG) of β interacts with helix C on the adjacent α subunit. The interactions between F/FG and C change upon O_2 binding.

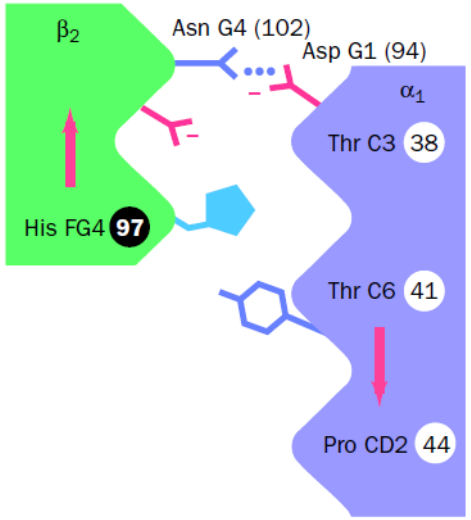
R and T states are governed by ridges into grooves packing



DeoxyHb (T state)

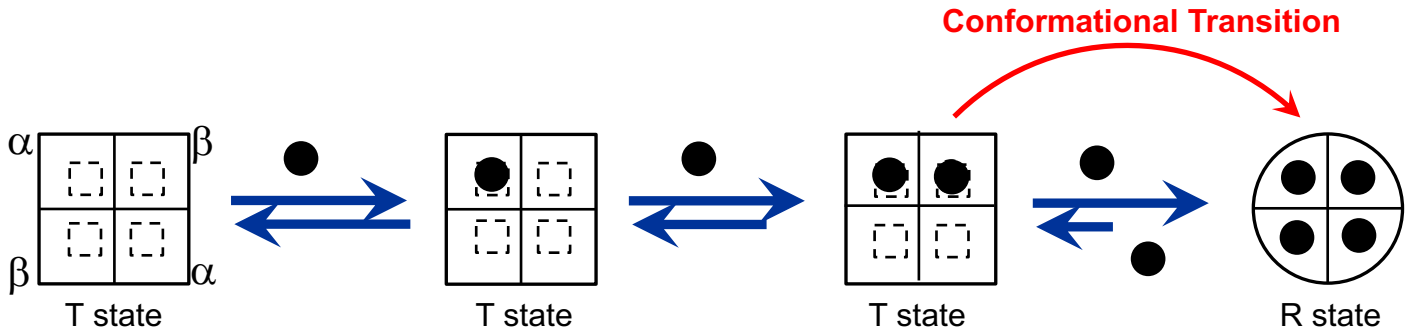


OxyHb (R state)

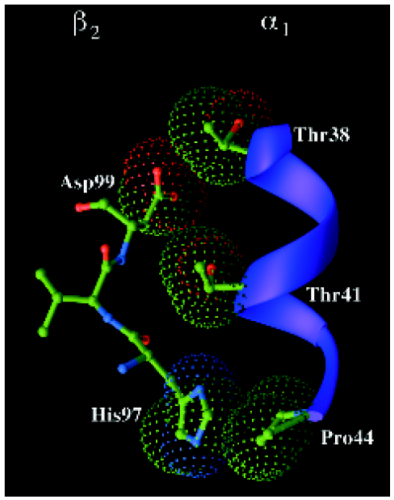


Upon O_2 binding, helix F shifts so that it interacts with helix C one turn further along. HisFG4 97 moves from groove between ProCD2 & ThrC6 in T state, to the groove between ThrC6 & ThrC3 in the R state. *There is a shift of the ridges into grooves packing*.

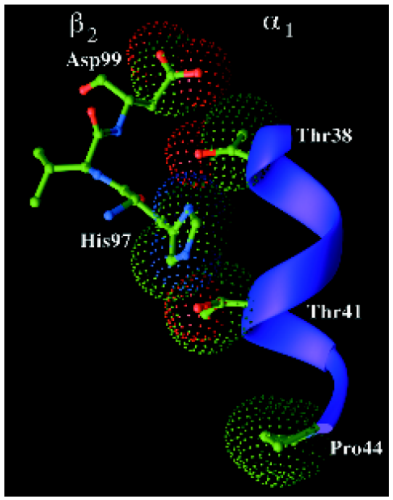
R and T states are governed by ridges into grooves packing



DeoxyHb (T state)



OxyHb (R state)



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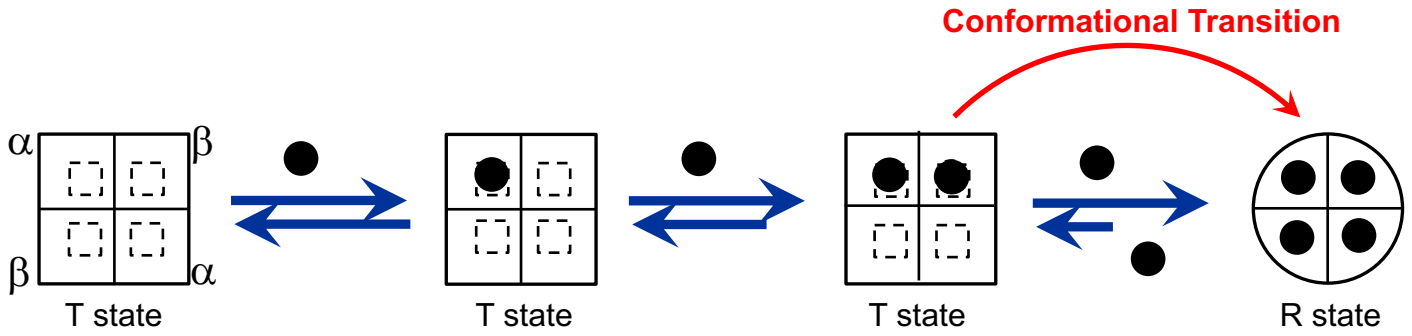
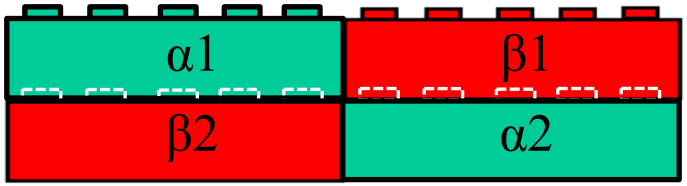
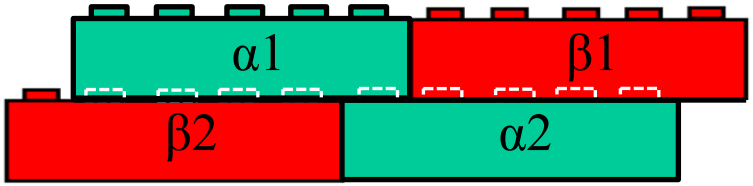
R and T states are governed by ridges into grooves packing



DeoxyHb (T state)

OxyHb (R state)

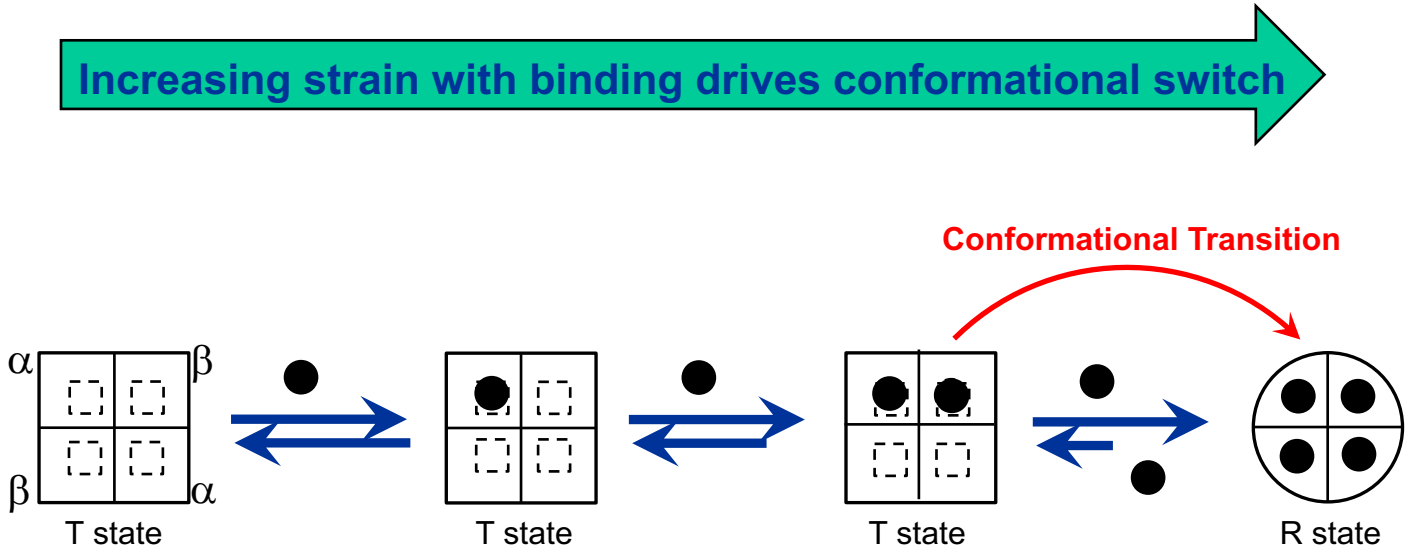




Ridges into grooves packing defines two states, as shown here schematically with the Lego blocks. Switching from T to R is all or nothing – **all four subunits must move together in concerted (i.e. all together) versus sequential (one subunit at a time) motions**.

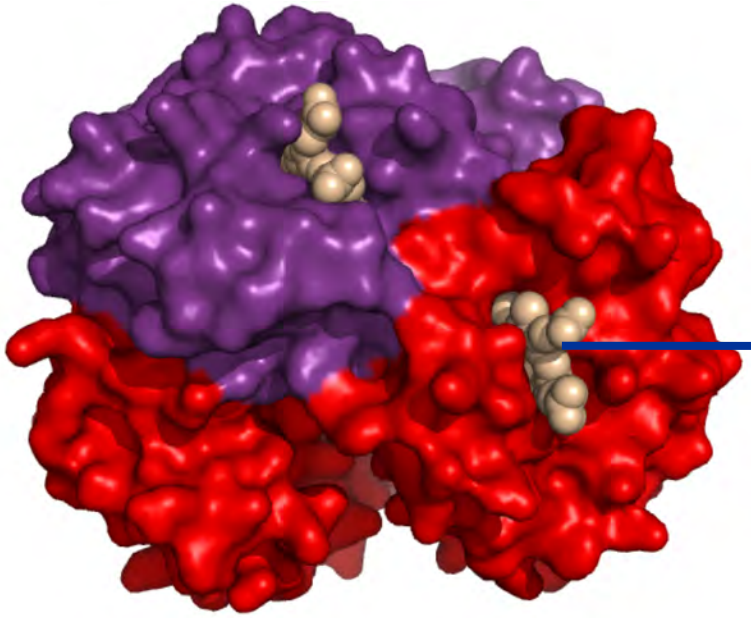
Why does O_2 binding drive the conformational switch?

O_2 binds with higher affinity (i.e. more energy is derived) to the R state. The energy derived from O_2 binding provides the driving force for the conformational switch:

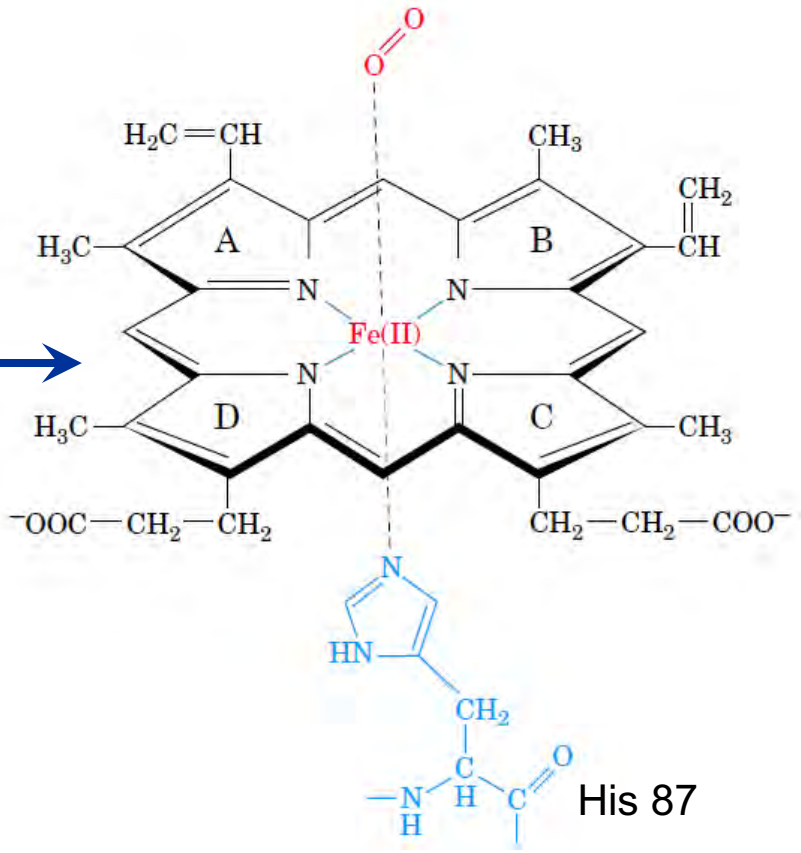


What is behind the driving force for the conformational switch? We need to understand this from both a *structural* and a *thermodynamic perspective*. Let's first look at how O_2 binding structurally promotes the conformational switch.

O_2 binds to heme – which is the porphyrin ring and Fe^{2+}



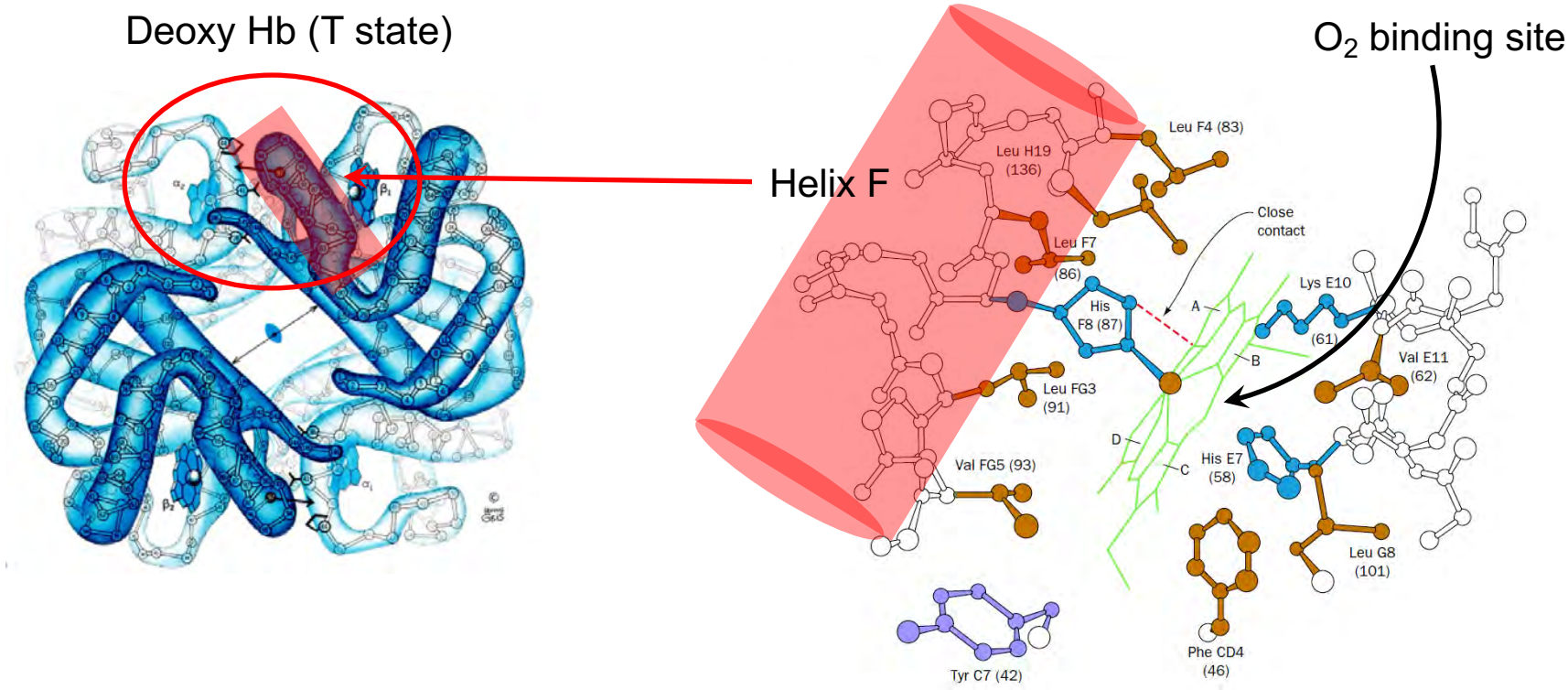
Hemoglobin



His 87

The Fe^{2+} (ferrous) ion is coordinated by 5 groups, 4 nitrogen atoms on the porphyrin ring and one nitrogen from the His87 side chain below the ring. His87 is located on helix F!

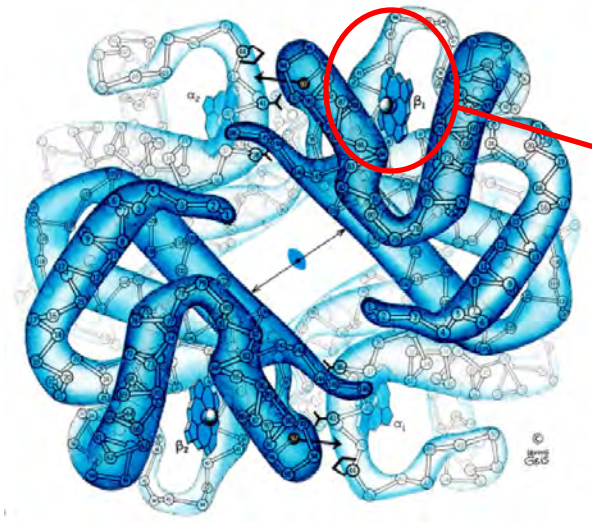
O_2 binds to heme – which is the porphyrin ring and Fe^{2+}



His87 is within van der Waals contact with the Fe^{2+} of the heme group – i.e. it forms a partial covalent bond with Fe^{2+}. Note that His87 is located on helix F (red), the helix that moves relative to helix C of the adjacent subunit upon transition from T to R!

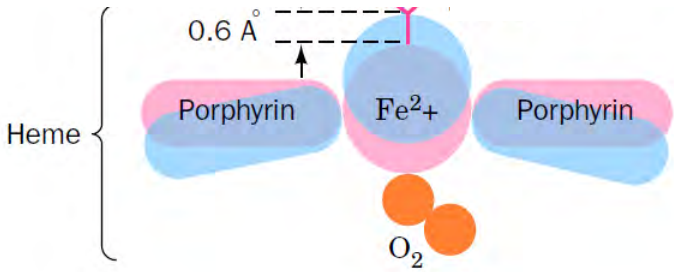
O_2 leads to a structural change in the heme group

Deoxy Hb (T state)



Oxy R state

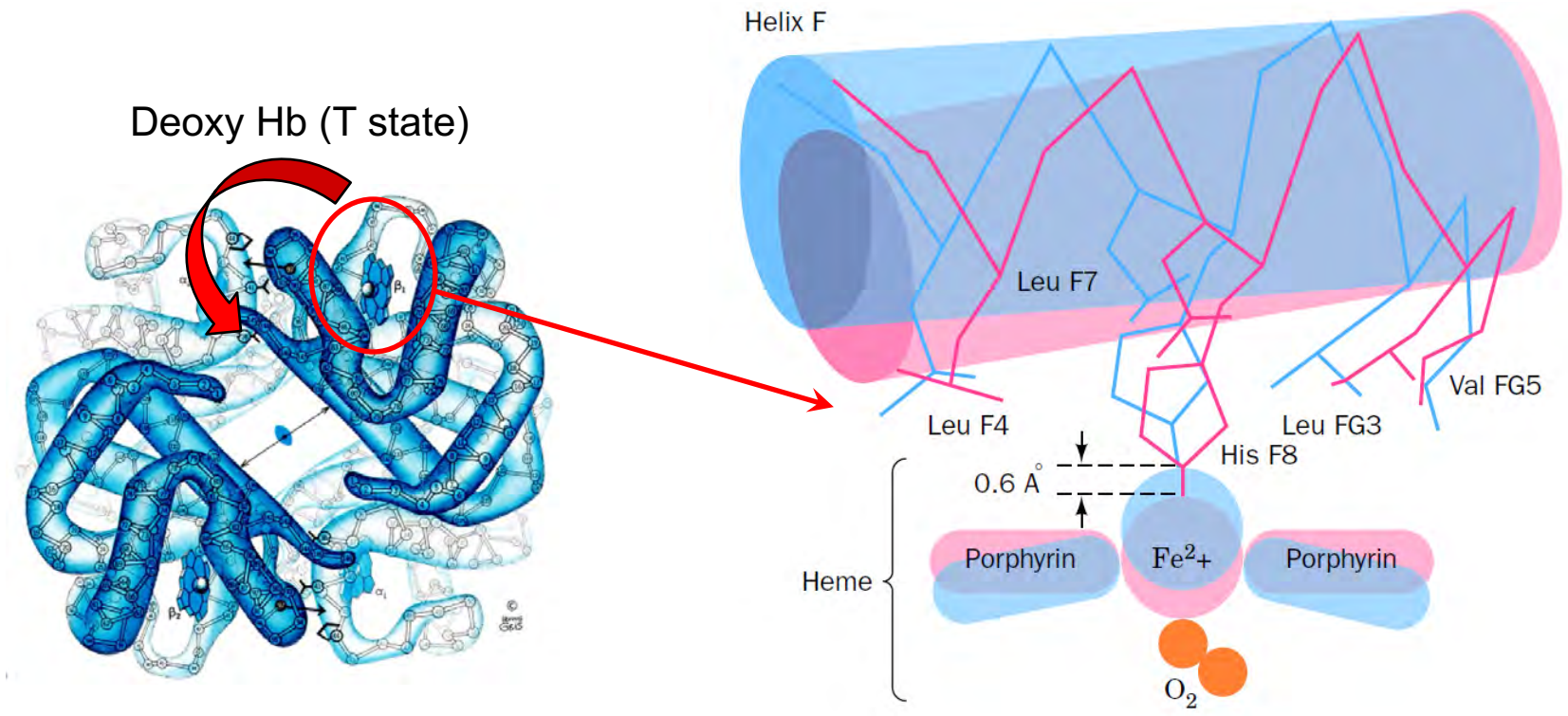
Deoxy T state



In the T state, the porphyrin ring is puckered, with the Fe^{2+} situated 0.6 Å above the plane of the heme – this is because the Fe – $N_{porphyrin}$ bonds are too long for the Fe^{2+} to remain in the plane of the ring.

When O_2 binds, the electronic structure of the Fe_{2+} changes so that the Fe – $N_{porphyrin}$ bonds decrease in length by 0.1 Å. The net effect is that the Fe^{2+} moves into the plane with the porphyrin ring.

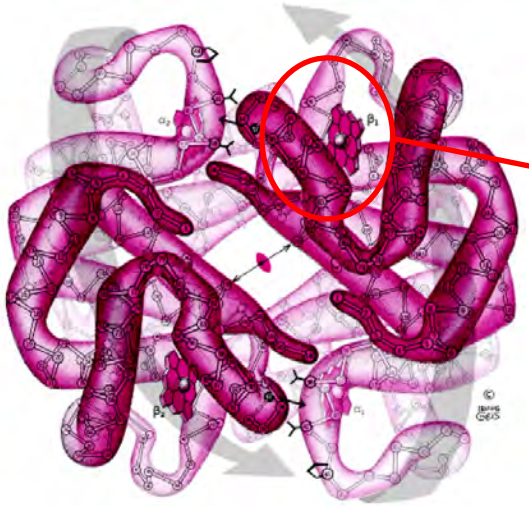
Structural change in the heme group pulls on HisF8…

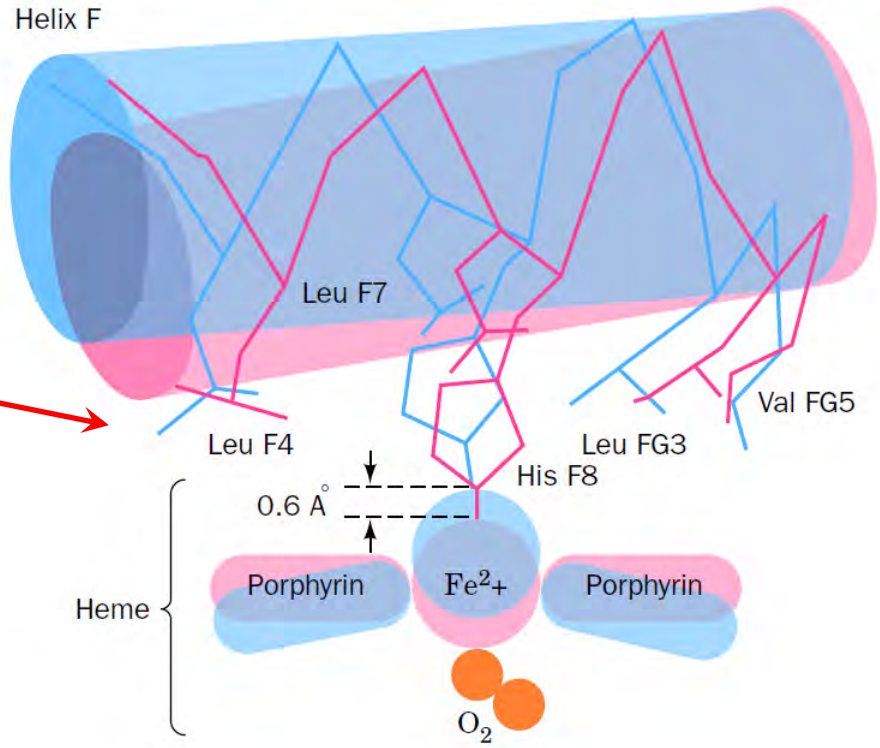


Due to the partial covalent bond between HisF8 (His87) and Fe^{2+}, movement of the Fe^{2+} into the plane of the porphyrin ring by 0.6 Å leads to movement of HisF8 (His87) and thus a tilt in the F helix. The tilt in helix F drives the "conformational switch" from T to R (helix F packs against helix C from the adjacent subunit).

Leading to a tilt of helix F, and the transition from the T to the R state

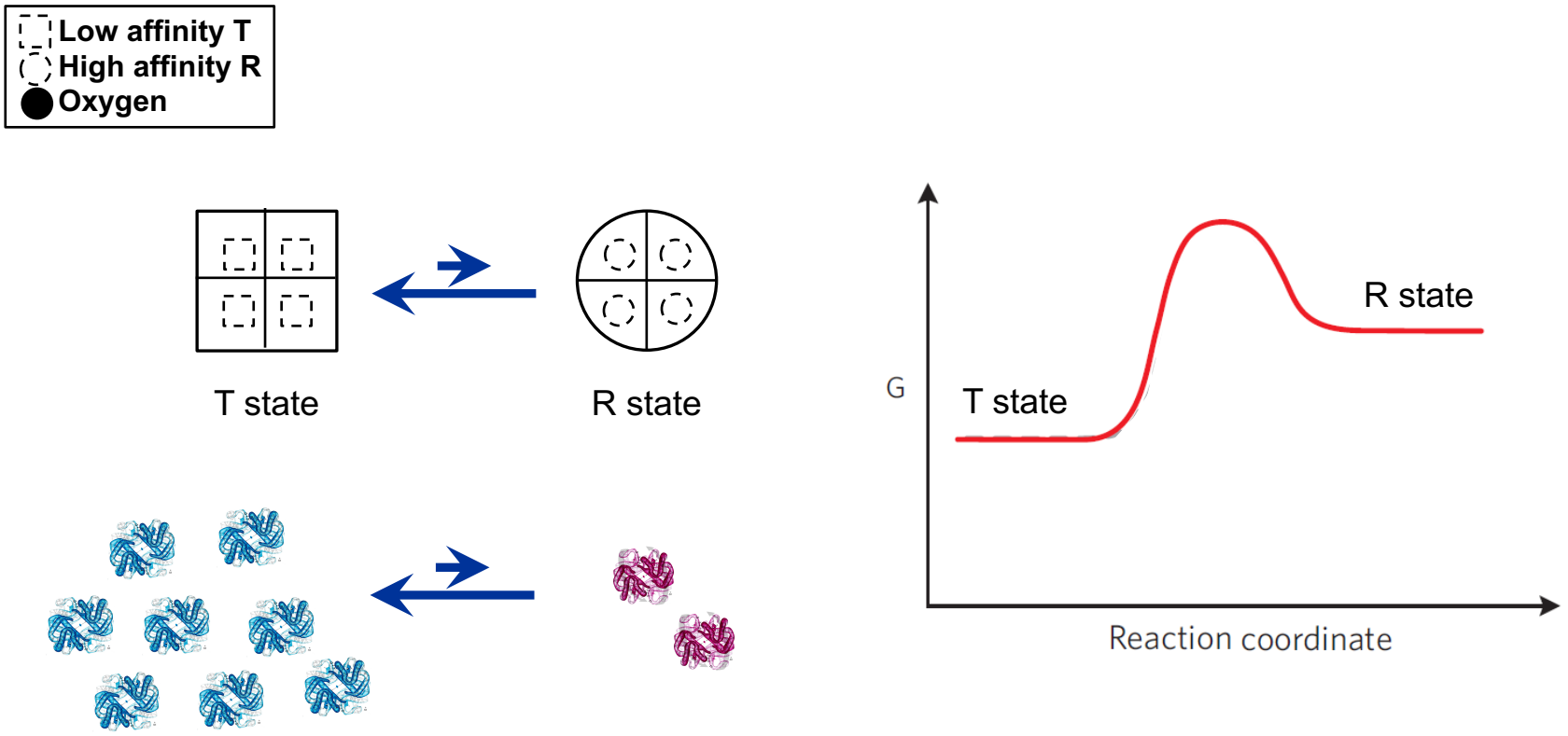
Oxy Hb (R state)





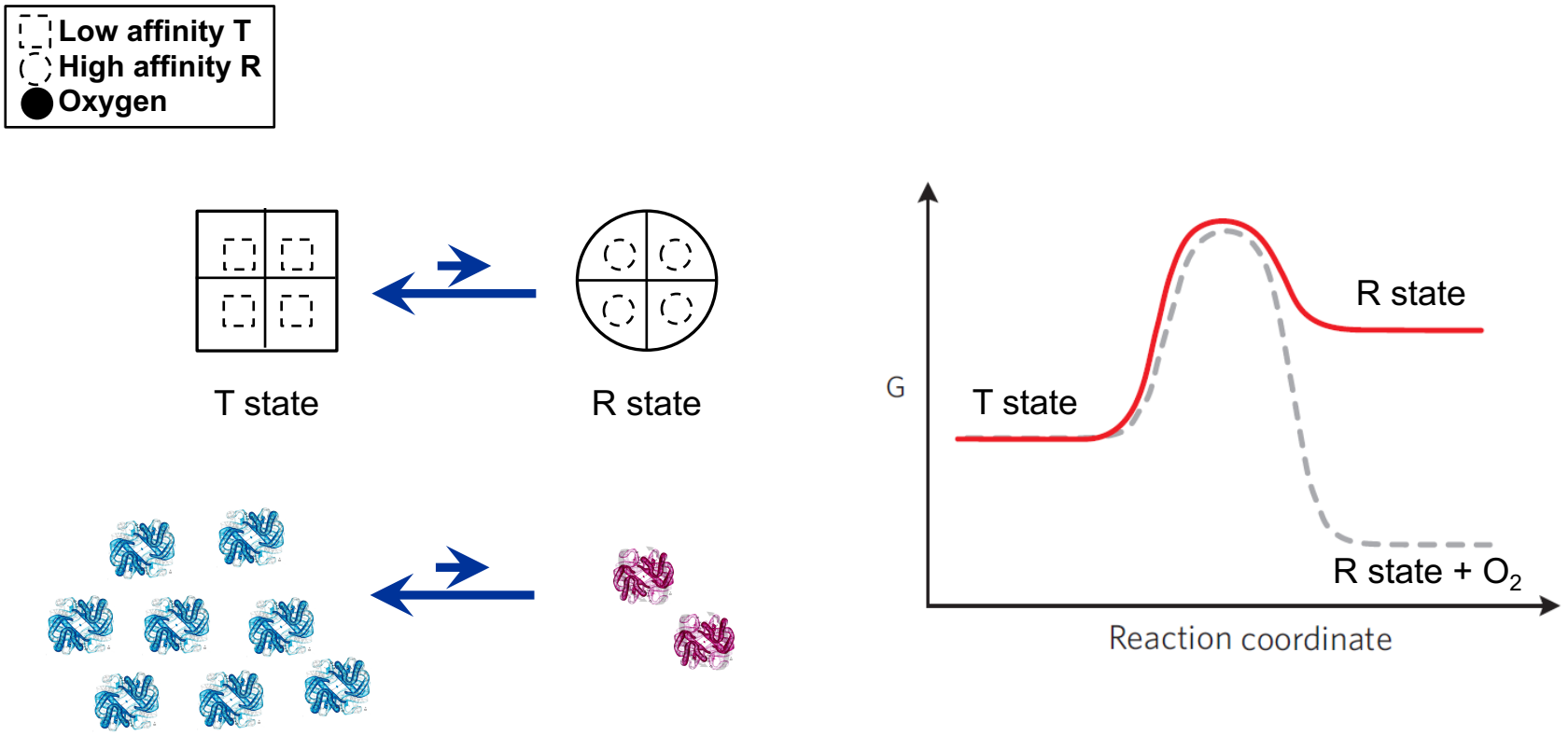
Due to the partial covalent bond between HisF8 (His87) and Fe^{2+}, movement of the Fe^{2+} into the plane of the porphyrin ring by 0.6 Å leads to movement of HisF8 (His87) and thus a tilt in the F helix. The tilt in helix F drives the “conformational switch” from T to R (helix F packs against helix C from the adjacent subunit).

Allosteric transitions from a thermodynamic perspective



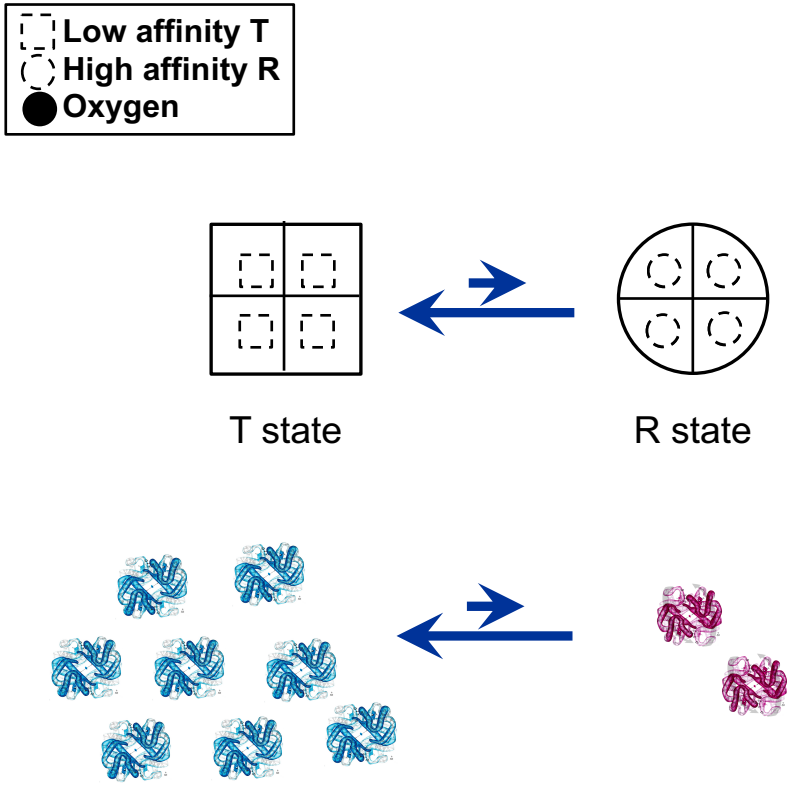
In the absence of O_2, Hb can exist in either T or R, but T has a much lower energy than R – so this form predominates. The proportion of molecules in the two states is governed by their relative energies – <u>but there are always some molecules of Hb in the R state</u>.

O_2 binds preferentially to the R state



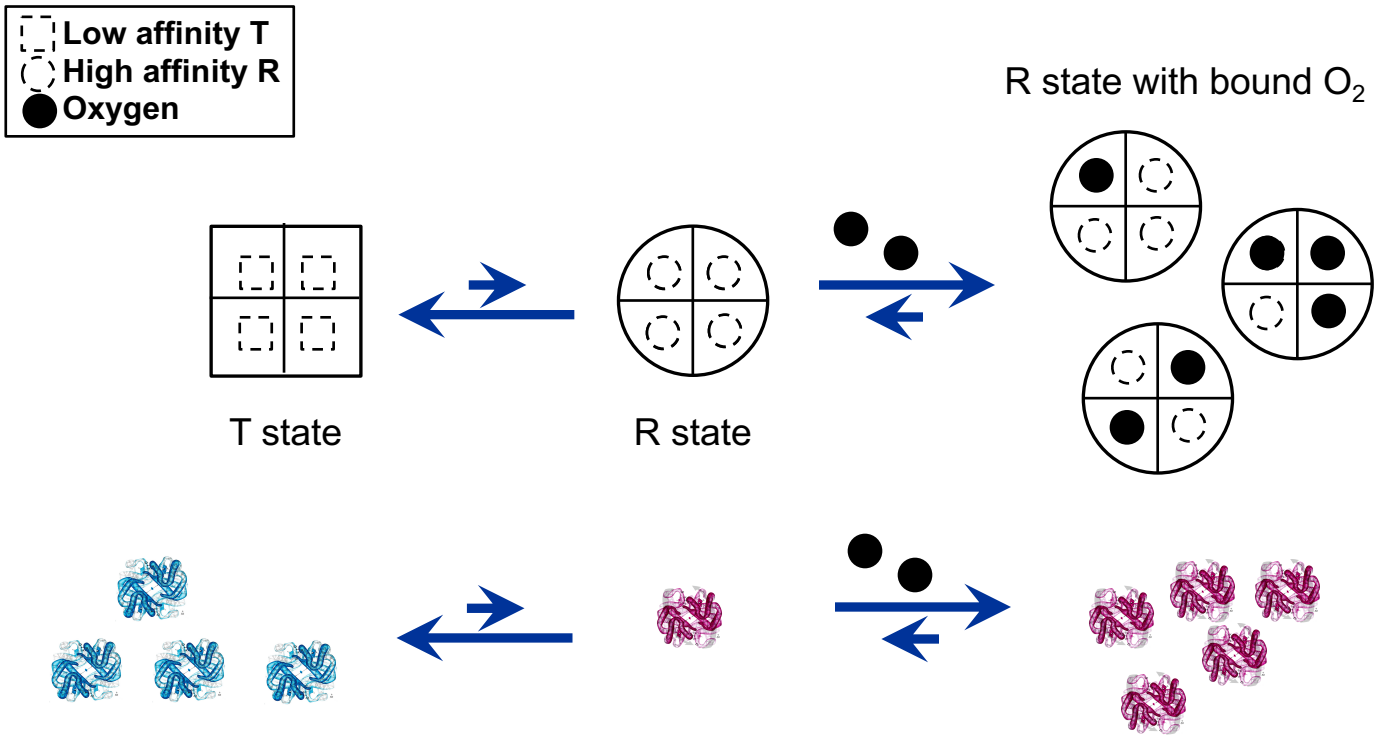
O_2 binds with a higher affinity to the R state than to the T state, so more energy is derived from O_2 binding to the R state. The energy of O_2 binding lowers the overall energy of the R state, so that R is now favored over T. The proportion of Hb molecules is thus shifted towards the R state.

O_2 binds with higher affinity to the R state



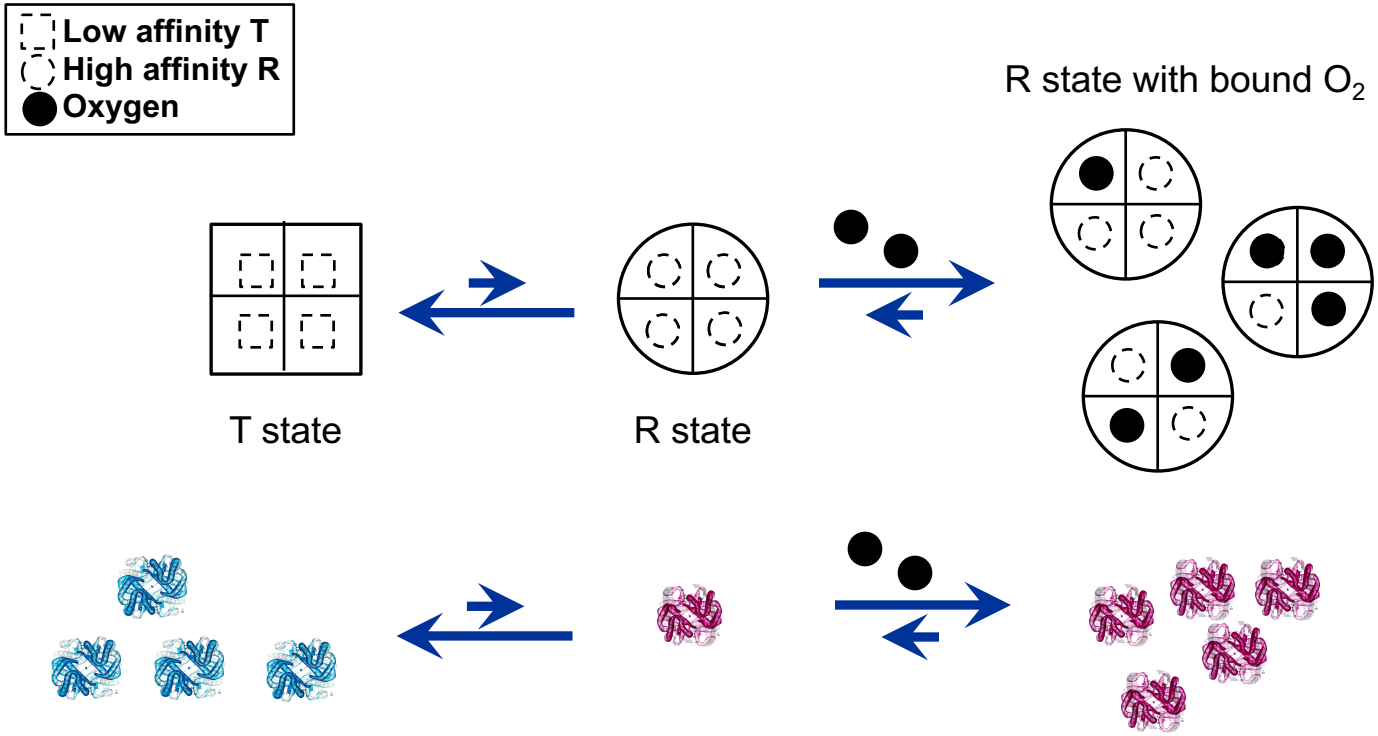
Another way of thinking about this is to consider affinity. The R state has a higher affinity for O_2 than the T state. So at low [O_2], O_2 can bind to the R state at concentrations where it will not bind to the T state…

O_2 binds preferentially to the R state



This creates a new species HbO_2, which disrupts the equilibrium between unbound T and R. The latter equilibrium will then re-establish, with the net effect being a shift in the number of molecules in the R state! By adding even more molecules of O_2, I can eventually shift the eqiulibrium between T and R states almost exclusively towards the R state.

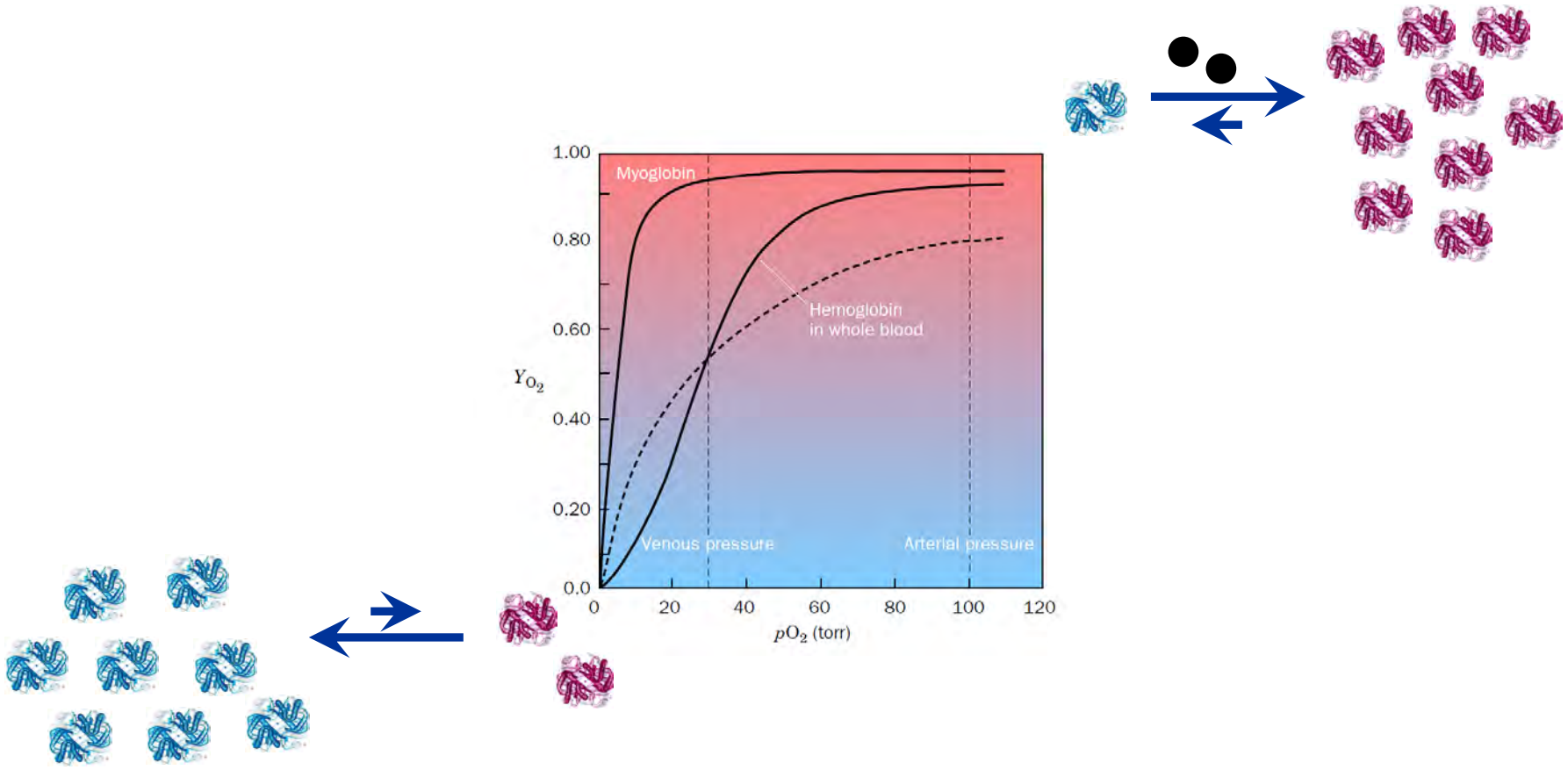
Why does conformational change lead to cooperativity?



The binding of one molecule of O_2 provides energy to tilt helix F. This creates stress within the Hb tetramer, but the binding of one O_2 is usually not enough to overcome the activation energy between T and R. With the binding of a 2nd or 3rd molecule of O_2, this internal stress builds to the point that the entire tetramer shifts from T to R.

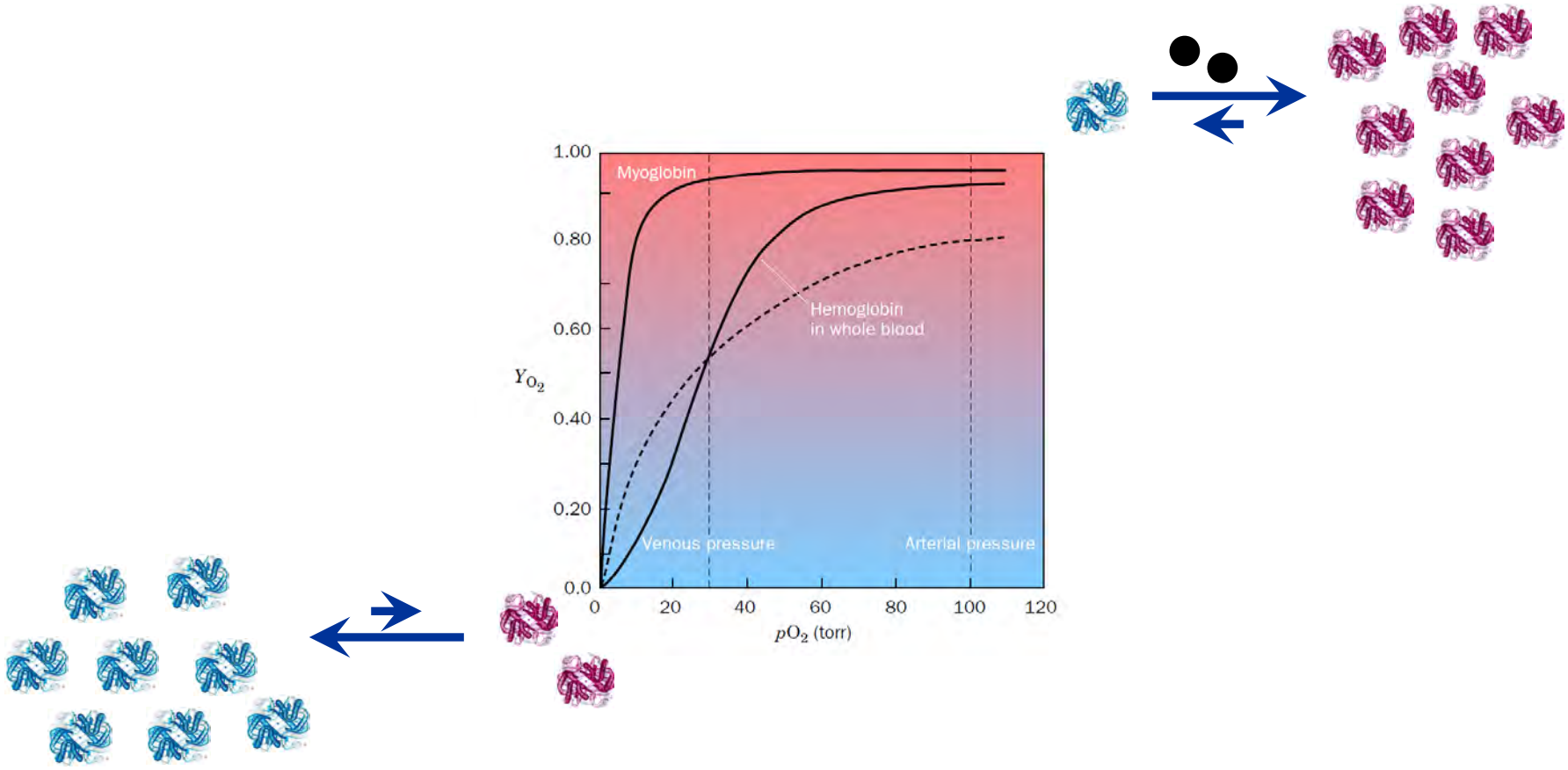
Because R has a higher affinity for O_2 than T, the conformational shift leads to a massive increase in O_2 binding to other sites!

Why does conformational change lead to cooperativity?



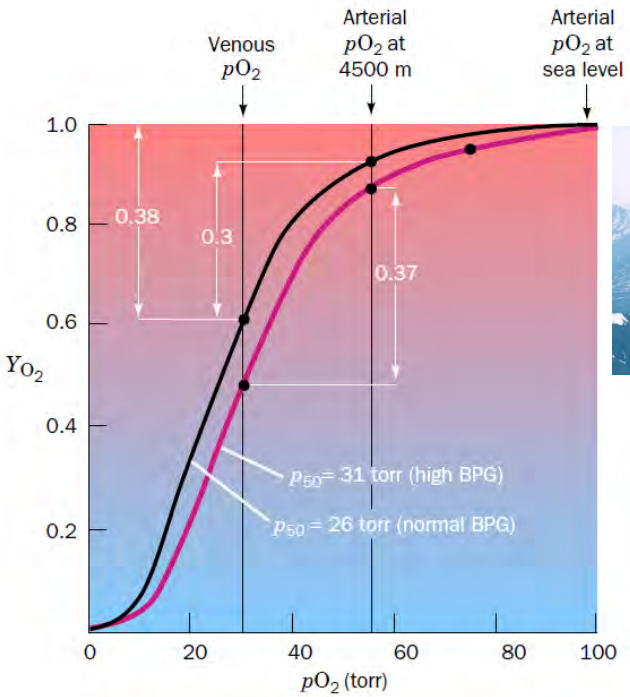
In a binding experiment, Hb starts mainly in the T state (low [O_2], where it binds O_2 with low affinity. As O_2 is added it binds to the available high affinity R state sites, and eventually leads to a conformation shifts from T to R. With a shift into the R state Hb, high affinity binding sites are formed that immediately bind O_2. The Sigmoidal nature of the curve reflects the appearance of these high affinity sites, which immediately bind O_2.

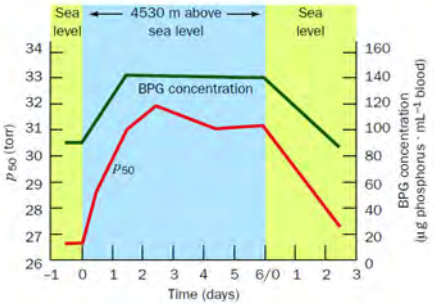
What does the Hill coefficient tell us?



The Hill coefficient tells us how easy it is to shift the conformation from T to R. If O_2 binding provides so much energy that it easily shifts the conformation from T to R, then the Hill coefficient will be close to n, the number of binding sites.

BPG binds to Hb shifting the p50 from 26 to 31 torr.

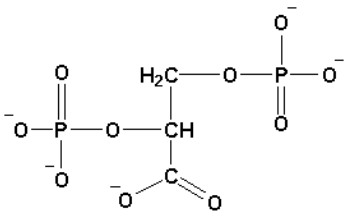




After several days at altitude, the levels of BPG increase in blood shifting the p50 for binding of O_2 from 26 torr to 31 torr. This allows Hb to more effectively deliver O_2 to peripheral tissues at high altitude.

How does BPG shift Hb O_2 binding affinity?

BPG binds to a cavity at the core of Hb in the T state



2,3-Bisphosphoglycerate

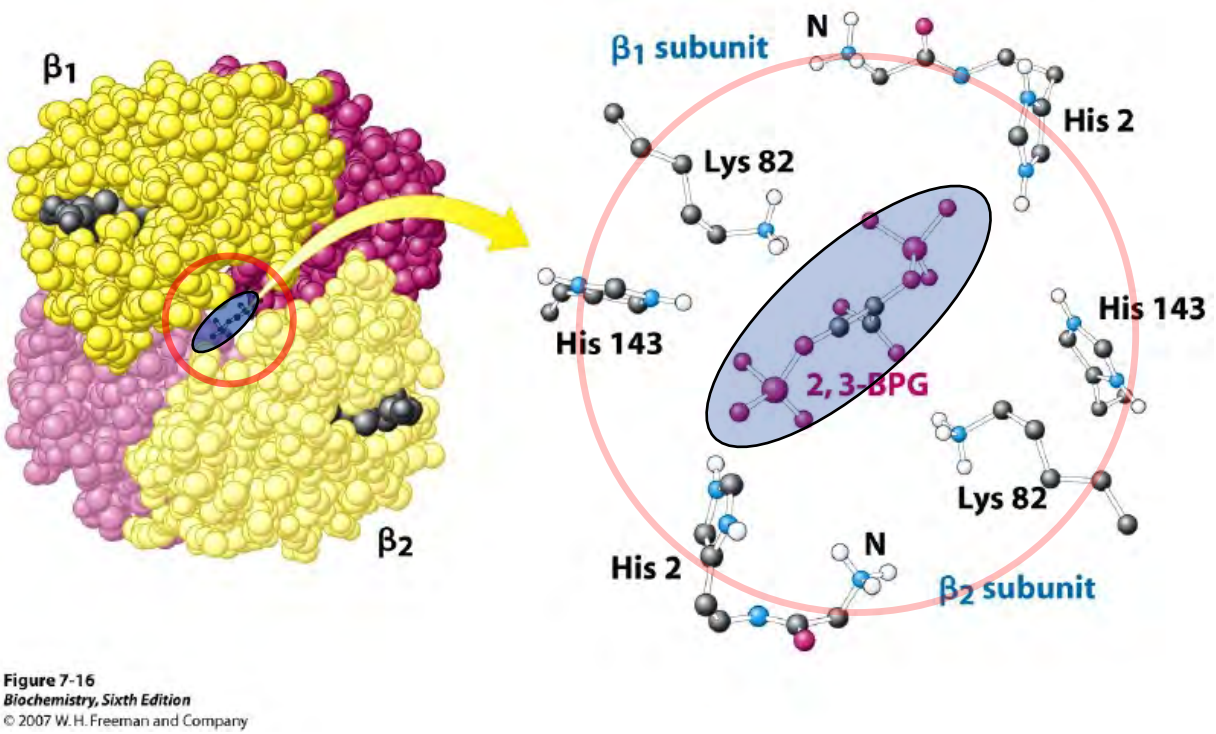
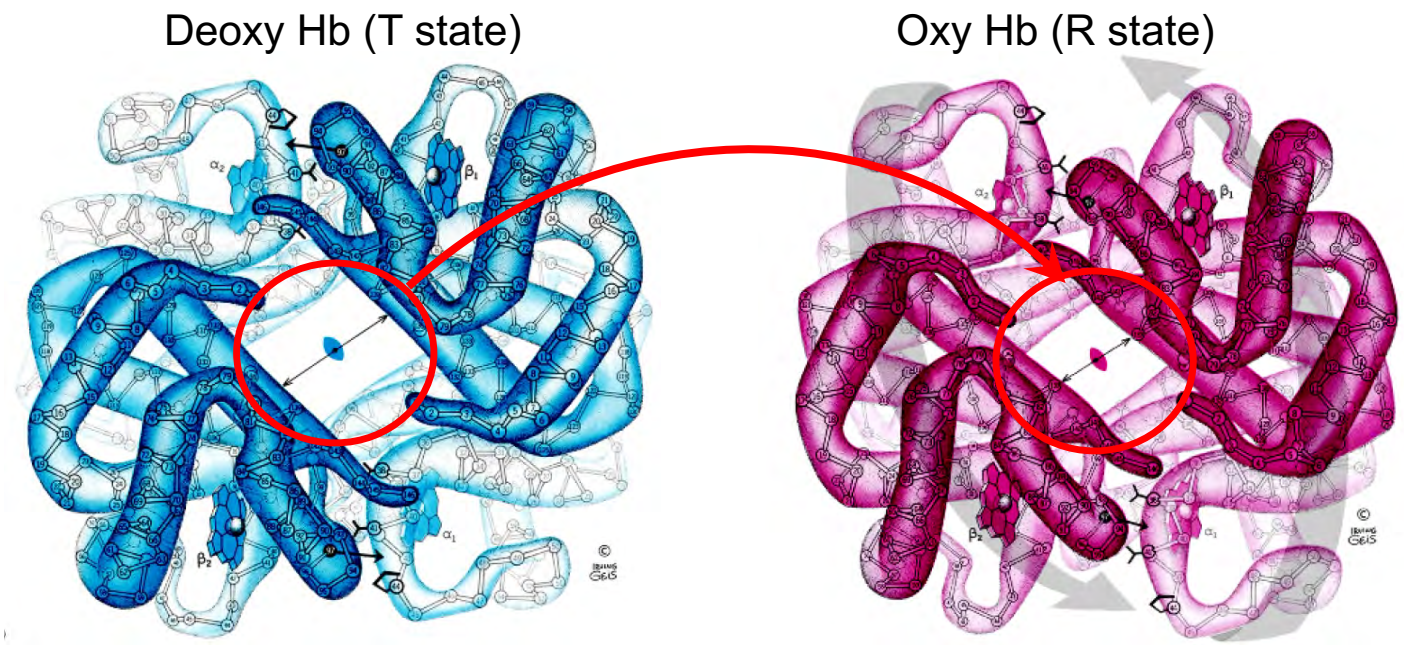


Figure 7-16
Biochemistry, Sixth Edition

Deoxy-Hb (T state) with BPG

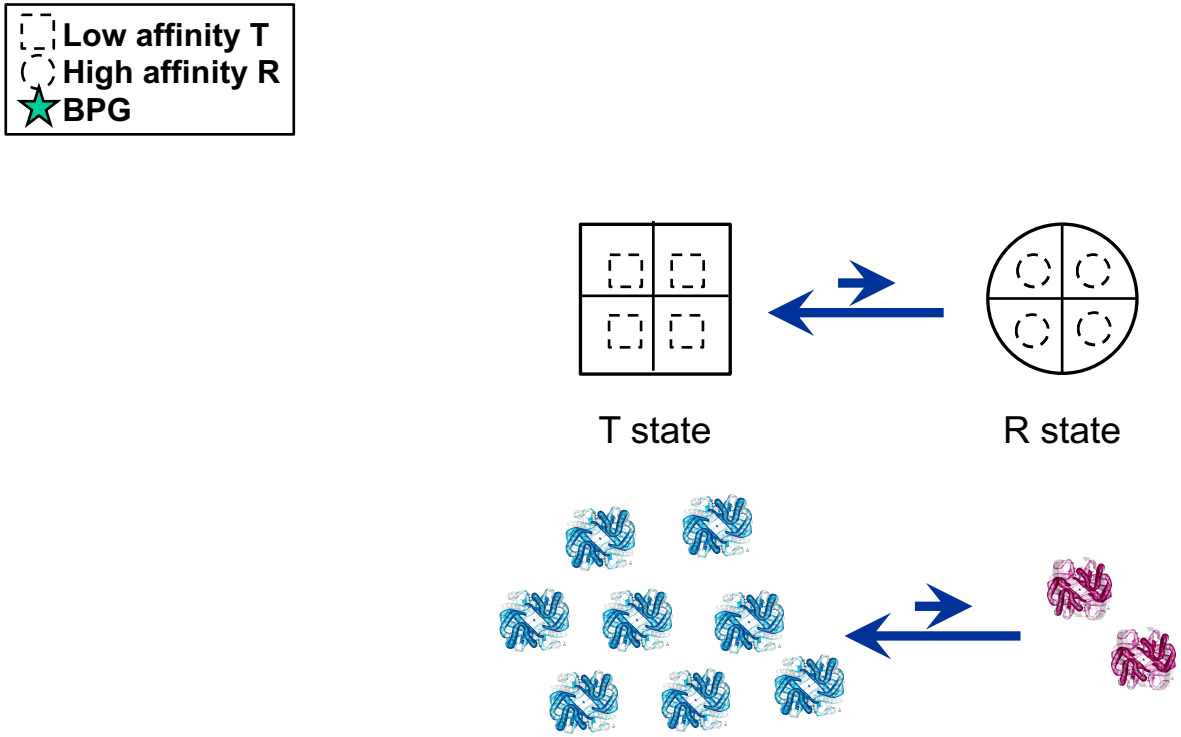
An important clue as to how BPG influences O_2 binding came from structural biology, which showed that BPG binds to only one site on Hb – a cavity at the center of Hb. There are a number of positive residues on the β-subunit that line the cavity and that interact with the highly negative BPG. *These ionic interactions are strong and stabilize the T state!*

BPG cannot bind to the R state, because the cavity is too small



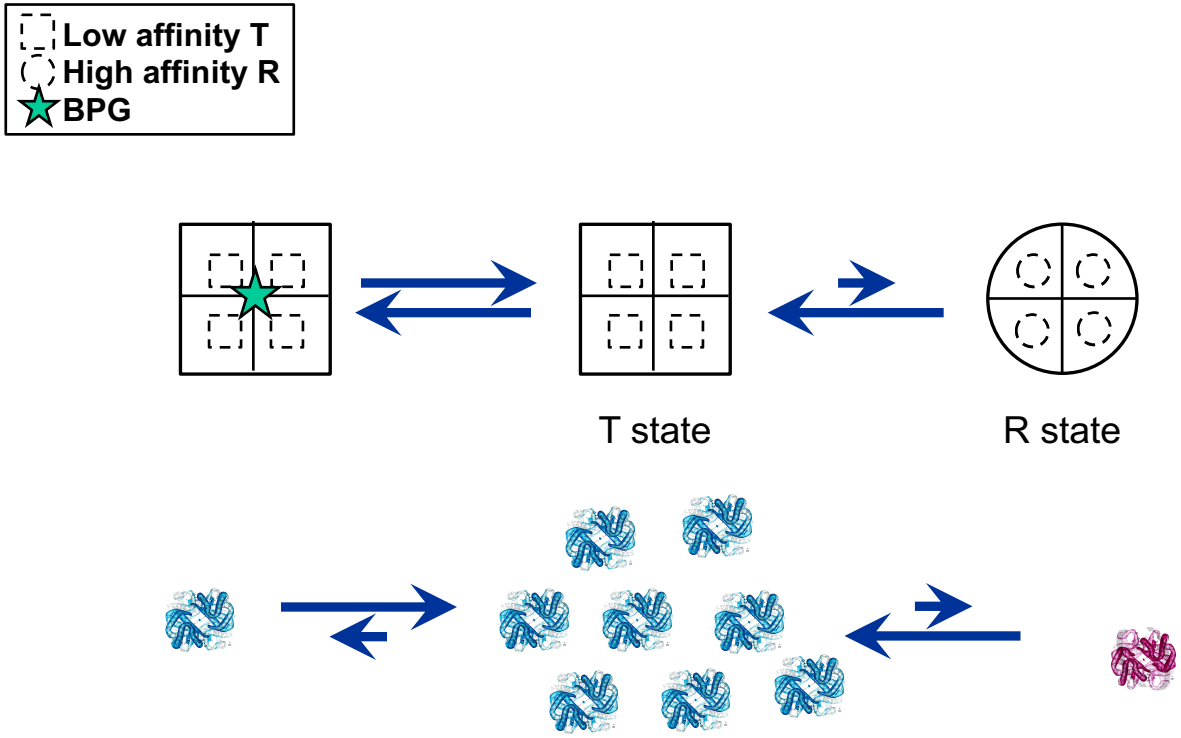
If you compare the structures of T and R, you see that the size of the central cavity decreases in the R state. In contrast to O_2, BPG binds with a much higher affinity to the T state than to the R state. BPG thus stabilizes the low O_2 affinity binding form of Hb. *BPG cannot bind to the cavity in the R state, because the cavity is too small.*

BPG binds with higher affinity to the T state



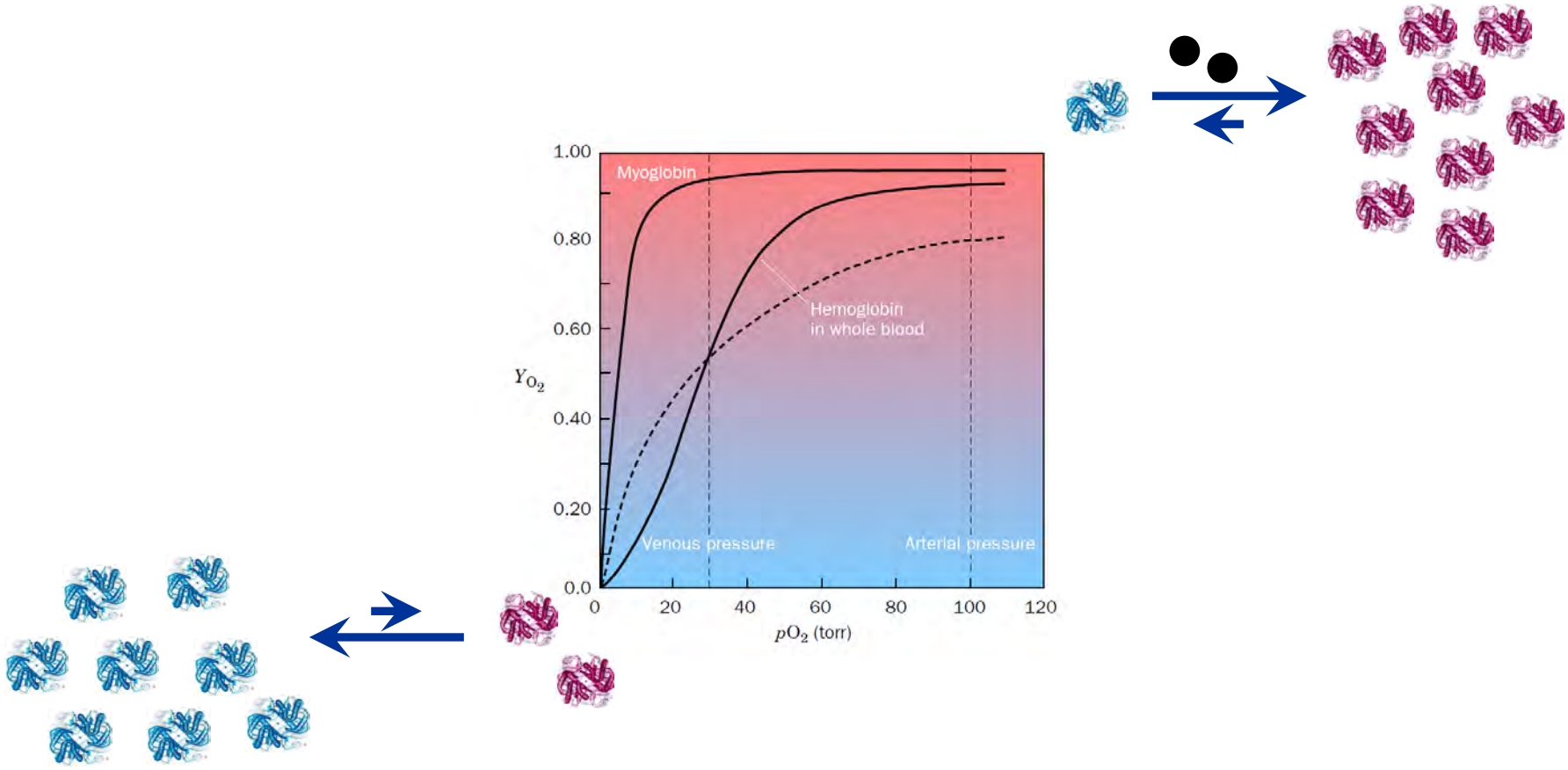
By binding to the T state, BPG creates another species – Hb-BPG. This creates a new equilibrium between BPG–bound Hb and BPG-free Hb…

BPG binds with higher affinity to the T state



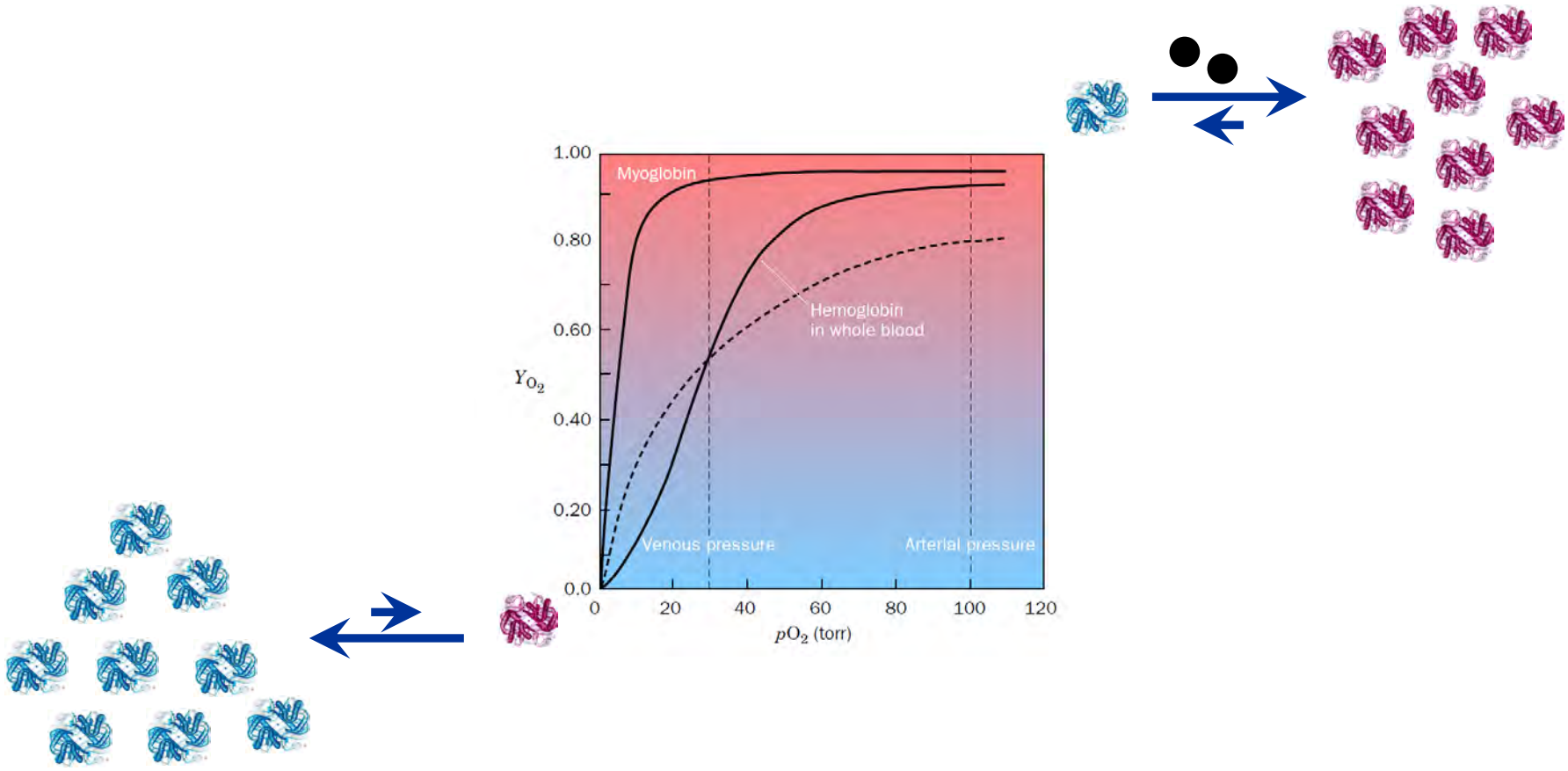
By binding to the T state, BPG creates another species – Hb-BPG. This creates a new equilibrium between BPG–bound Hb and BPG-free Hb… So overall the equilibrium between R and T states shifts in favor of the T state, which binds O_2 with low affinity

BPG binds preferentially to the T state



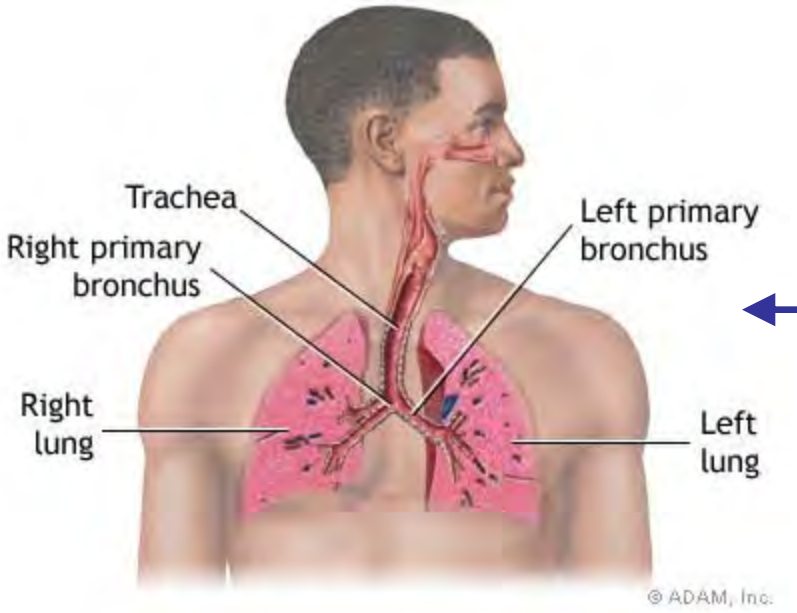
At normal concentrations of BPG (i.e. at sea level), there is an equilibrium between R and T states. At high elevation, more BPG is produced that binds to the T state…

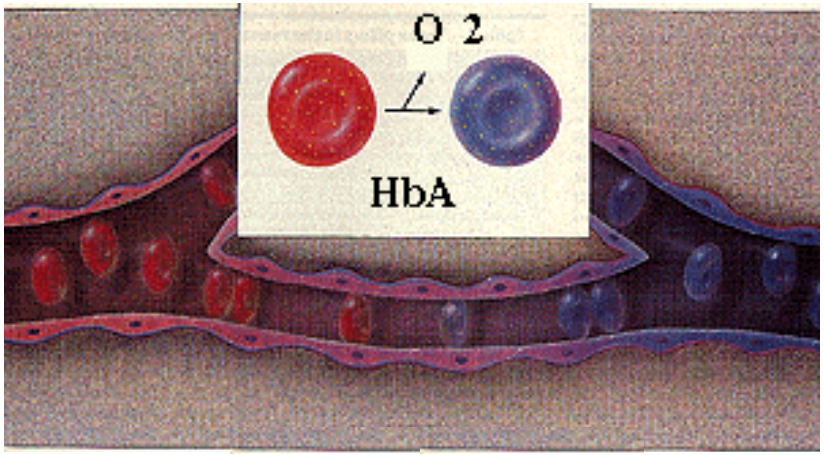
BPG binds preferentially to the T state



At normal concentrations of BPG (i.e. at sea level), there is an equilibrium between R and T states. At high elevation, more BPG is produced that binds to the T state, which shifts the equilibrium even more in favor of the T state. So there are even fewer high affinity R state Hb molecules. It thus take a higher concentration of O_2 to get sufficiently binding to R state Hb molecules to shift the equilibrium in favor of the R state. The binding curve is thus shifted to the right – leading to a higher effective *p50*.

BPG binds preferentially to the T state



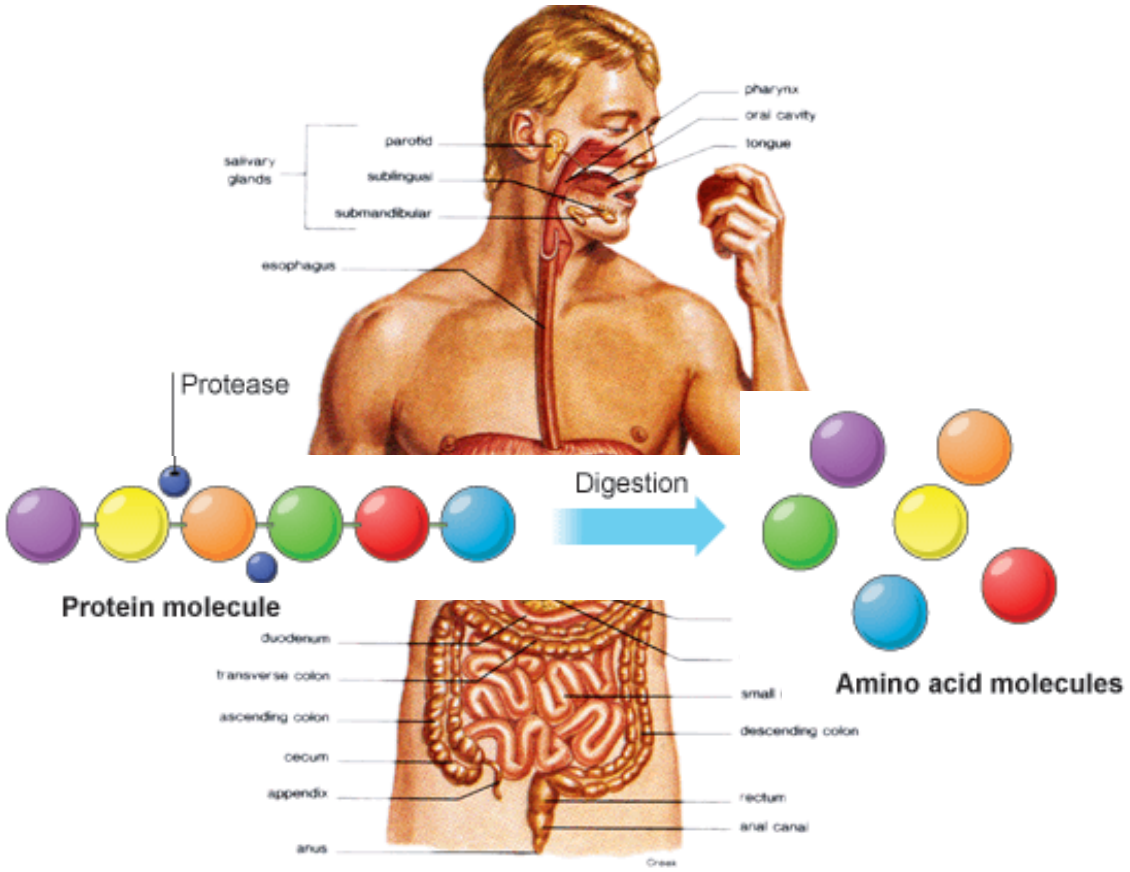


Hb is a finely tuned O_2 delivery machine, which is efficient and adapts to changes in environment. Cooperative O_2 binding is key to both oxygen delivery and the modulation of activity. Cooperativity arises because Hb exits in two states – a low affinity O_2 binding state that is more stable than the high O_2 affinity binding state.

By stabilizing more or less of the high affinity binding state, ligands, such as O_2 and BPG, shift the O_2 binding curves to lower or higher binding affinity.

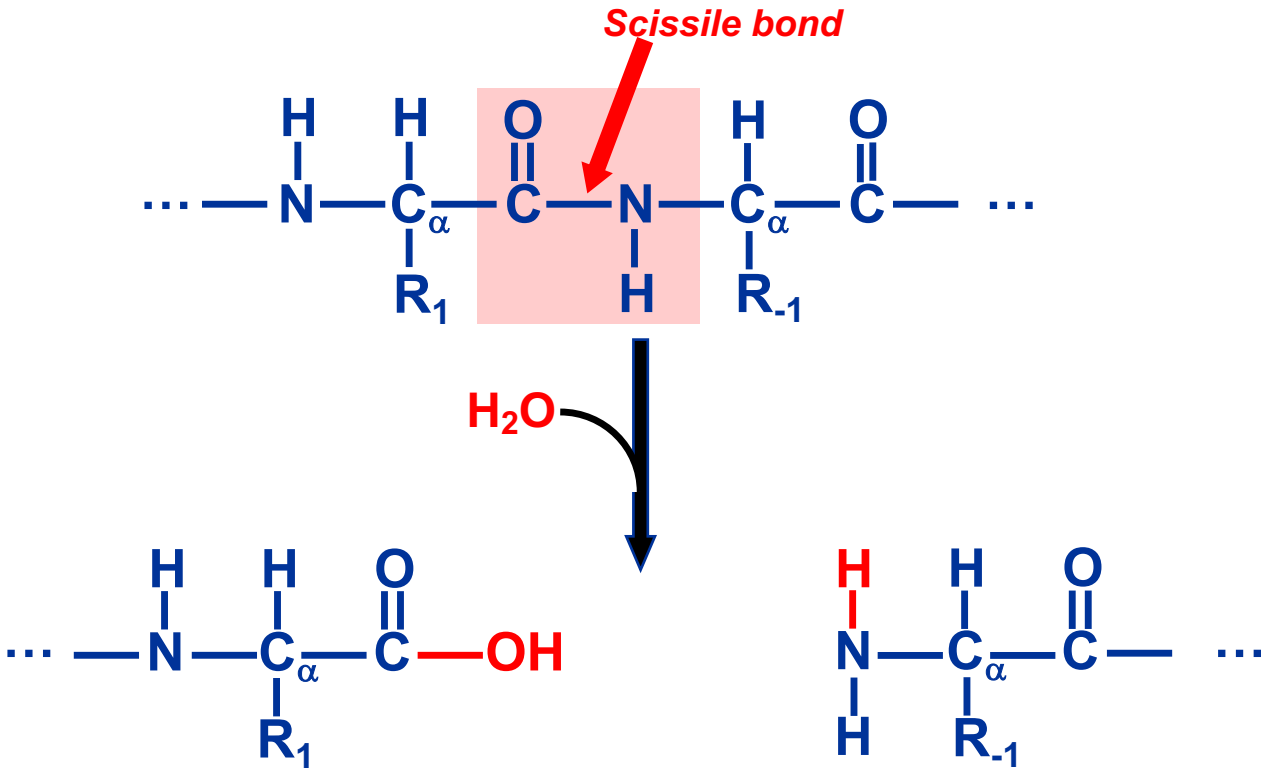
This type of mechanism is common to all (multi-subunit) proteins whose activity is allosterically (cooperatively) regulated.

Role of Water: Ligand Perspective
(Catalytic water molecules)



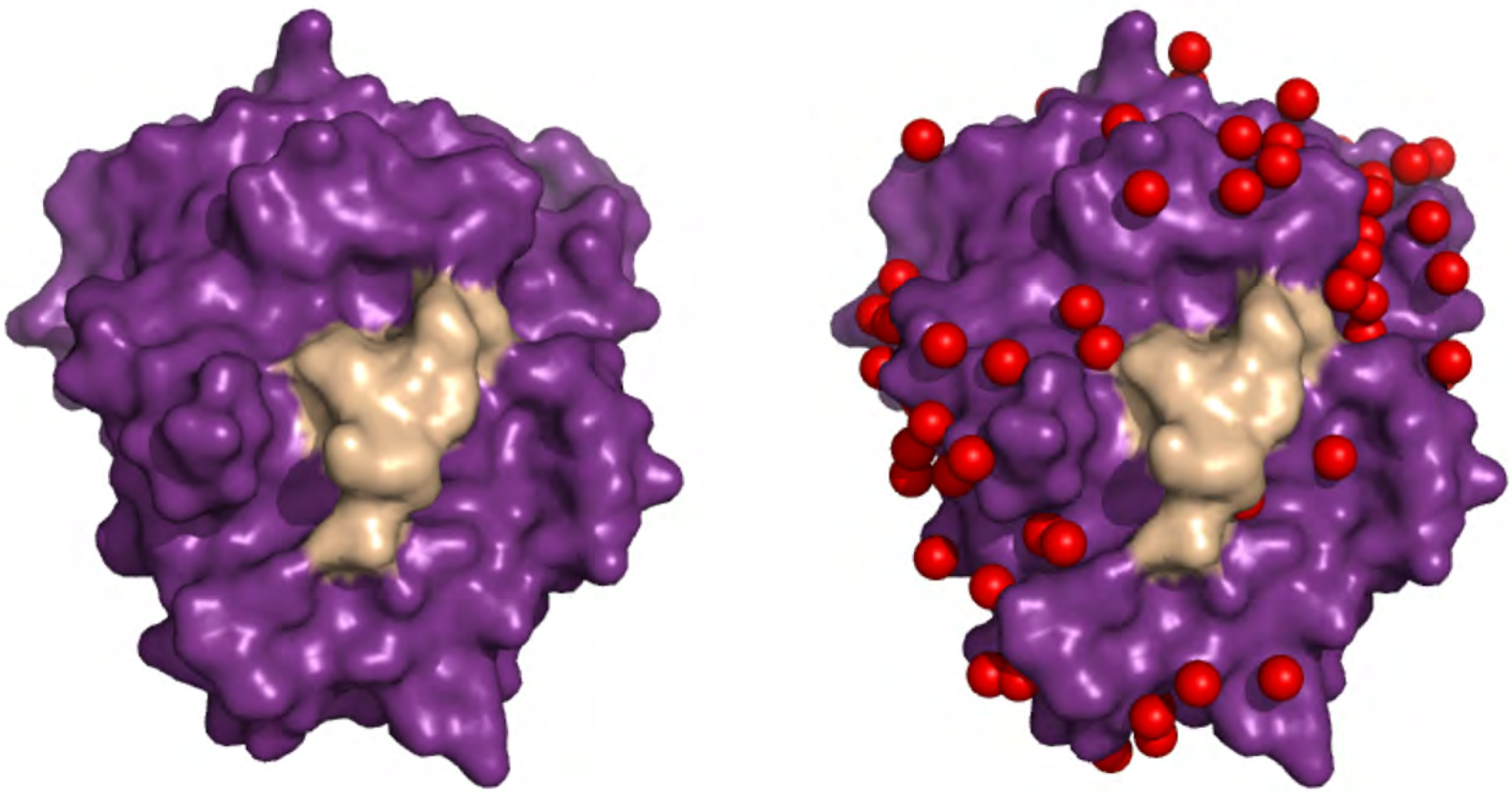
Serine proteases are…

…a family of digestive enzymes that catalyze the hydrolysis of peptide bonds



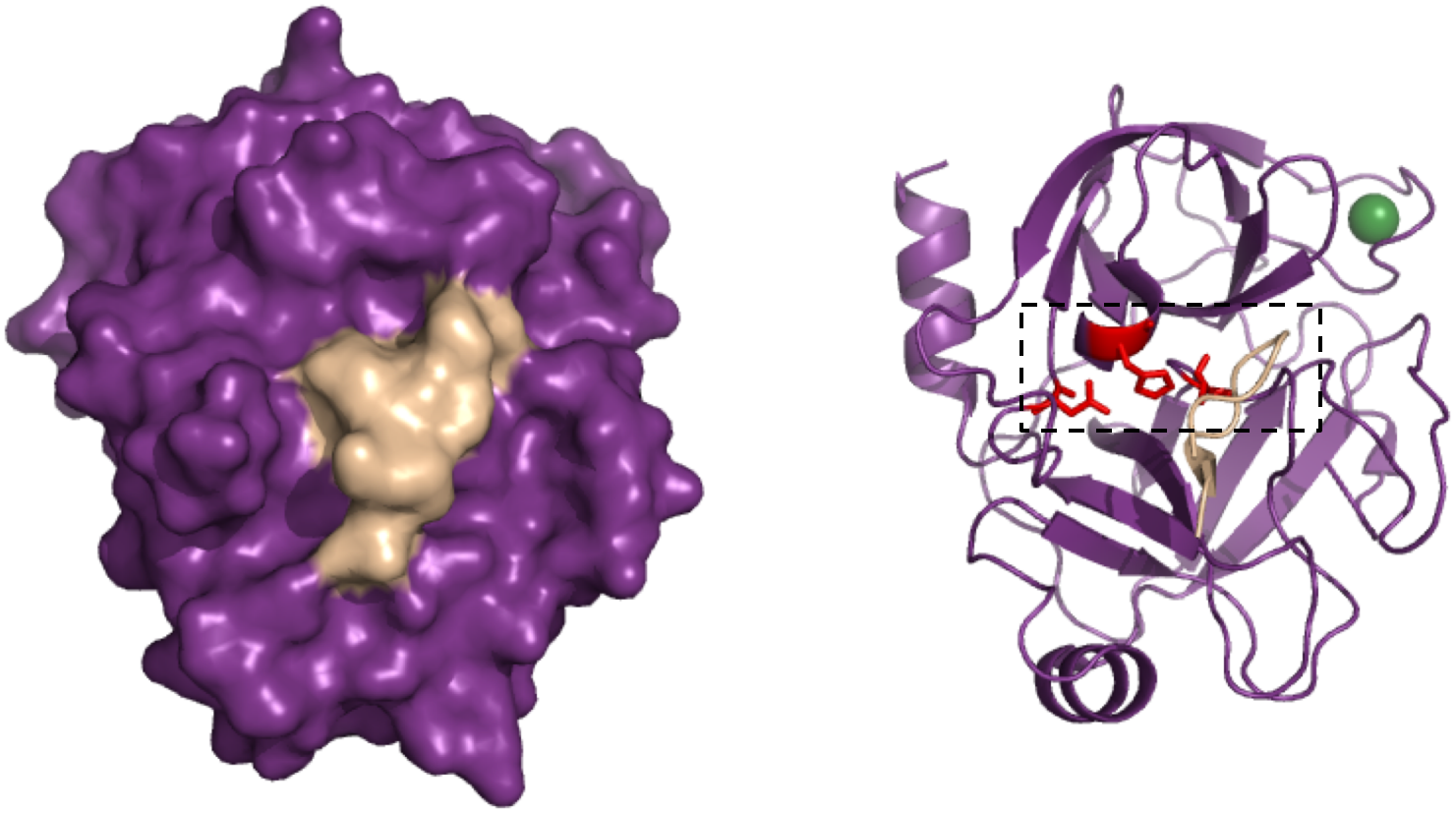
Serine proteases increase hydrolysis rates by ~10^{10}-fold. How do they do this?

Trypsin: a serine protease



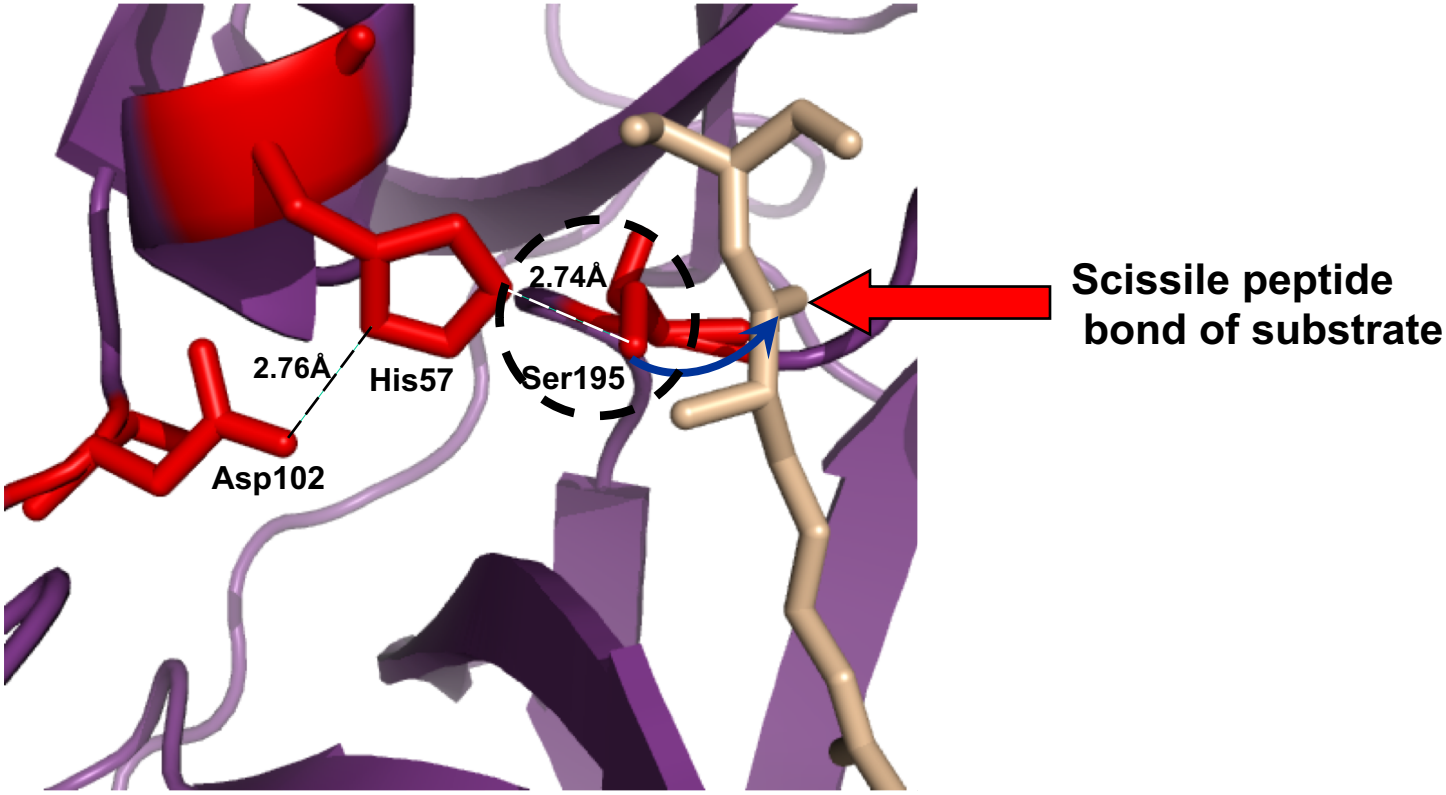
The crystal structure of trypsin was solved in 1967 and gave immediate insight into the mechanism of catalysis. The surface diagram on the left is of trypsin (purlple) bound to an 11 residue peptide inhibitor (tan). The same structure on the right shows the bound water molecules (red).

Trypsin: a serine protease



Surface (left) and cartoon/ribbon (right) representations of the trypsin structure. A bound Ca^{2+} is shown in green. Key residues in the active site are highlighted in red. We will zoom in on the box, which highlights residues close to the tan inhibitor

Trypsin: catalytic site



His57 hydrogen bonds with Ser195, which is perfectly positioned for catalytic attack on the peptide bond. Ser195 is highly reactive, suggesting it is an excellent nucleophile. *Why is Ser195 so reactive?*

Trypsin: water molecule at the catalytic site

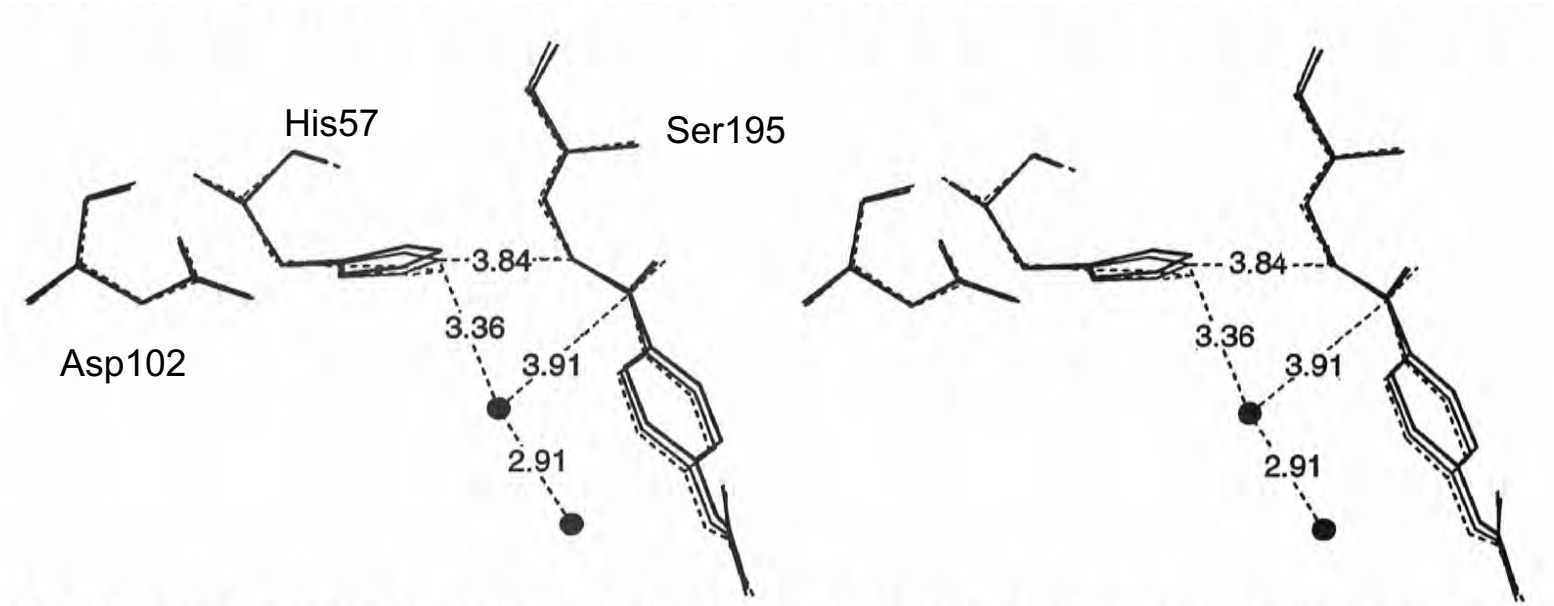
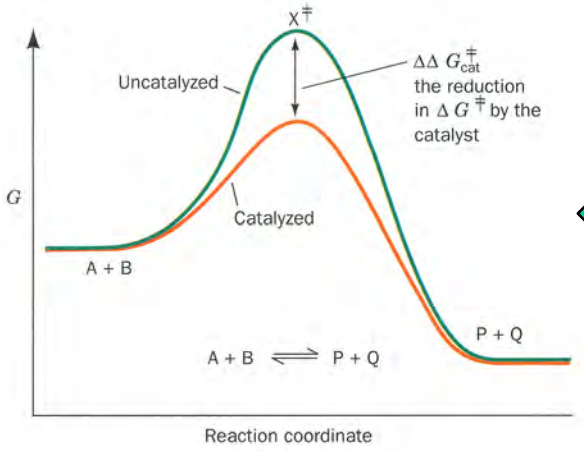
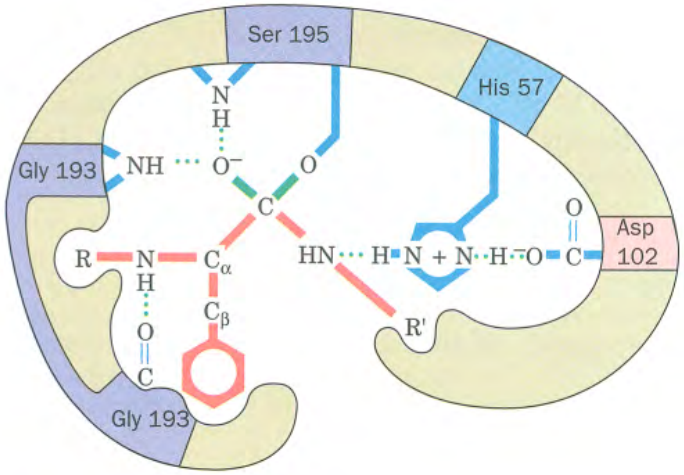


Fig. 3. Stereo images of contacts in the active-site region showing interatomic distances in angstroms for three structures: dashed, t_0; solid, t_3; and heavy line, t_{90}. Residue Asp^{102} is on the left, His^{57} in the middle, and guanidinobenzoyl Ser^{195} is to the right. The two water molecules shown are 1082 (upper dot) and 1051 (lower dot) from the t_3 structure (see Fig. 2B).

(Singer et al, Science, 1993)

Trypsin: catalytic mechanism

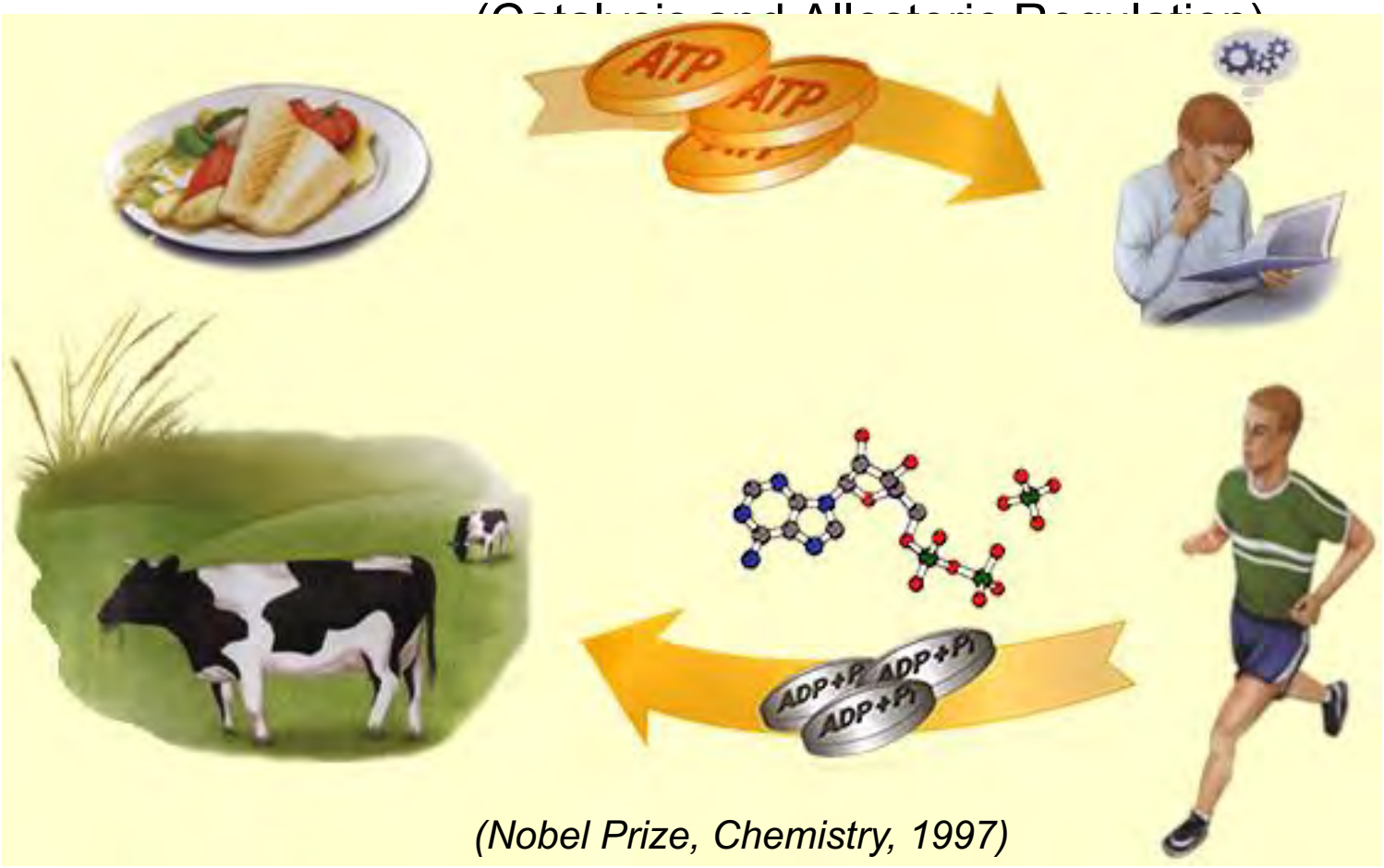




10^{10}-fold rate enhancement = 57.1 kJmol^{-1}

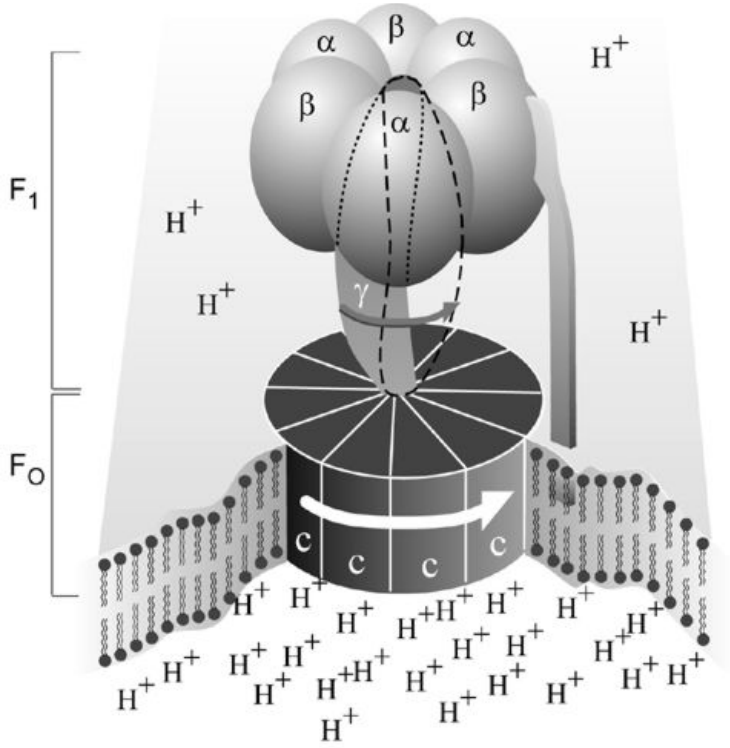
Enzyme-Substrate Transition State

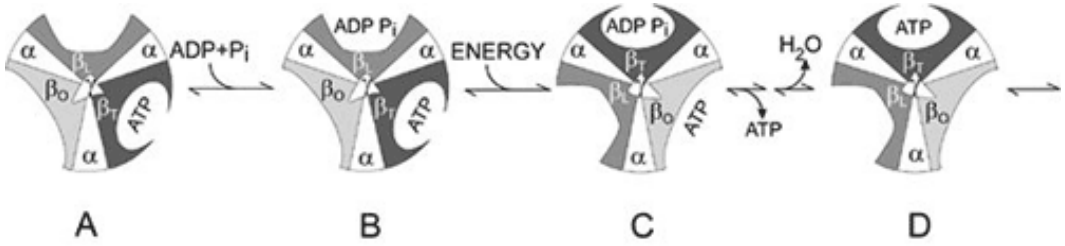
Role of ATP: Ligand Perspective



(Nobel Prize, Chemistry, 1997)

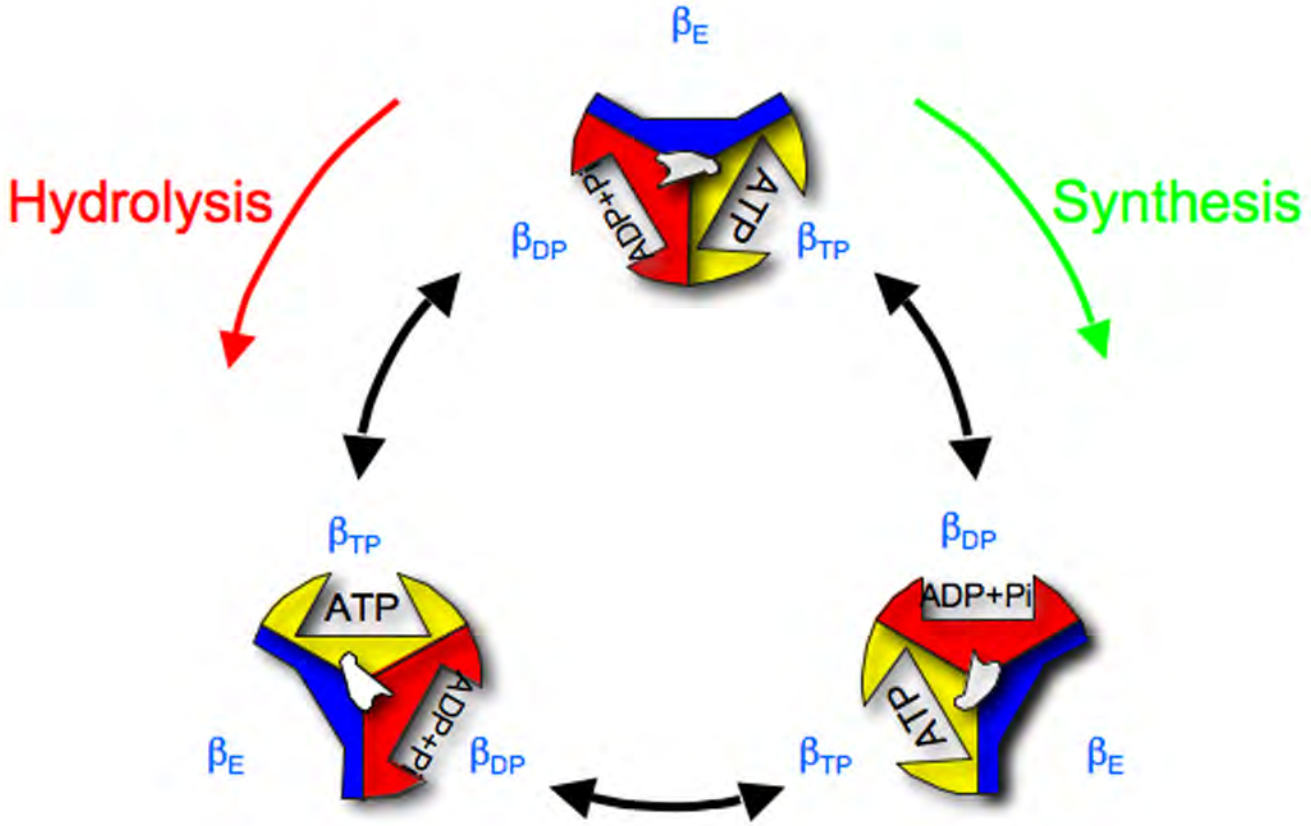
ATP Synthase (F-ATPase)





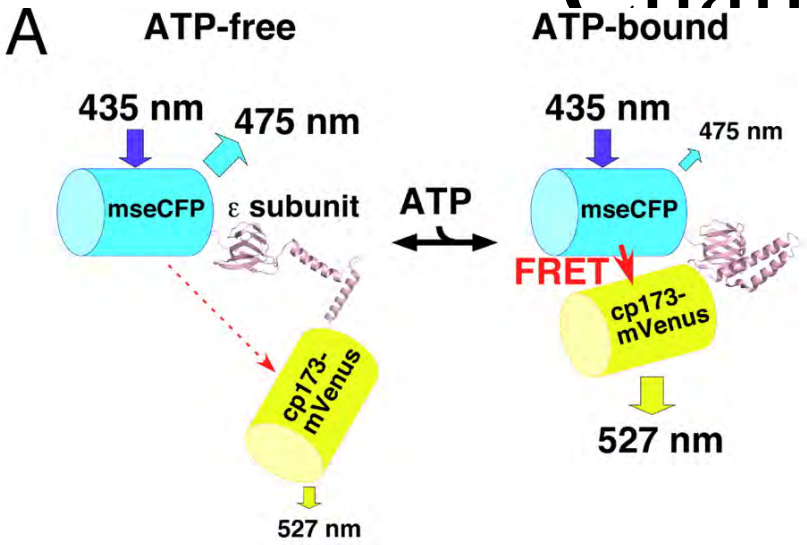
(Nobel Prize, Chemistry, 1997)

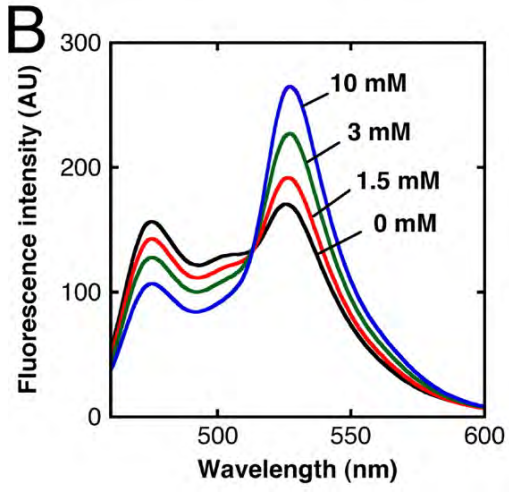
ATP Synthesis or Hydrolysis

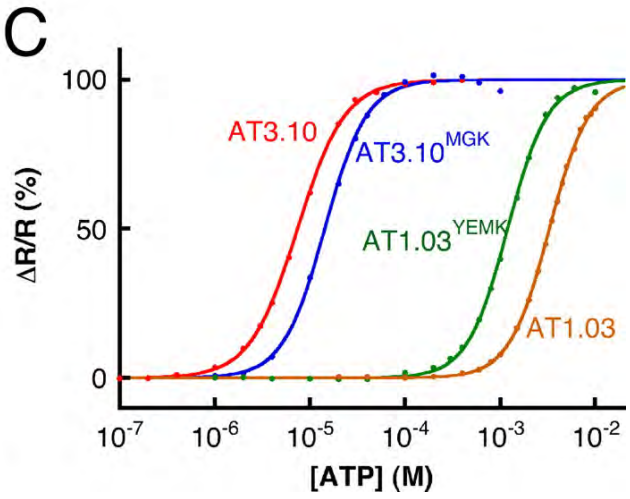


(Nakamoto et al, ABB, 2008)

ATP v.s. Protein Structural Change

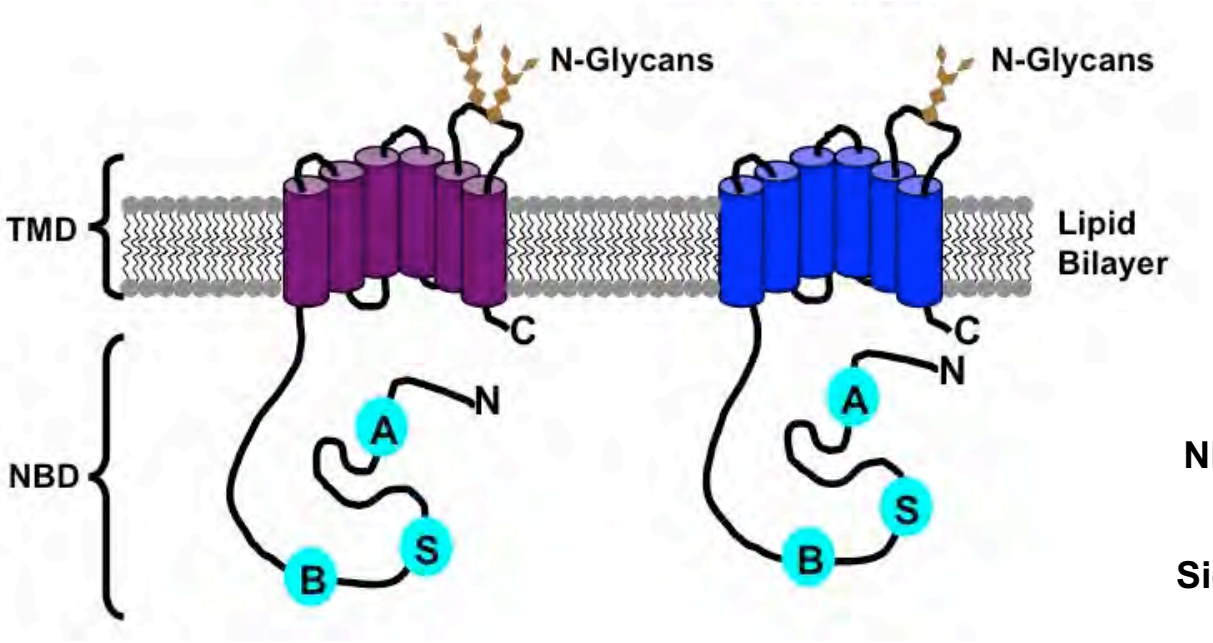


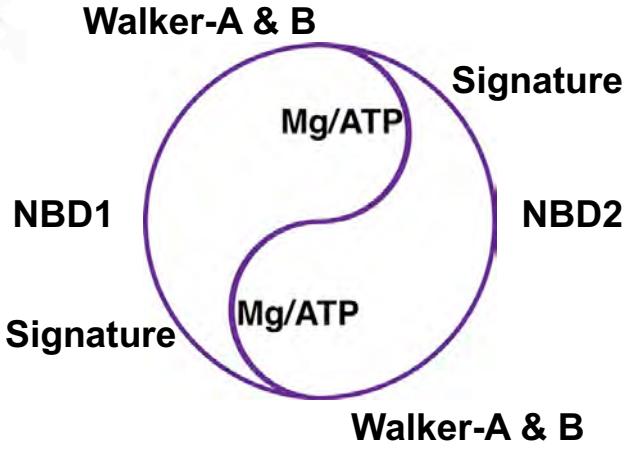




(Imamura et al, PNAS, 2009)

ATP-Binding Cassette (ABC) Proteins

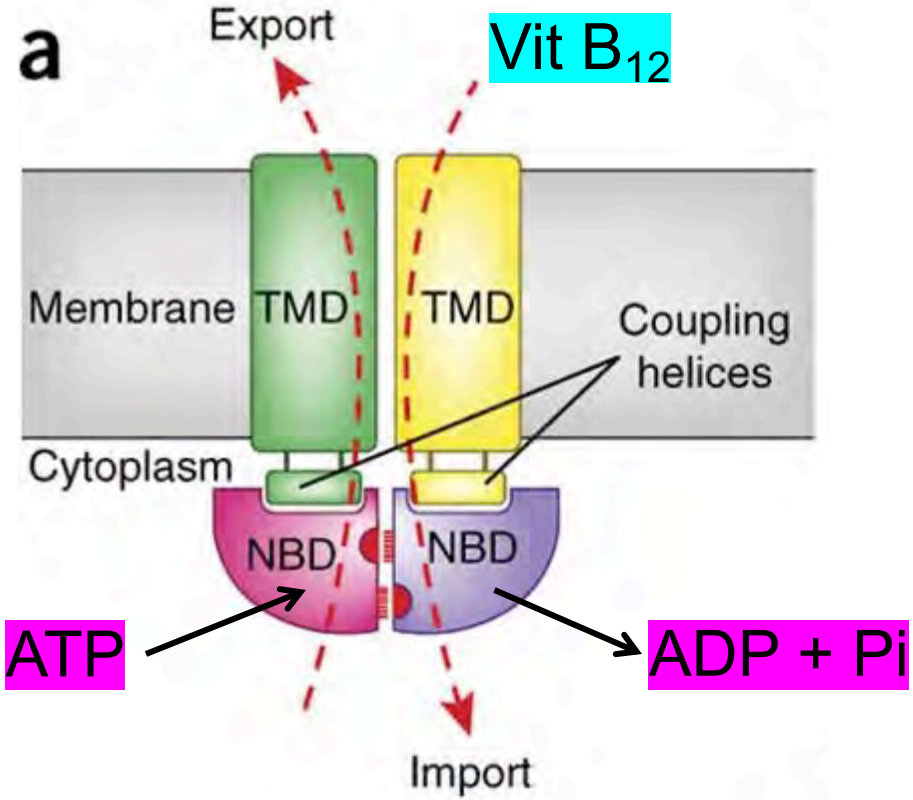




A: Walker A motif
(GxxGxGKS/T)
B: Walker B motif
(φφφφDE)

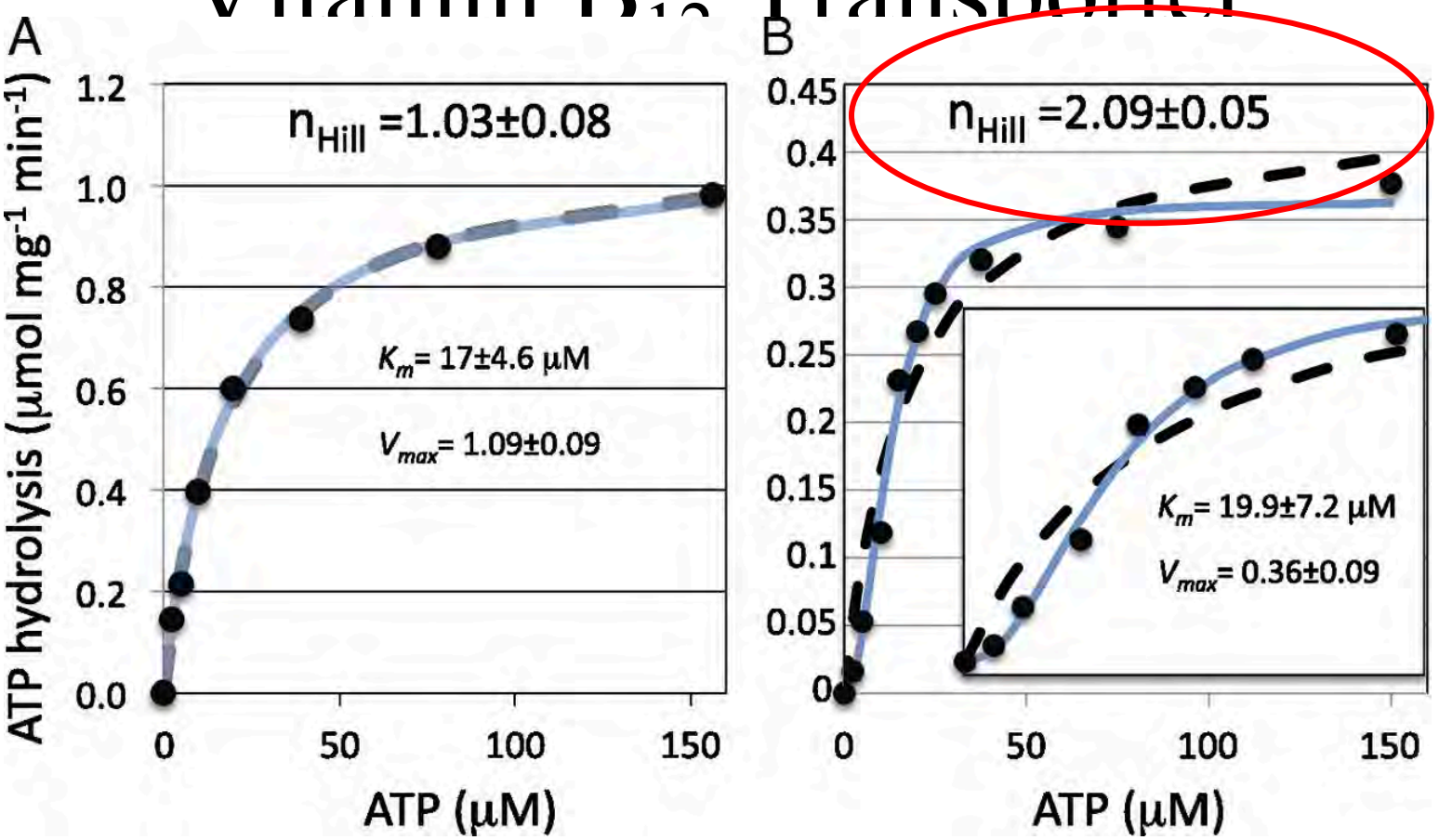
S: ABC signature motif
(φSGGQ/E)
φ: hydrophobic amino acids

Vitamin B_{12} Transporter



(Locher, Nat Struct Mol Biol, 2016)

Vitamin B_{12} Transporter



(Tal et al, PNAS, 2013)