Protein Structure, Function and Disease

Energetics of protein function: 1) hemoglobin and 2) serine protease

(Partially adopted from Prof. John Baenziger's former lectures)

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PART I: THERMODYNAMICS OF O2 BINDING TO HEMOGLOBIN

Protein Interaction with Other Molecules

- When ligands bind to proteins, conformational changes occur to permit tighter binding of the ligands:

  Induced Fit

- In multi-subunit proteins, conformational change of one subunit often affect others.

- Enzymes (protein catalysts) bind and transform other molecules. These ligands are also called substrates.

- The ligand (substrate)-binding site is called active site (or catalytic site).

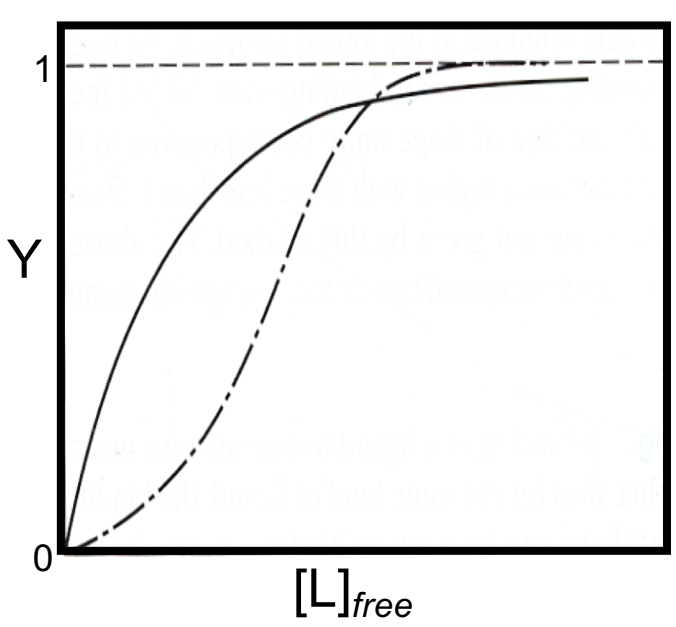
Mb and Hb classic examples of independent vs cooperative binding

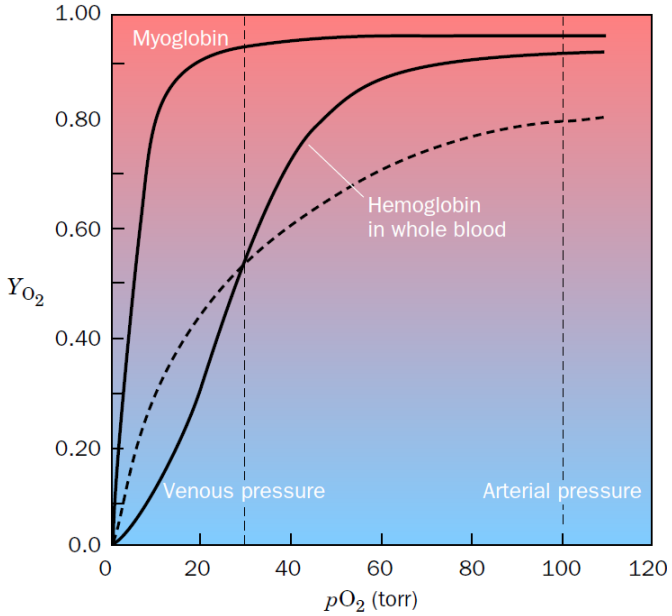
$$r = \frac{n[L]_{free}}{K_D + [L]_{free}}$$

——— 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.

Gibb's free energy

$$A + B \longrightarrow C$$

$$\Delta G_{rxn} = \Delta H - T\Delta S$$

The Gibb's free energy equation governs all biological phenomenon that occur inside a cell. This equation tells us that a reaction or interaction A+B interacting to form C will proceed if the product of the reaction/interaction has a lower energy ($\Delta G_{rxn} < 0$) than the initial reactants.

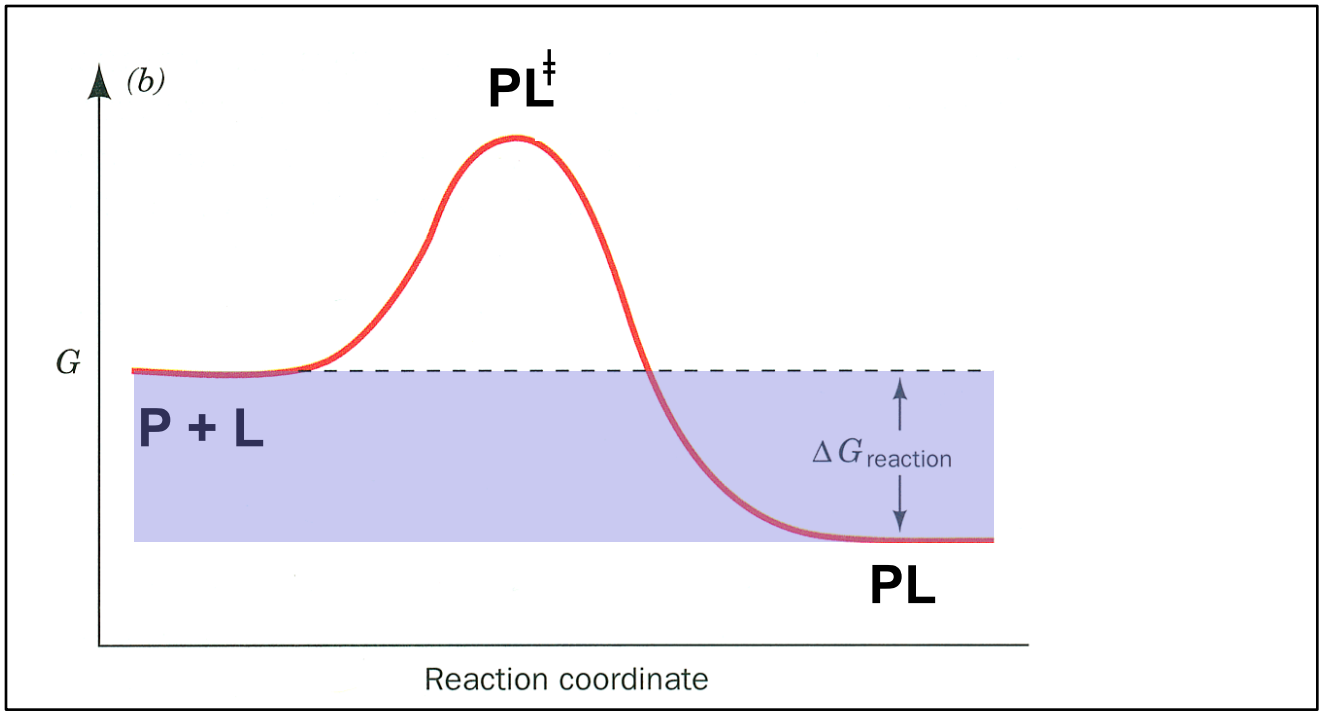
If $\Delta G_{rxn} < 0$, then the reaction favors the formation of C

For $\Delta G_{rxn} < 0$, the sum of the change in enthalpy and the change in entropy (i.e. the totality of the interactions) must be less than zero.

(ΔH: enthalpy; ΔS: entropy)

Activation Energy, K_{eq} and K_D

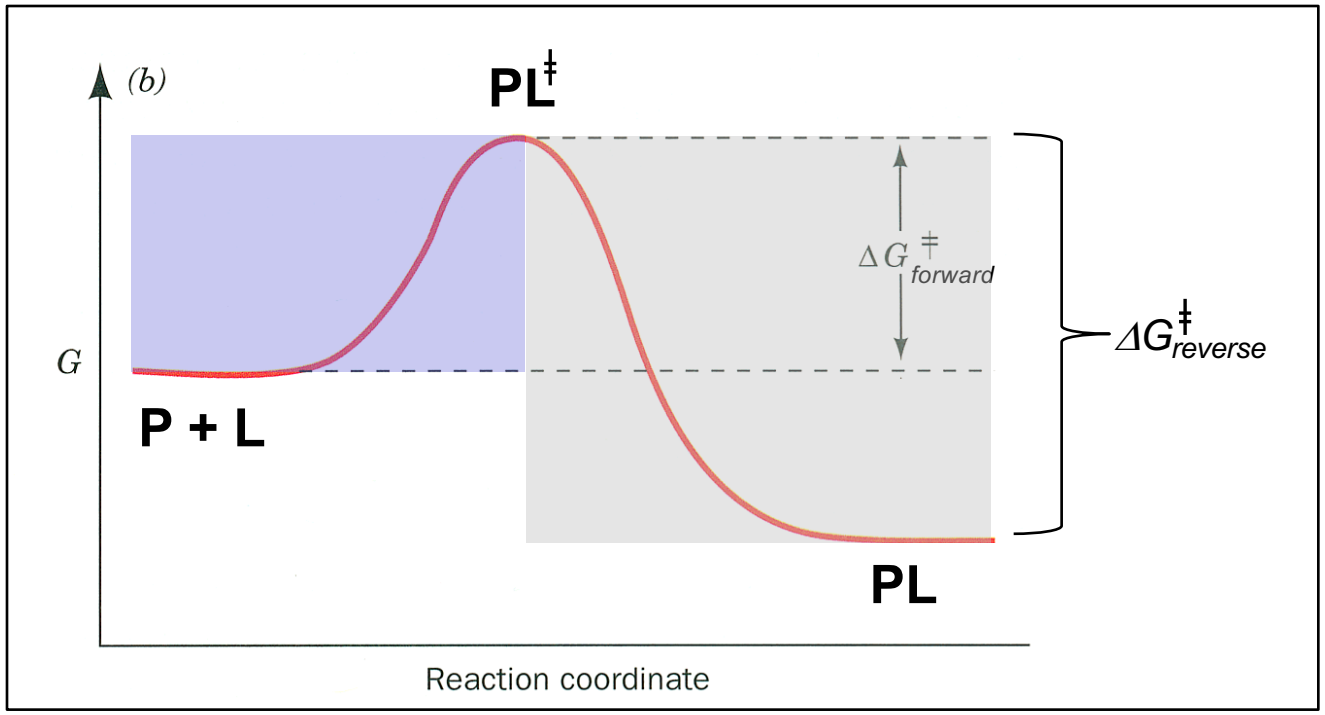
$$P + L \underset{}{\overset{K_{eq}}{\rightleftharpoons}} PL$$



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

Activation Energy, K_{eq} and K_D

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

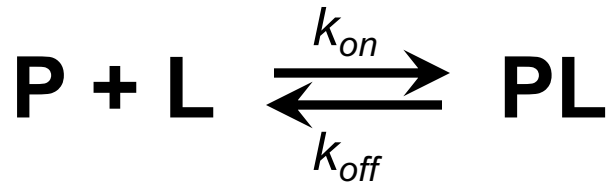


k_{on} is related to the "activation energy", $\Delta G^{\ddagger}_{forward}$

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 ($PL^{\ddagger}$)

Activation Energy, K_{eq} and K_D

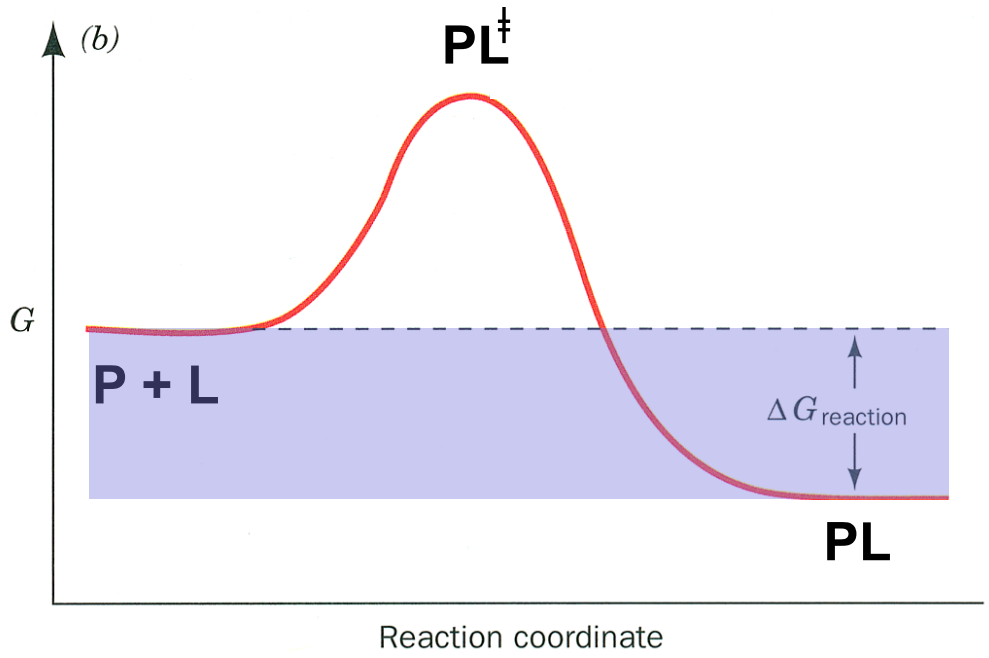


$$K_{eq} = \frac{[PL]}{[P]_{free}[L]_{free}}$$

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

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).

Gibbs Free Energy (ΔG_{rxn}) and Equilibrium Constant (K_{eq} or $1/K_D$)

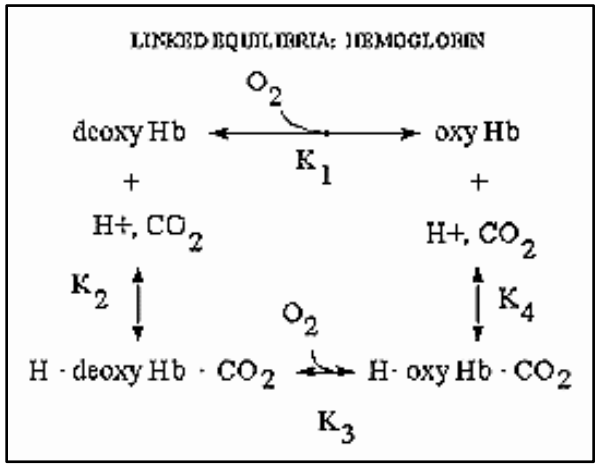


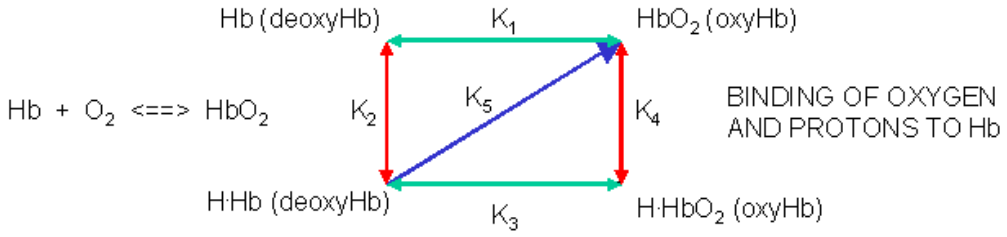
$$P + L \underset{}{\overset{K_{eq}}{\rightleftharpoons}} PL$$

"Thermodynamics" refers to the equilibrium condition defined by the equilibrium constant, K_{eq}. K_{eq} is mathematically related to the change in Gibbs free energy between reactants and the products, ΔG_{rxn}:

$$\Delta G_{rxn} = -RT\ln K_{eq}$$ (T= temperature in K, R=8.3145 $Jmol^{-1}$).

Binding and Linked Equilibria

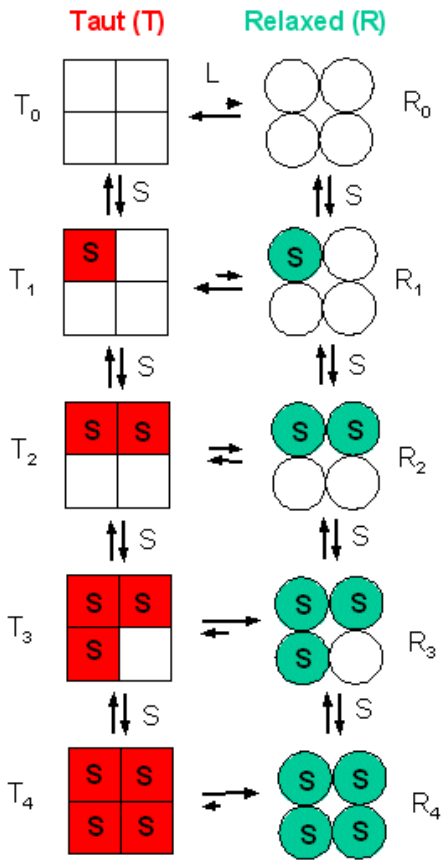




E.g., ΔG_{rxn} for K_1, K_2, K_3, …?

Cooperative Binding (induced fit) in Haemoglobin

MWC Model



$L = [To]/[Ro] >> 1$

$K_T = [T_n][S]/[T_{n+1}]$ n=#S bound

$K_R = [R_n][S]/[R_{n+1}]$

$K_R << K_T$

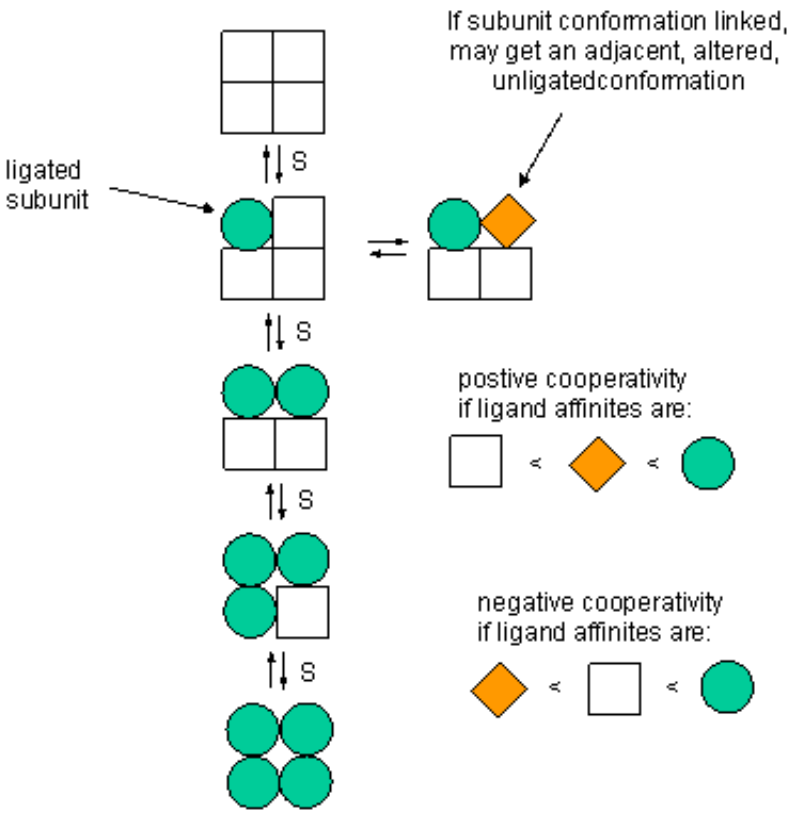
Substrate binding
Pulls equilibrium
From T → R

$c = K_R/K_T$

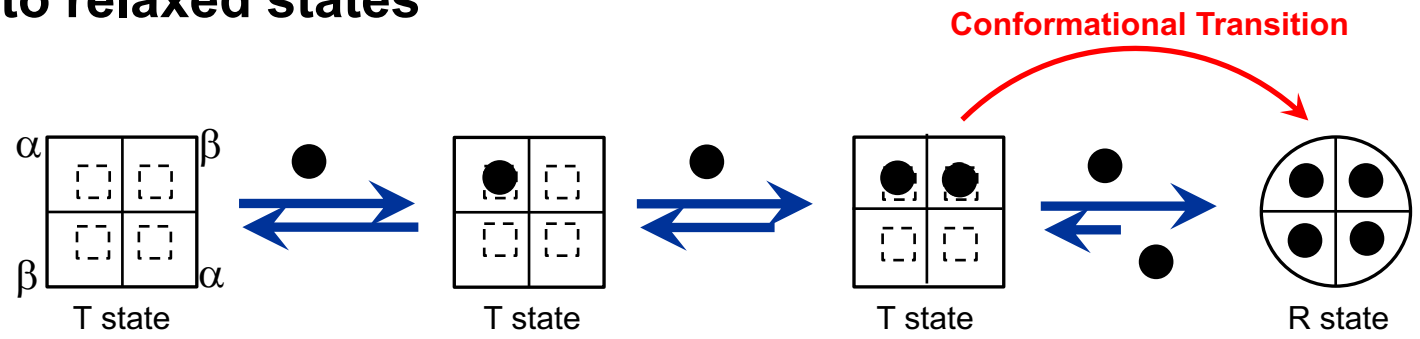
$[O2]/K_R = \alpha$

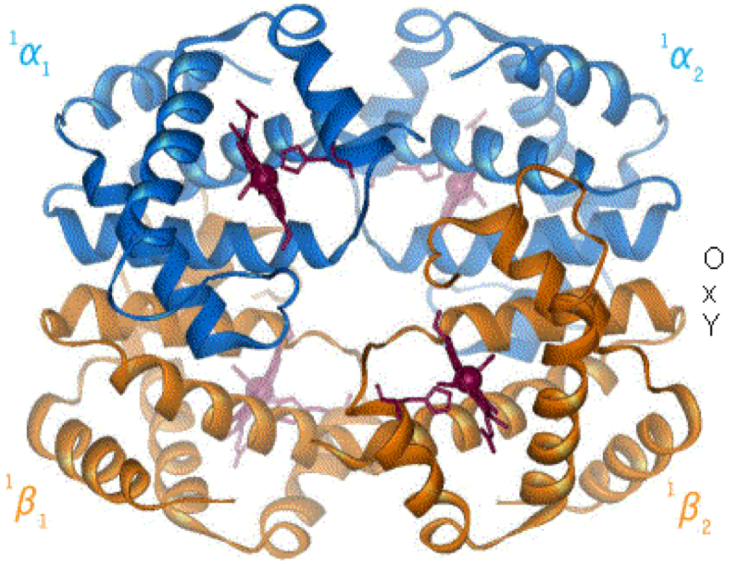
$$Y = \frac{(\alpha)(1-\alpha)^{n-1} + Lc\alpha(1+c\alpha)^{n-1}}{(1+\alpha)^n + L(1+c\alpha)^n}$$

KNF Model



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

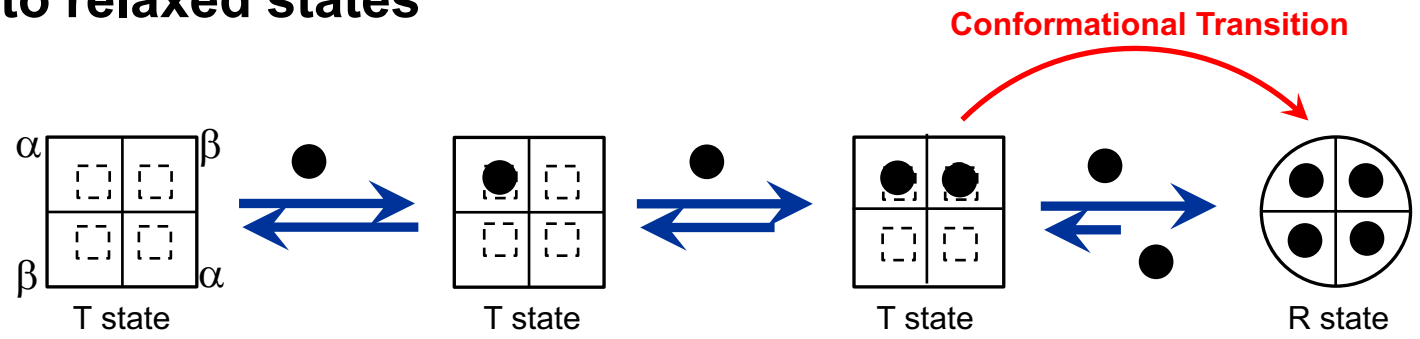


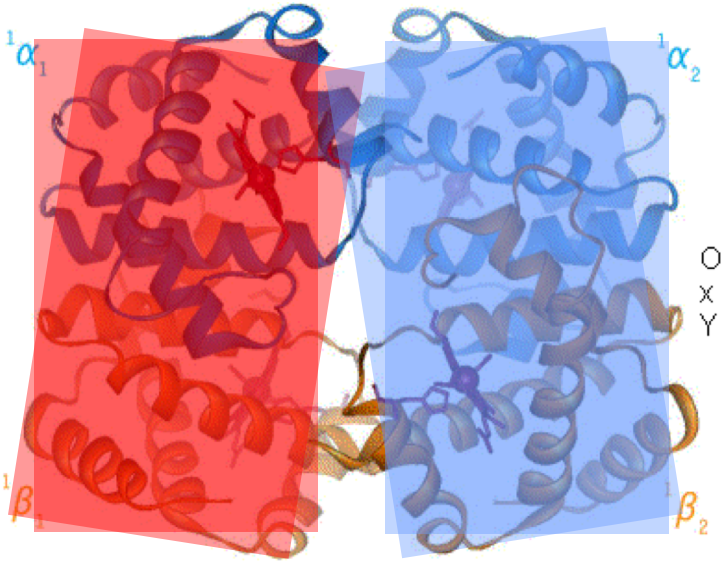


Hb is a dimer of αβ dimers ($\alpha_1\beta_1$ and $\alpha_2\beta_2$). The α_1/β_1 and α_2/β_2 interfaces are tighter than the interface between the $\alpha_1\beta_1$ and $\alpha_2\beta_2$ dimers. O_2 binding causes the $\alpha_1\beta_1$ dimer to move relative to the $\alpha_2\beta_2$ dimer. This change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





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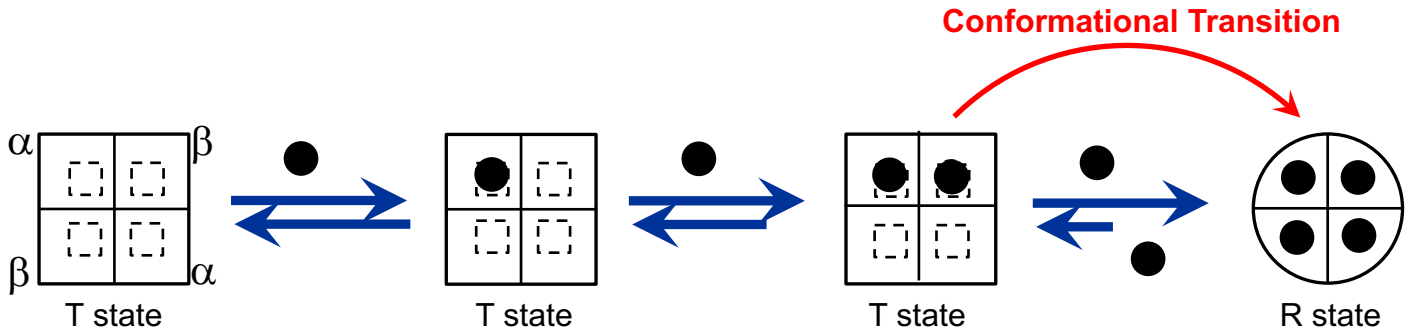
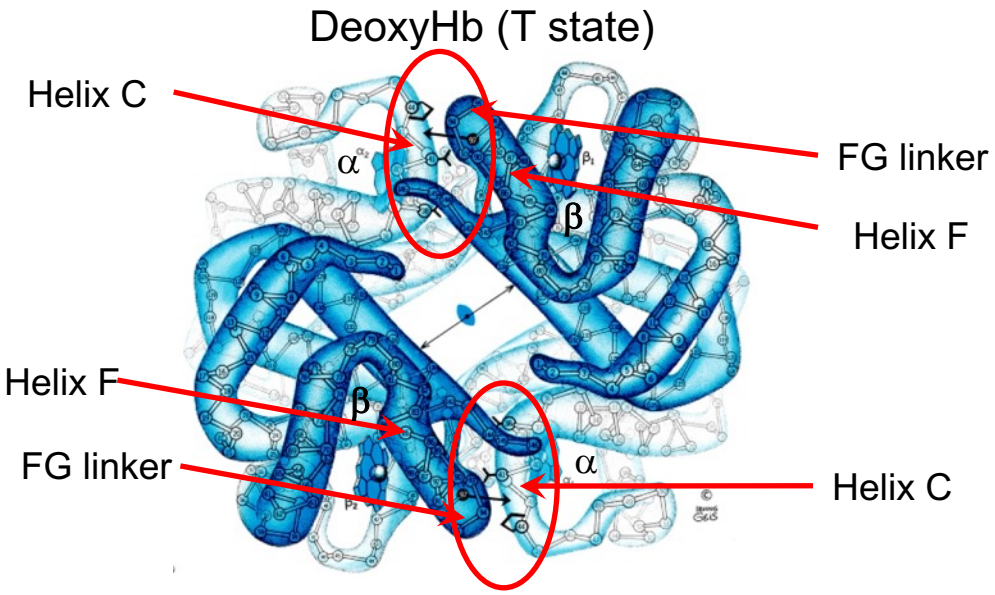
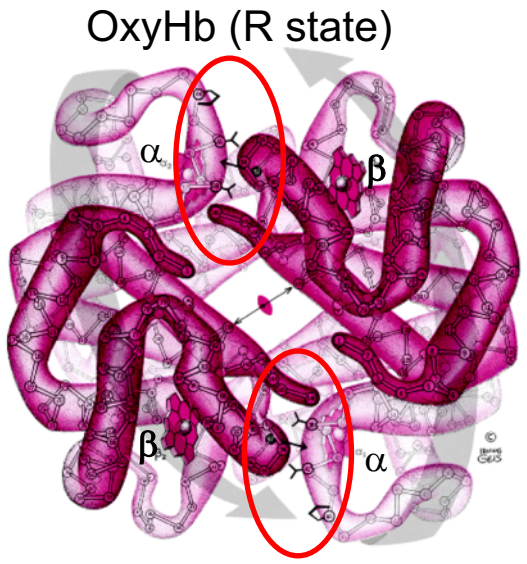
R and T states are governed by ridges into grooves packing

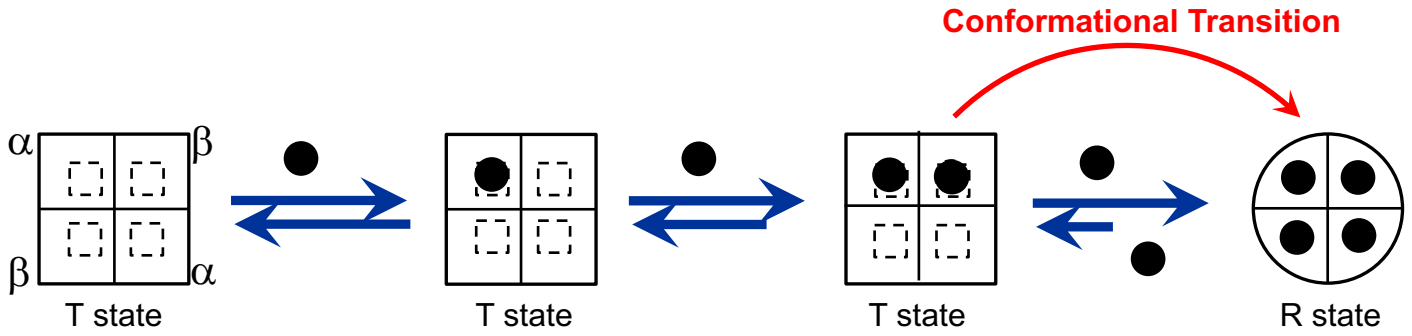




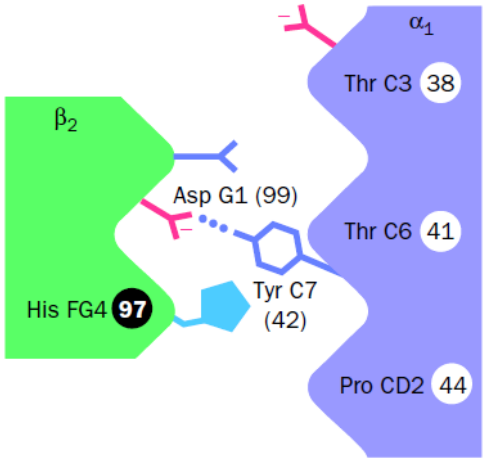


The linker between helix F and G (FG) of β interacts with helix C on the adjacent α subunit. The interactions between F/FG and C change upon O_2 binding.

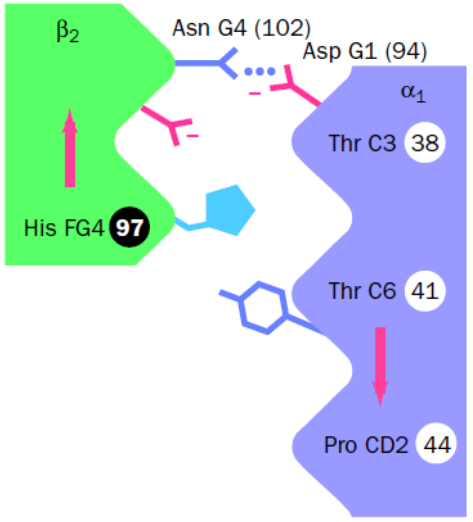
R and T states are governed by ridges into grooves packing



DeoxyHb (T state)

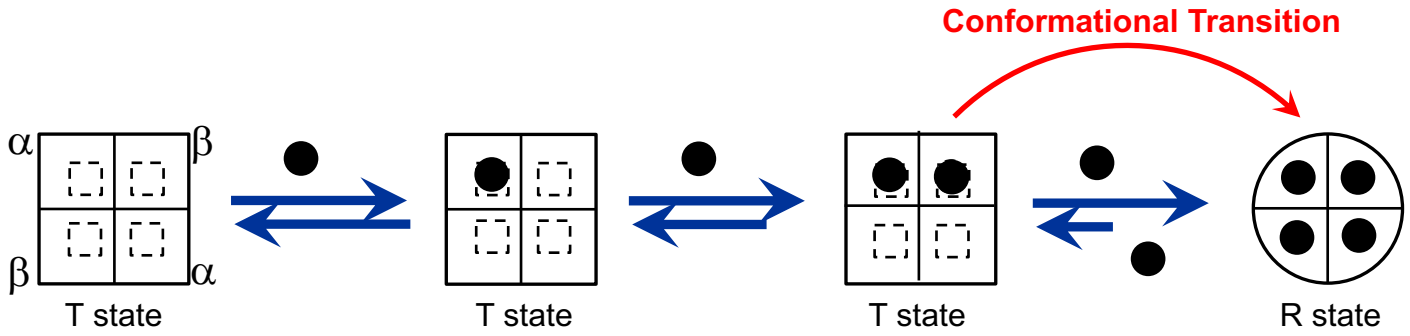


OxyHb (R state)

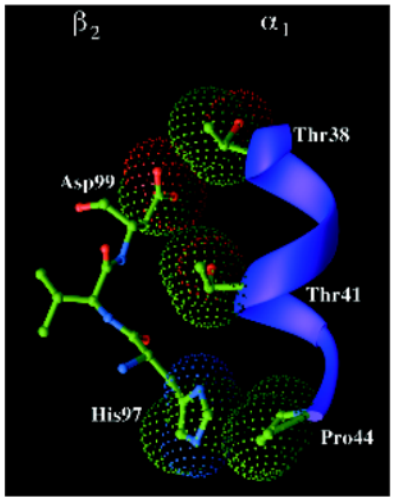


Upon O_2 binding, helix F shifts so that it interacts with helix C one turn further along. HisFG4 97 moves from groove between ProCD2 & ThrC6 in T state, to the groove between ThrC6 & ThrC3 in the R state. *There is a shift of the ridges into grooves packing*.

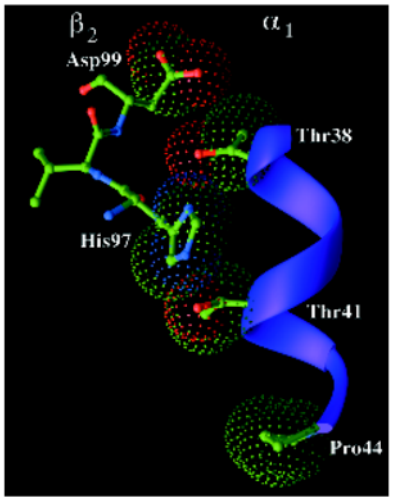
R and T states are governed by ridges into grooves packing



DeoxyHb (T state)



OxyHb (R state)



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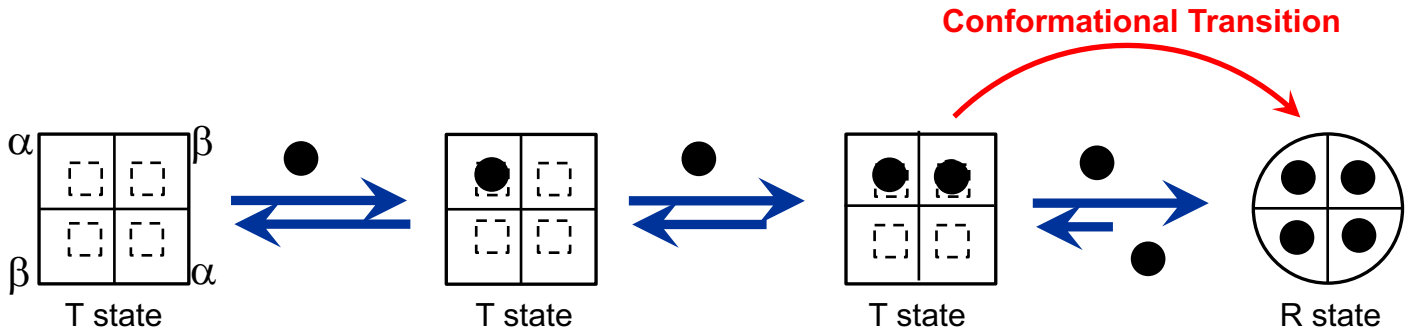
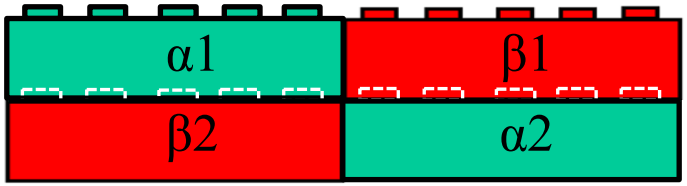
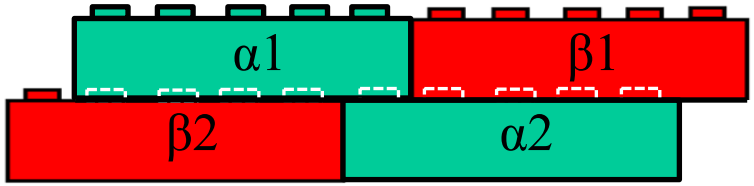
R and T states are governed by ridges into grooves packing



DeoxyHb (T state)

OxyHb (R state)

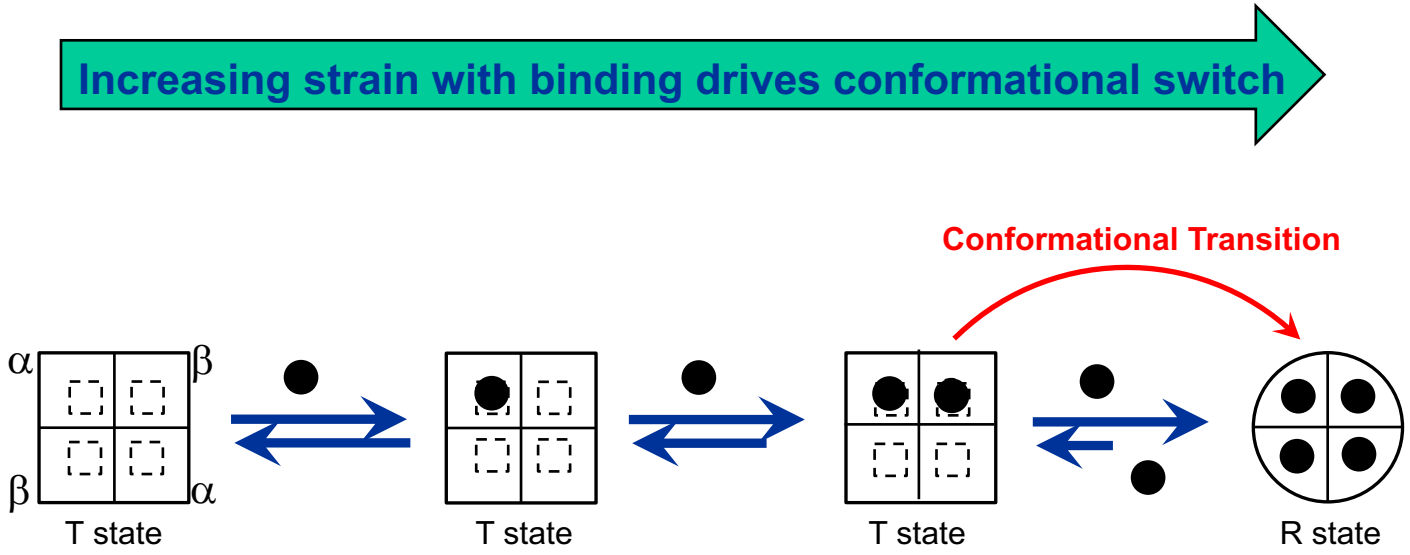




Ridges into grooves packing defines two states, as shown here schematically with the Lego blocks. Switching from T to R is all or nothing – **all four subunits must move together in concerted (i.e. all together) versus sequential (one subunit at a time) motions**.

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:



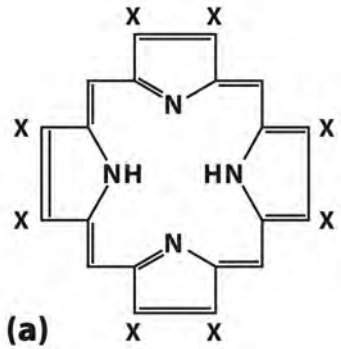
What is behind the driving force for the conformational switch? We need to understand this from both a *structural* and a *thermodynamic perspective*. Let's first look at how O_2 binding structurally promotes the conformational switch.

O_2-Binding to Myoglobin and Hemoglobin

- O_2 dissolves poorly in aqueous solutions.
- Its diffusion is not effective.
- Evolution of larger animals needed evolution of proteins that carry O_2.
- However, none of the aa can reversibly bind O_2.
- There is a need for transition elements like Fe and Cu which can do such job.
- Fe is incorporated into a protein-bound prosthetic group called "**heme**".

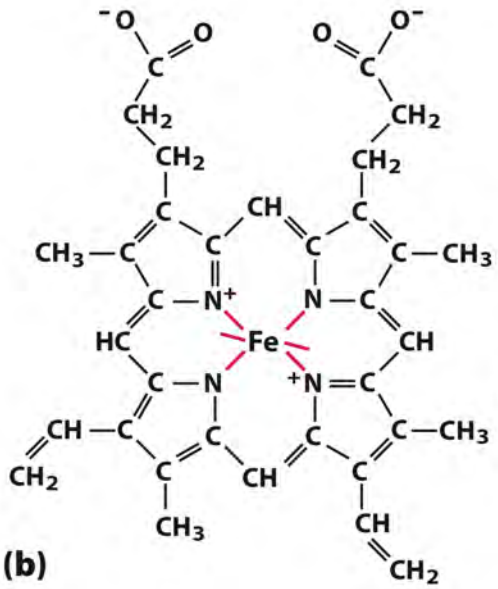
Porphyrin and Heme

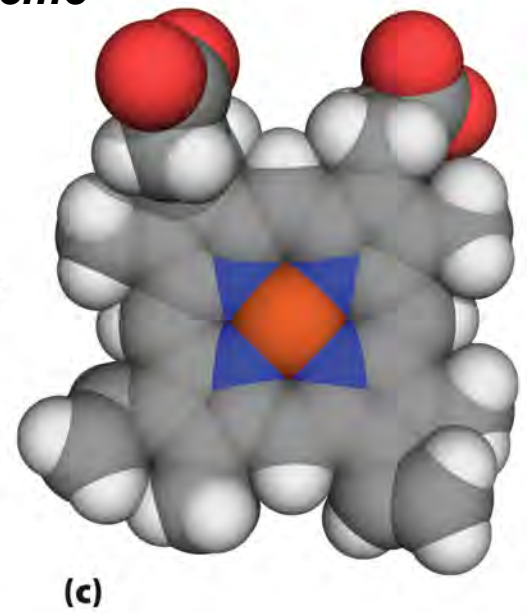
Porphyrin family

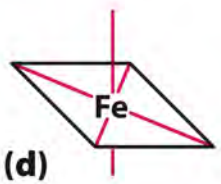


four pyrrole rings linked by methene bridges

Heme







The iron atom of heme has six coordination bonds: four in the plane of, and bonded to, the flat porphyrin ring system, and two perpendicular to it

Figure 5-1
Lehninger Principles of Biochemistry, Sixth Edition

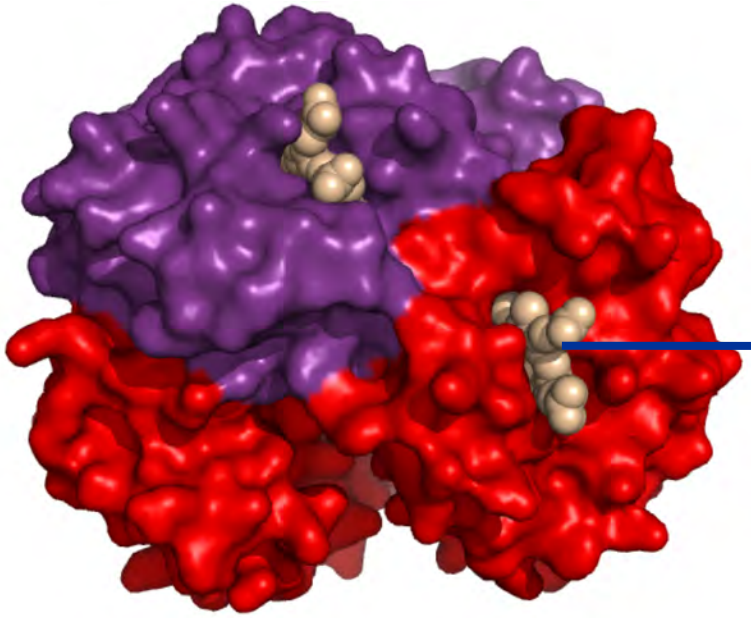
Carbon monoxide (CO) and Heme

- CO has similar size and shape to O_2.
- It can fit to the same binding site.
- CO binds over 20,000 times more than O_2 because the carbon in CO has a filled lone electron pair that can be donated to vacant d-orbitals on the Fe^{2+}.
- Protein pocket decreases affinity for CO, but it still binds about 250 times better than oxygen.
- CO is highly toxic as it competes with oxygen. It blocks the function of heme-bound proteins, such as myoglobin, hemoglobin, and mitochondrial cytochromes that are involved in oxidative phosphorylation.

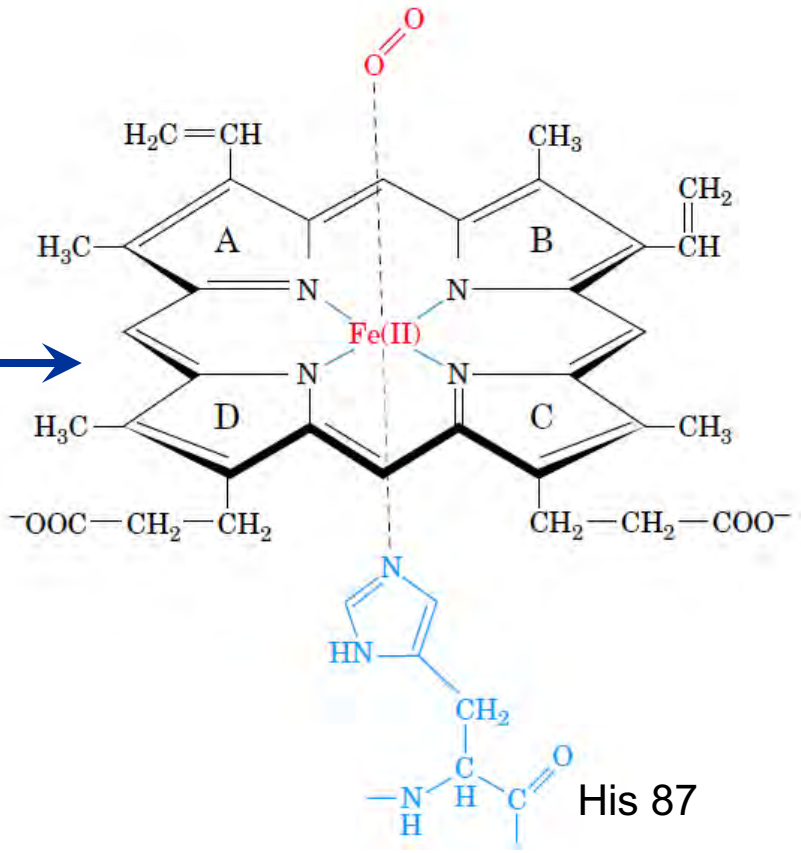
Carbon monoxide (CO) and Heme

Figure 5-5ab
Lehninger Principles of Biochemistry, Sixth Edition

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



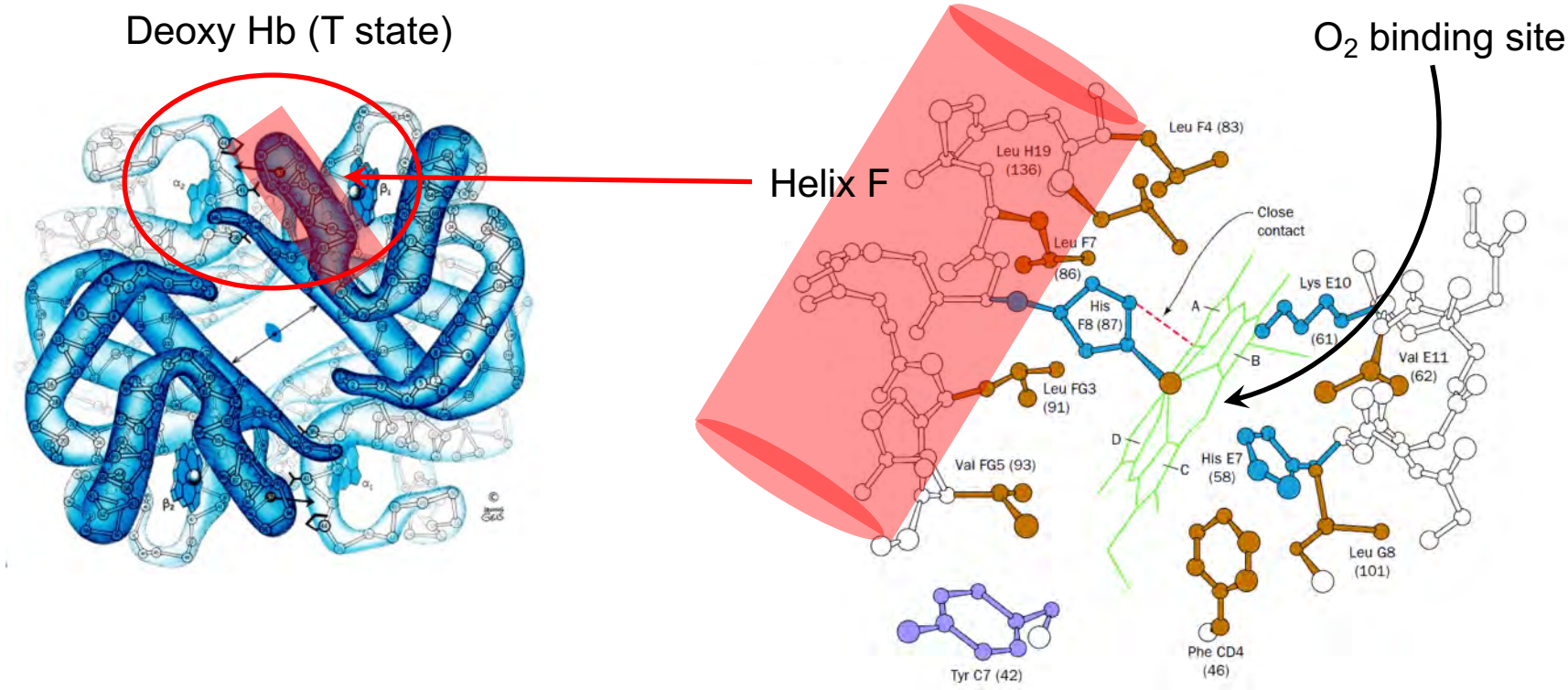
Hemoglobin



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!

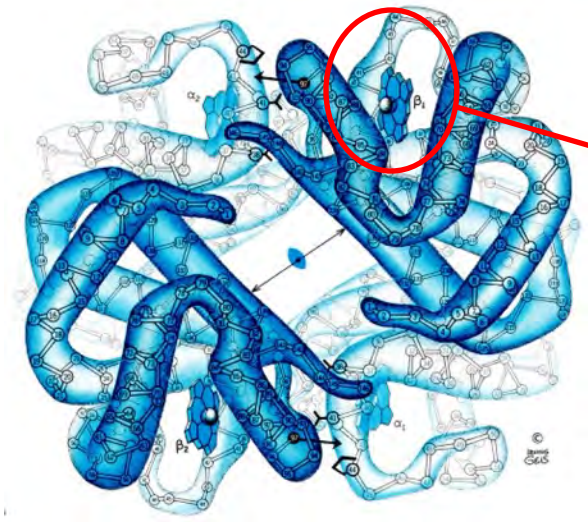
O_2 binds to heme – which is the porphyrin ring and Fe^{2+}



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!

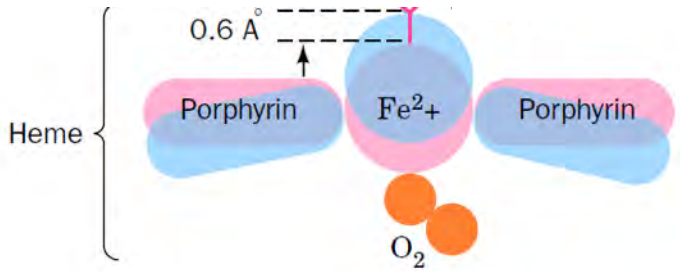
O_2 leads to a structural change in the heme group

Deoxy Hb (T state)



Oxy R state

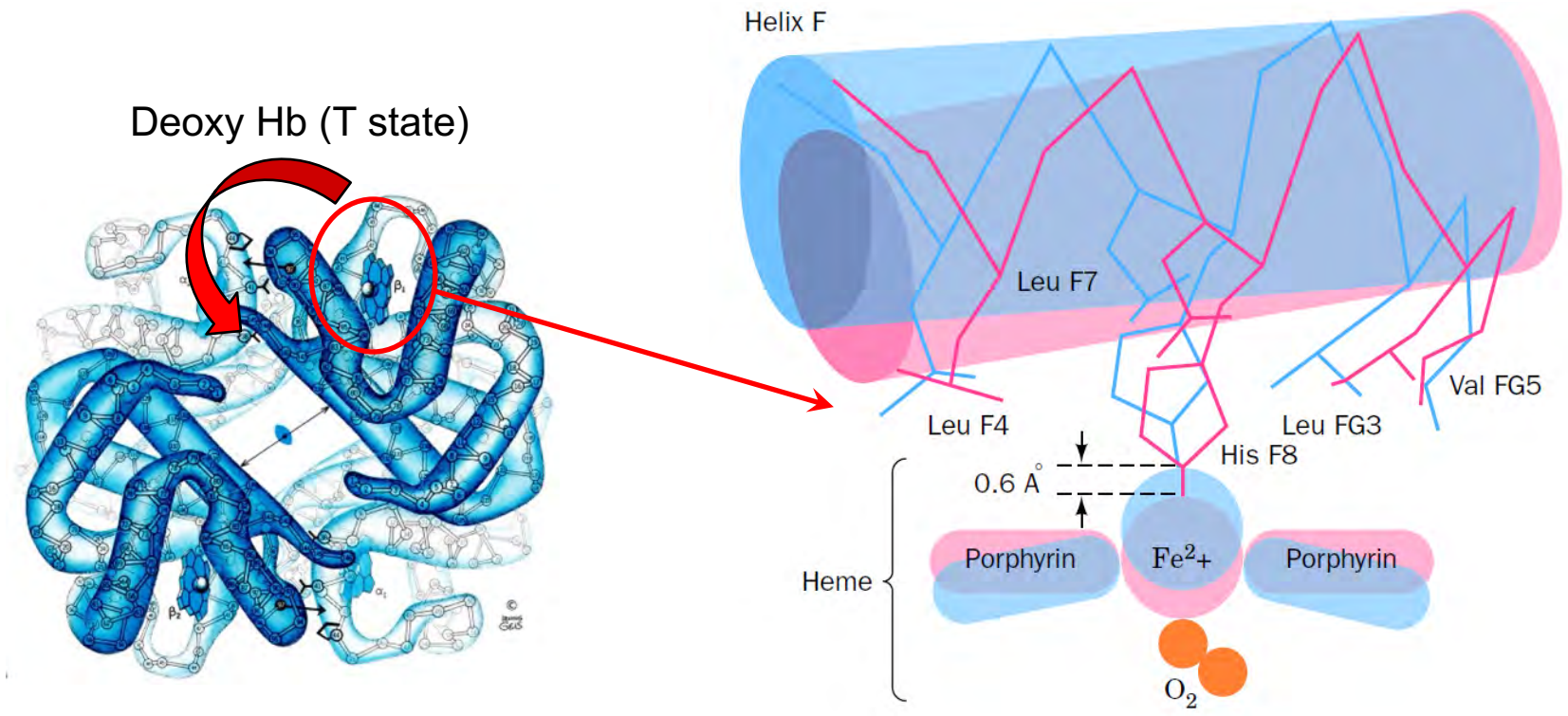
Deoxy T state



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.

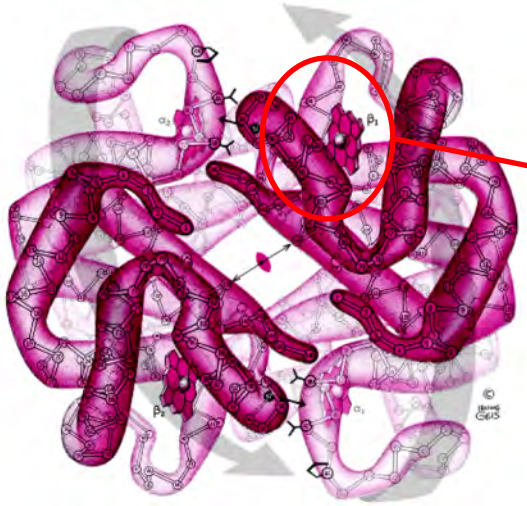
Structural change in the heme group pulls on HisF8…

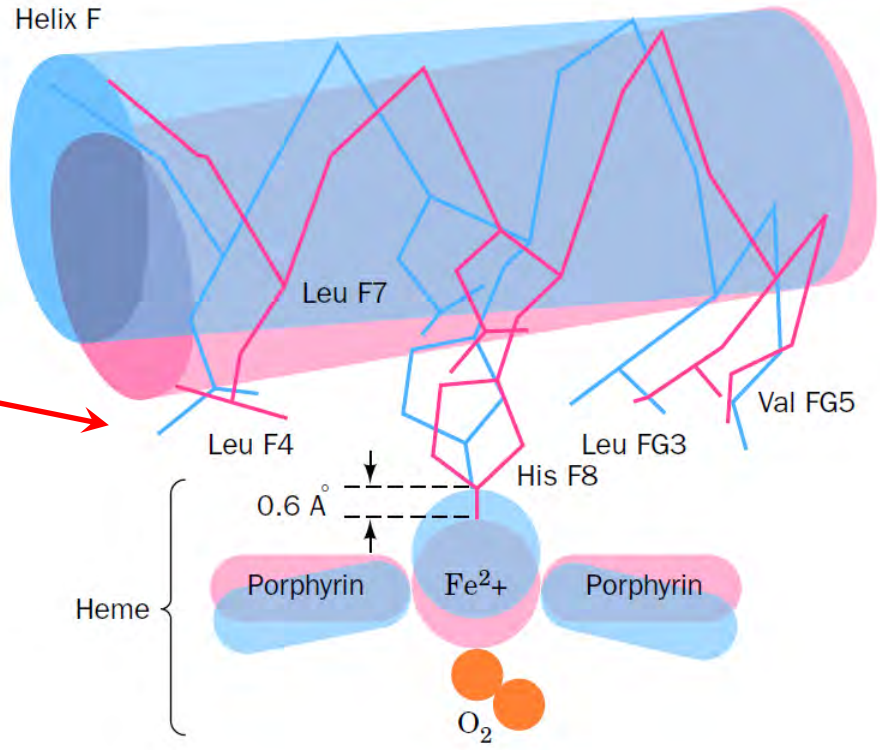


Due to the partial covalent bond between HisF8 (His87) and Fe^{2+}, movement of the Fe^{2+} into the plane of the porphyrin ring by 0.6 Å leads to movement of HisF8 (His87) and thus a tilt in the F helix. The tilt in helix F drives the “conformational switch” from T to R (helix F packs against helix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





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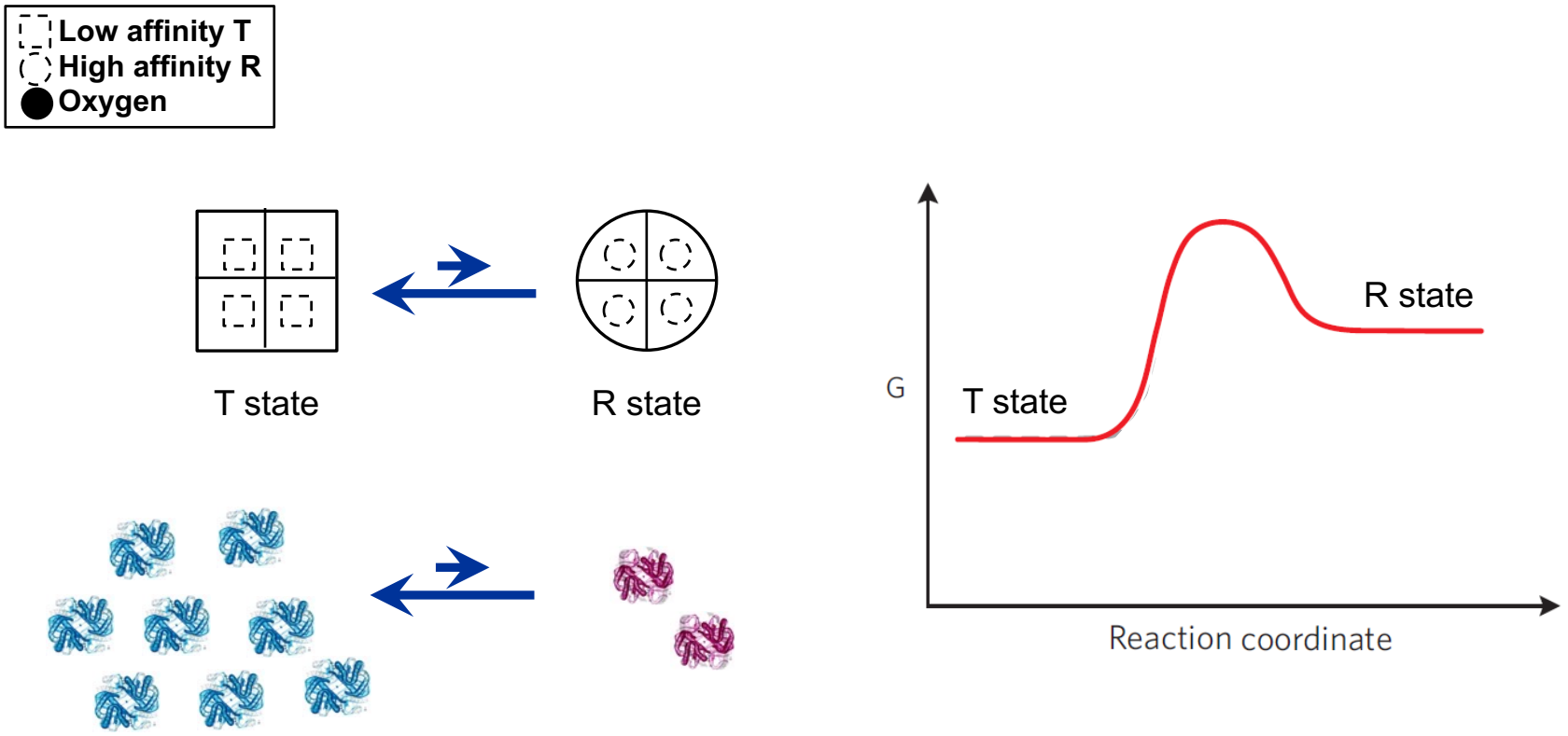
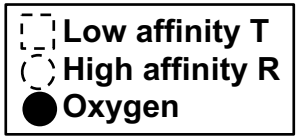
Allosteric transitions from a thermodynamic perspective

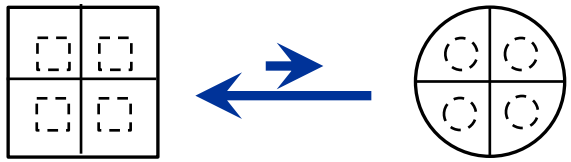


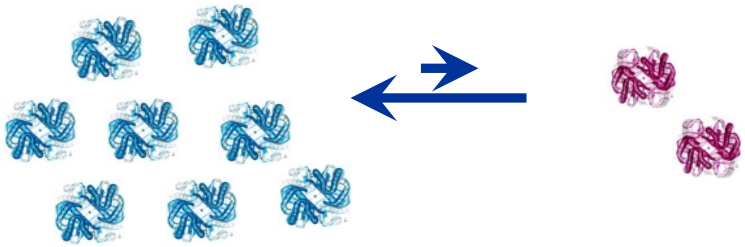
In the absence of O_2, Hb can exist in either T or R, but T has a much lower energy than R – so this form predominates. The proportion of molecules in the two states is governed by their relative energies – but there are always some molecules of Hb in the R state.

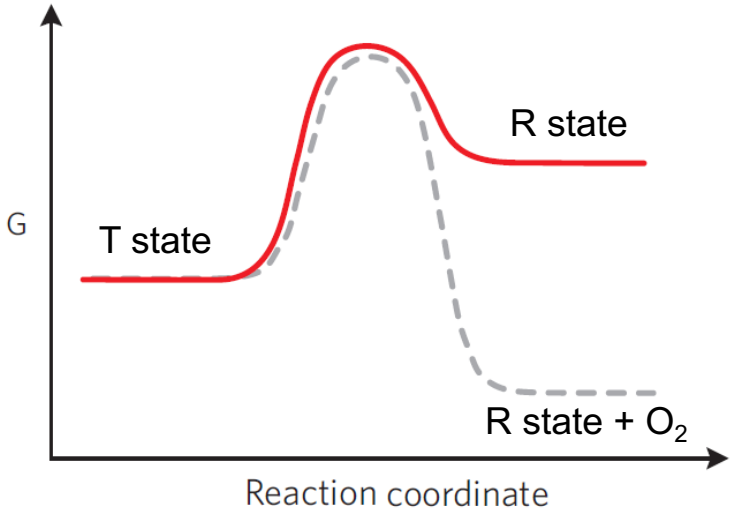
O_2 binds preferentially to the R state



$\Delta G_{rxn} = -RT\ln K_{eq}$?

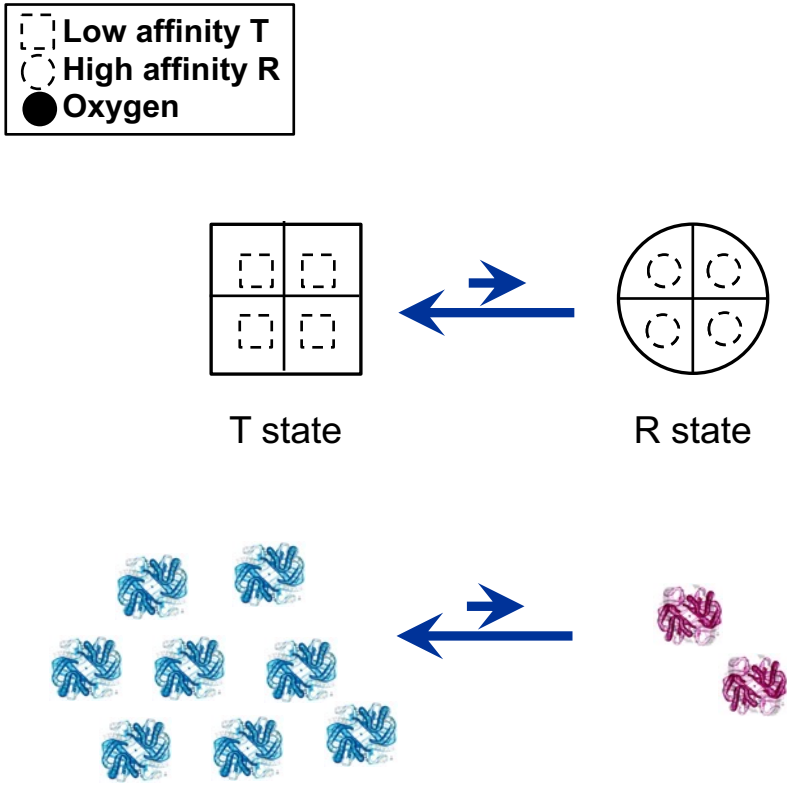






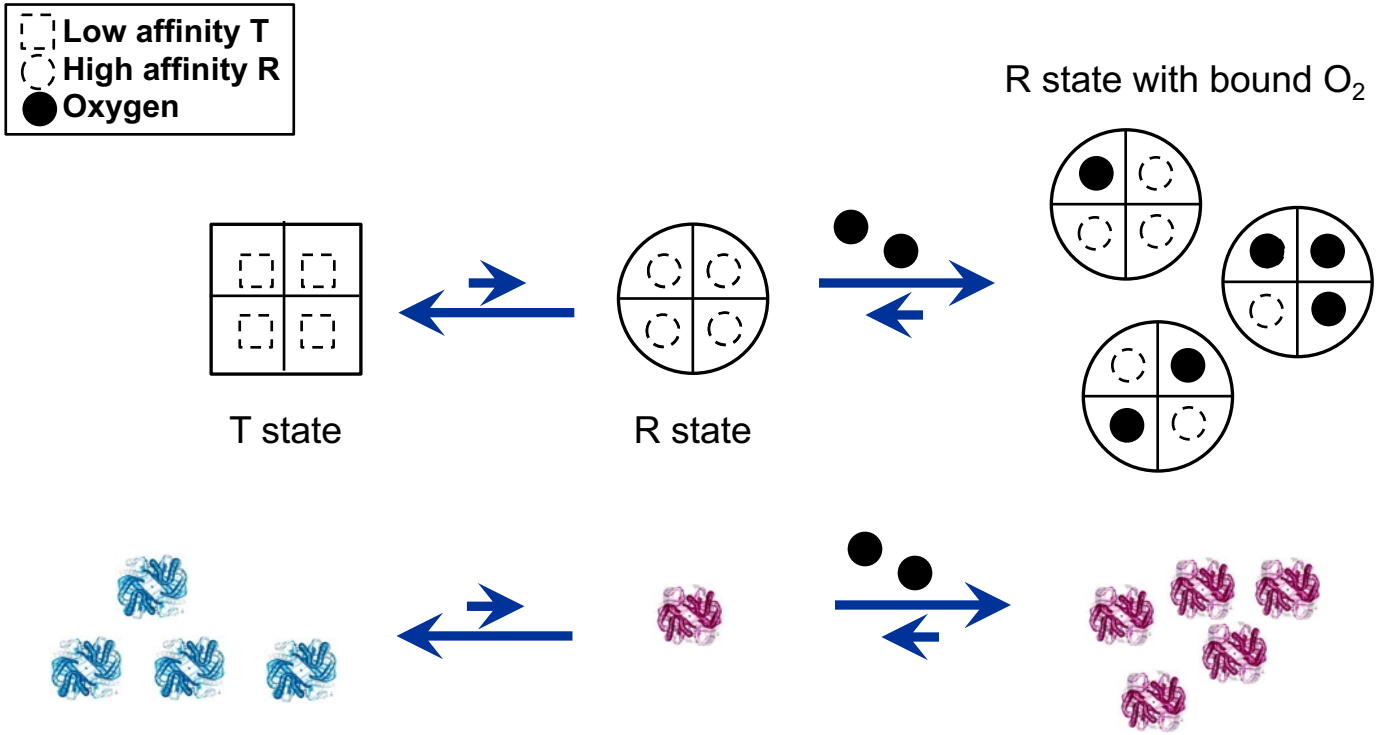
O_2 binds with a higher affinity to the R state than to the T state, so more energy is derived from O_2 binding to the R state. The energy of O_2 binding lowers the overall energy of the R state, so that R is now favored over T. The proportion of Hb molecules is thus shifted towards the R state.

O_2 binds with higher affinity to the R state



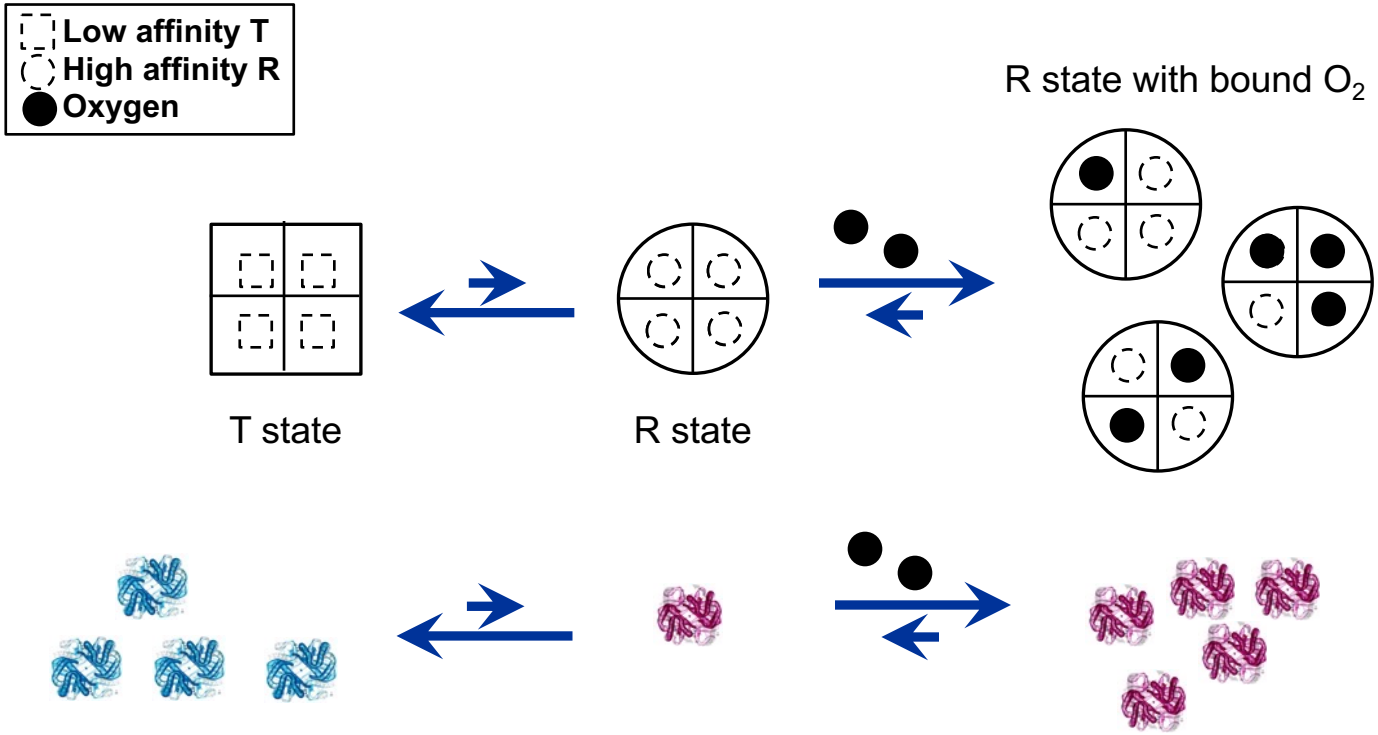
Another way of thinking about this is to consider affinity. The R state has a higher affinity for O_2 than the T state. So at low [O_2], O_2 can bind to the R state at concentrations where it will not bind to the T state…

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.

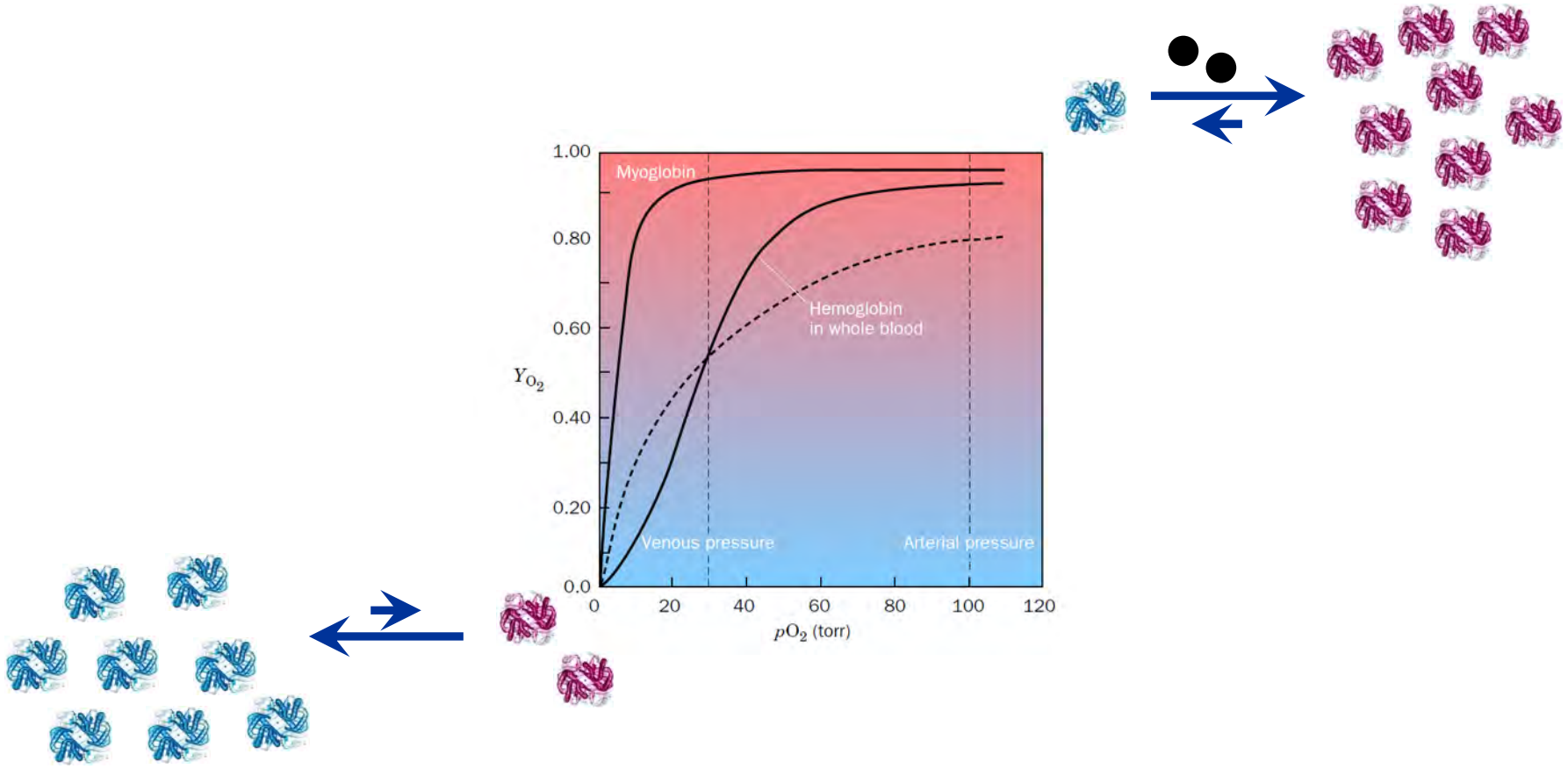
Why does conformational change lead to cooperativity?



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.

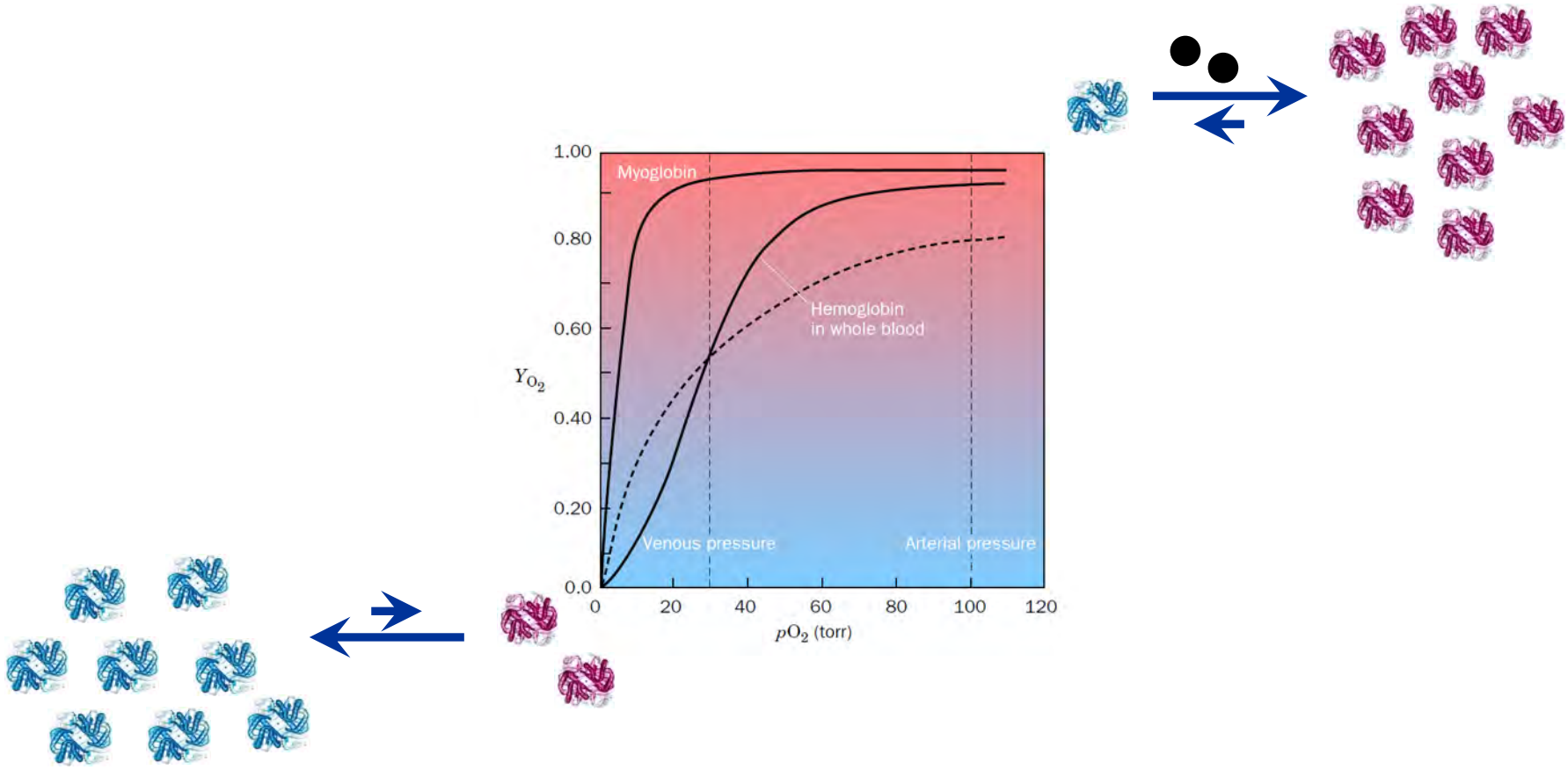
Because R has a higher affinity for O_2 than T, the conformational shift leads to a massive increase in O_2 binding to other sites!

Why does conformational change lead to cooperativity?



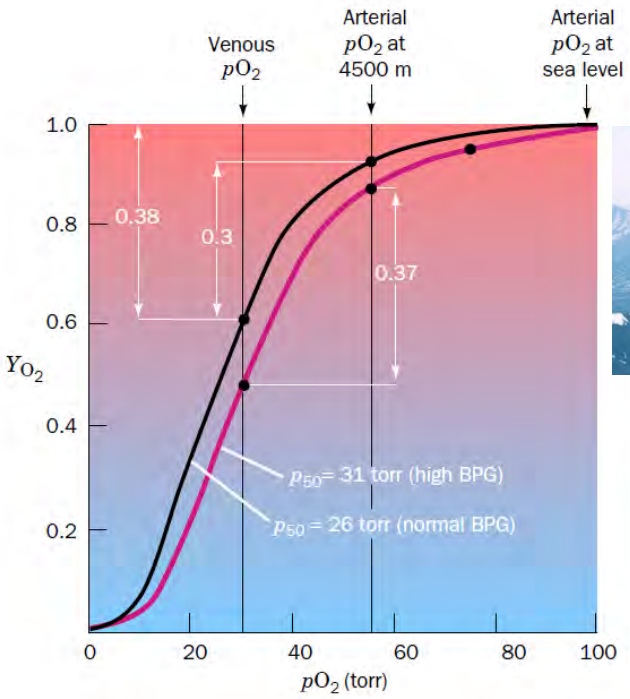
In a binding experiment, Hb starts mainly in the T state (low [O_2], where it binds O_2 with low affinity. As O_2 is added it binds to the available high affinity R state sites, and eventually leads to a conformation shifts from T to R. With a shift into the R state Hb, high affinity binding sites are 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.

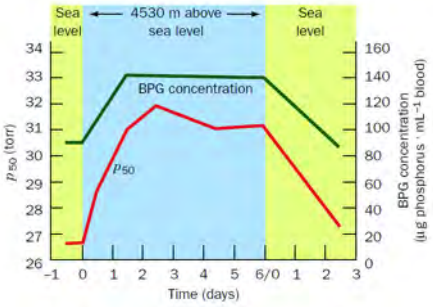
What does the Hill coefficient tell us?



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.

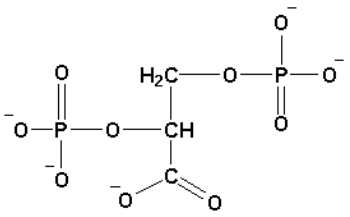




After several days at altitude, the levels of BPG increase in blood shifting the p50 for binding of O_2 from 26 torr to 31 torr. This allows Hb to more effectively deliver O_2 to peripheral tissues at high altitude.

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

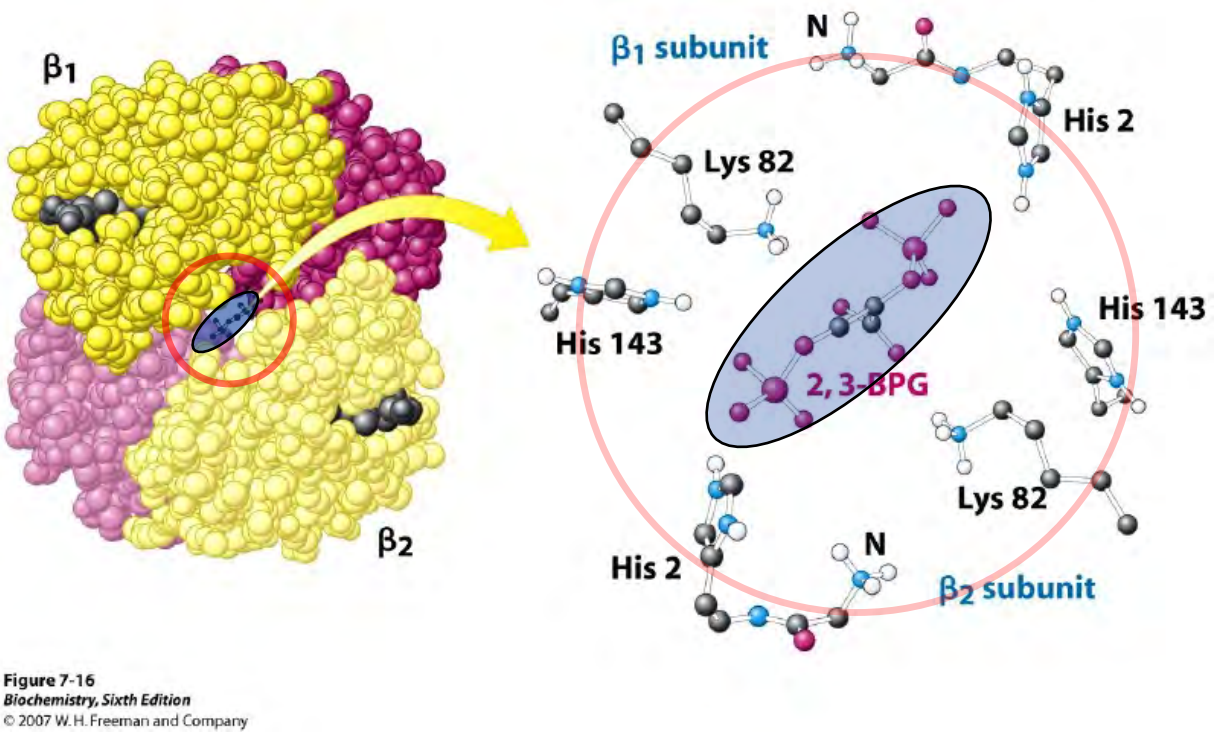
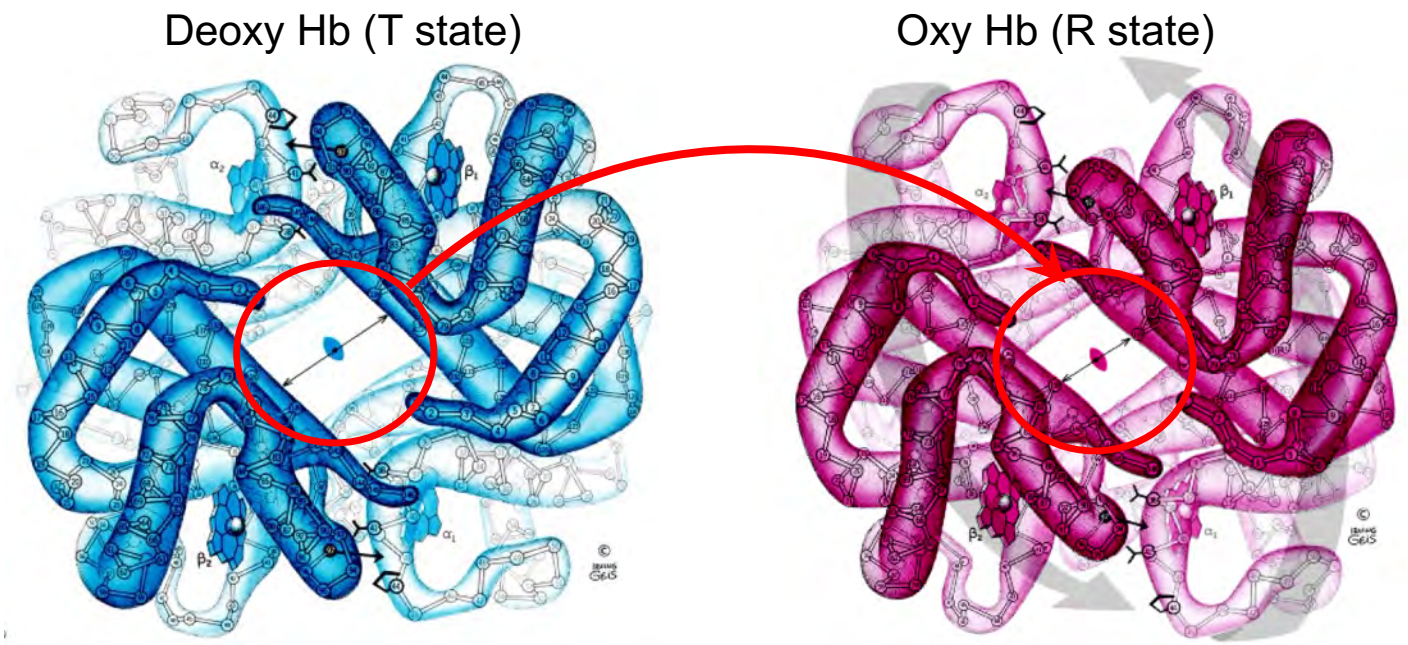


Figure 7-16
Biochemistry, Sixth Edition

Deoxy-Hb (T state) with BPG

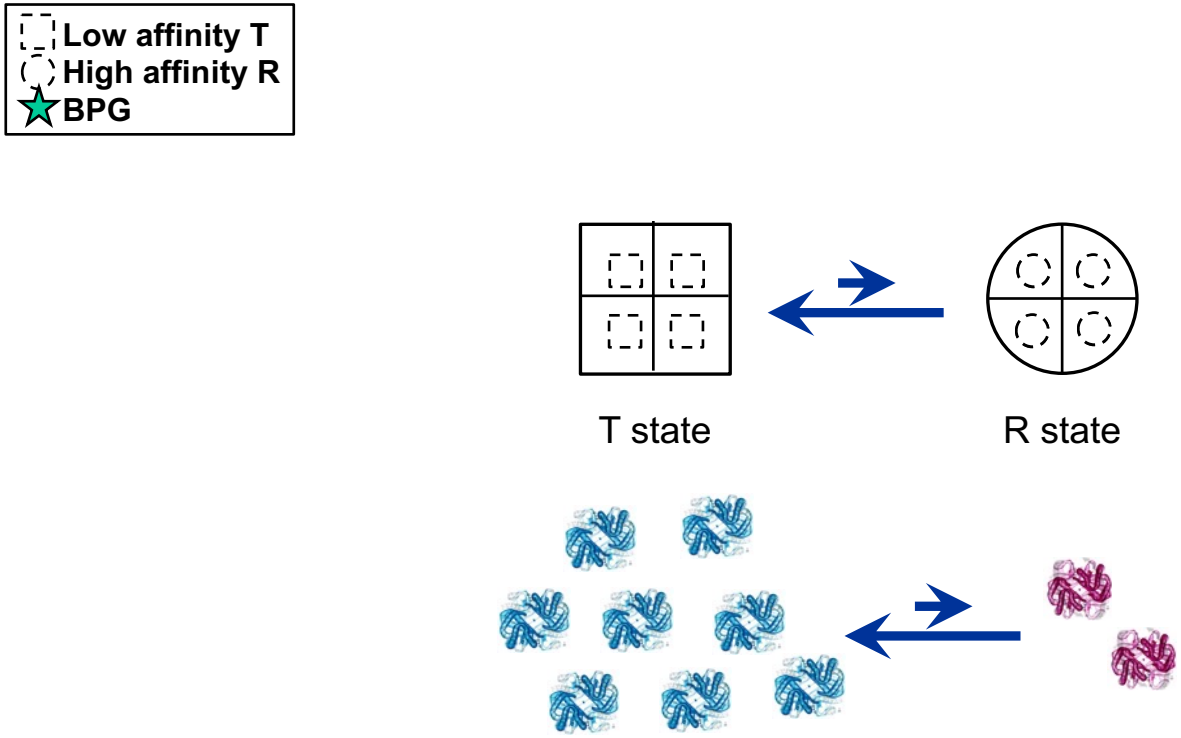
An important clue as to how BPG influences O_2 binding came from structural biology, which showed that BPG binds to only one site on Hb – a cavity at the center of Hb. There are a number of positive residues on the β-subunit that line the cavity and that interact with the highly negative BPG. *These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

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



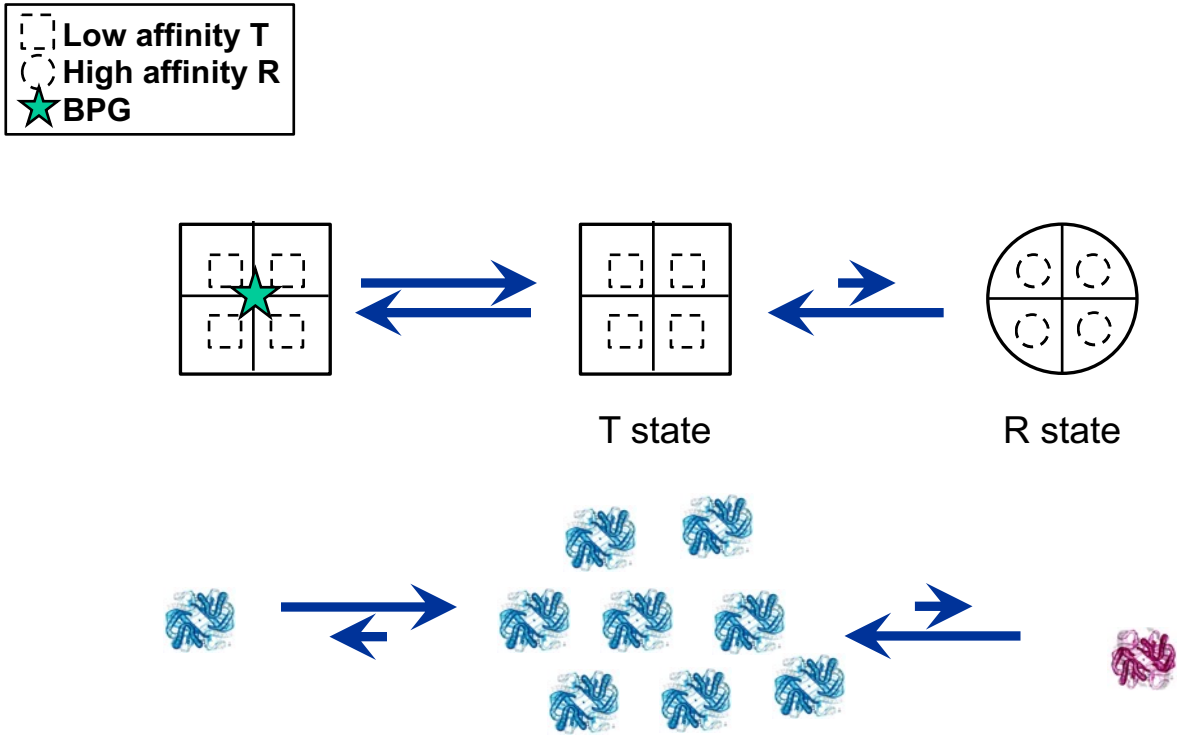
If you compare the structures of T and R, you see that the size of the central cavity decreases in the R state. In contrast to O_2, BPG binds with a much higher affinity to the T state than to the R state. BPG thus stabilizes the low O_2 affinity binding form of Hb. *BPG cannot bind to the cavity in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

BPG binds with higher affinity to the T state



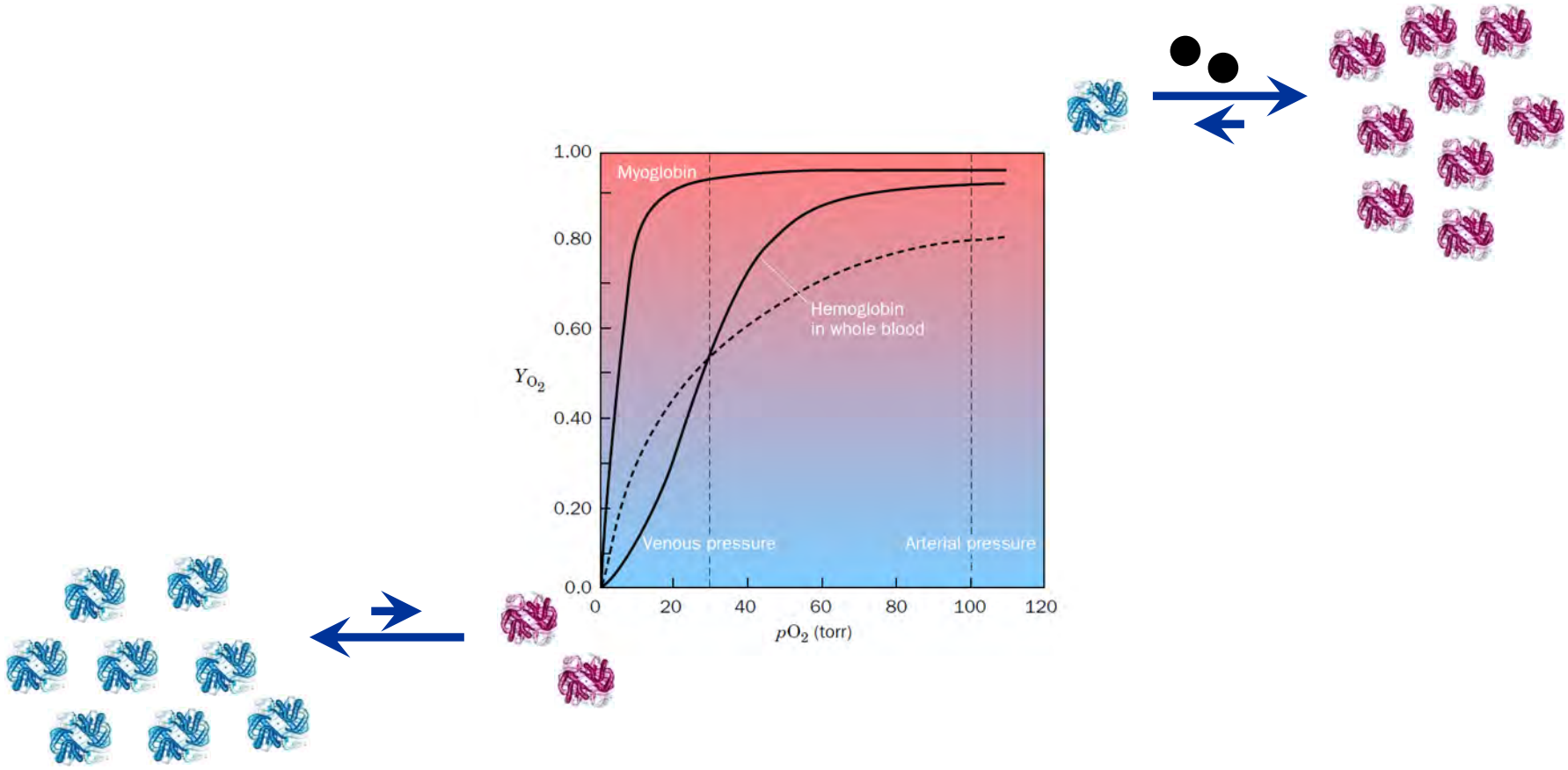
By binding to the T state, BPG creates another species – Hb-BPG. This creates a new equilibrium between BPG–bound Hb and BPG-free Hb…

BPG binds with higher affinity to the T state



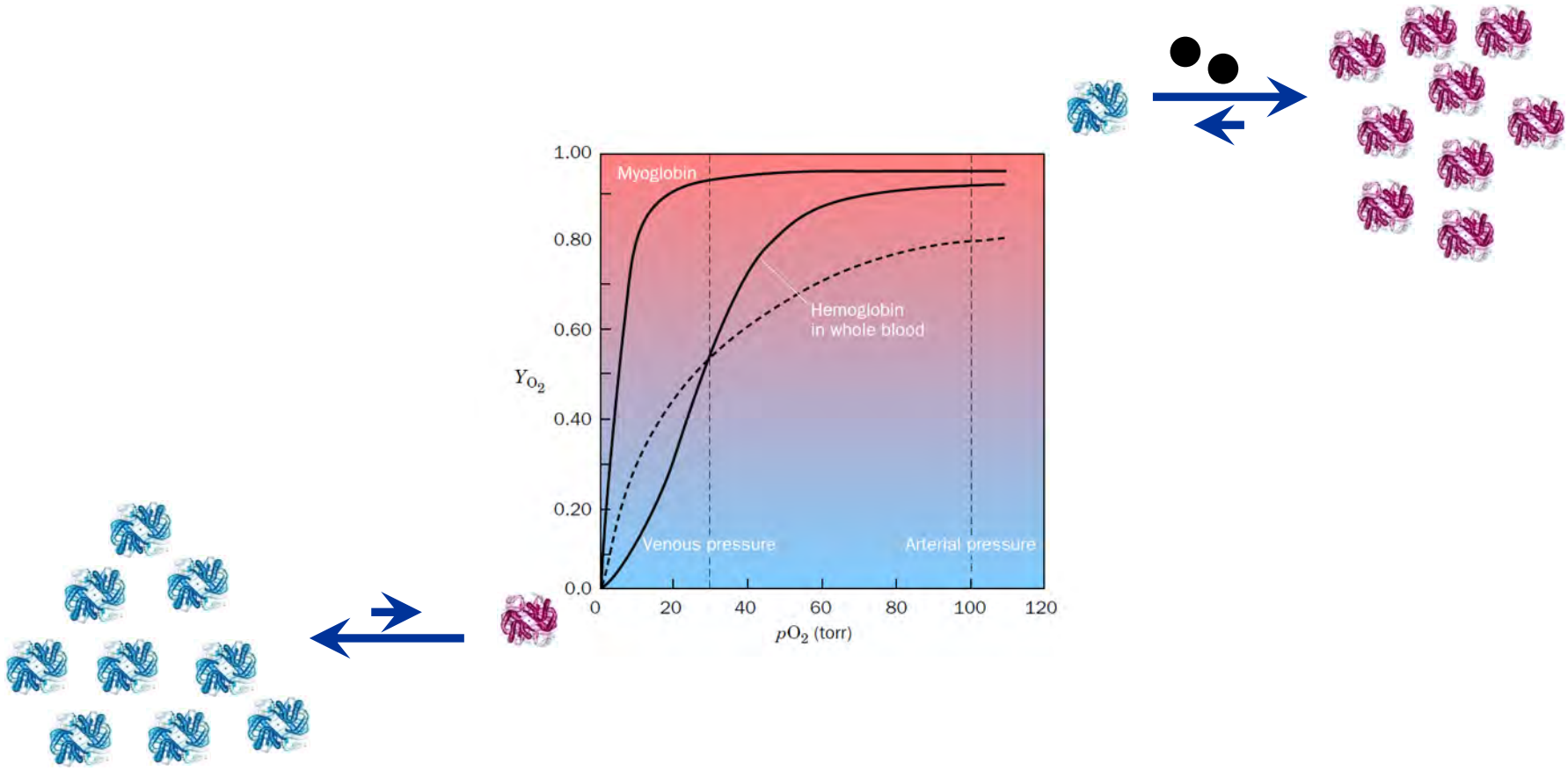
By binding to the T state, BPG creates another species – Hb-BPG. This creates a new equilibrium between BPG–bound Hb and BPG-free Hb… So overall the equilibrium between R and T states shifts in favor of the T state, which binds O_2 with low affinity

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…

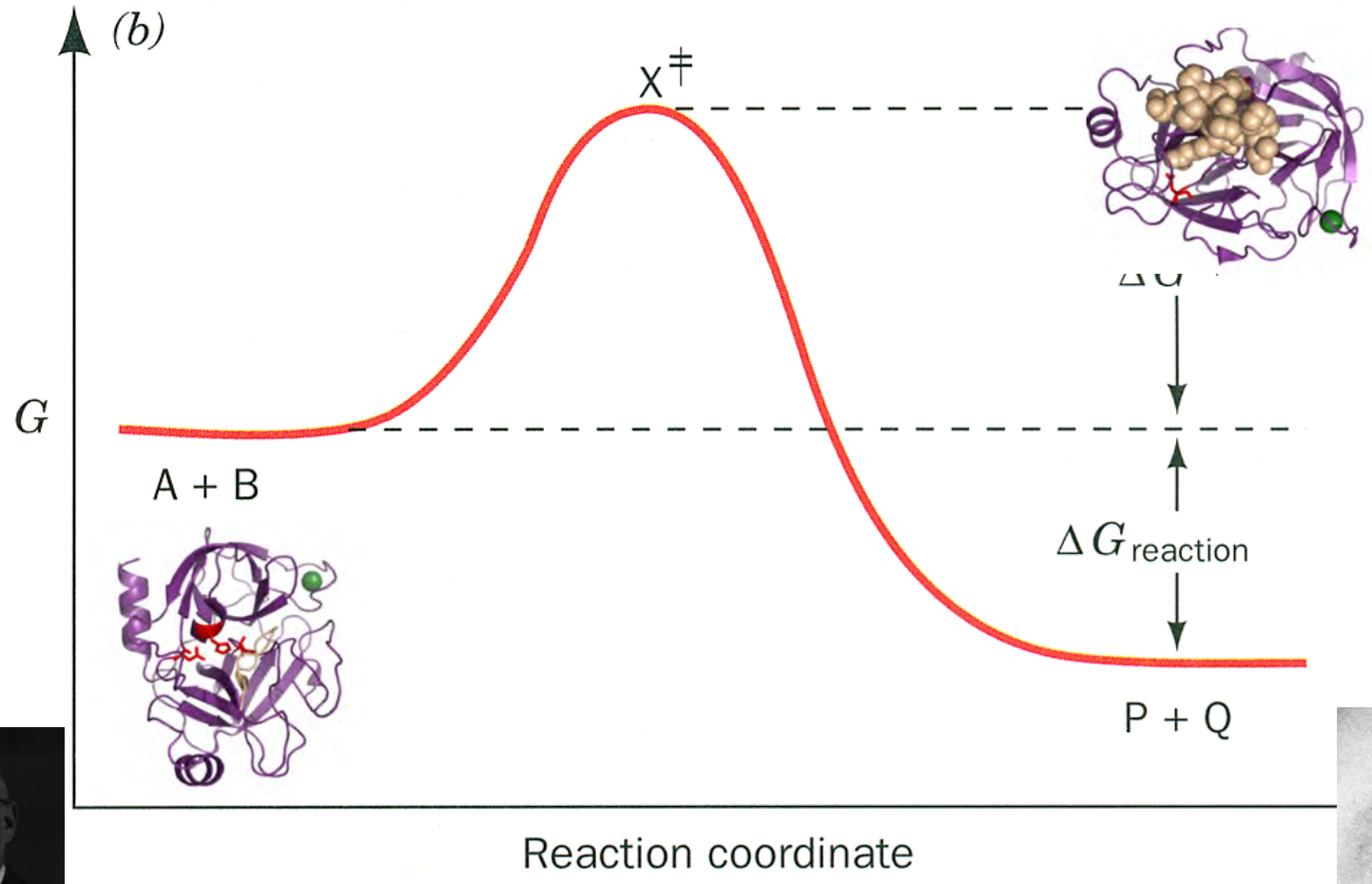
BPG binds preferentially to the T state



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*.

PART II: ENZYMOLOGY AND CATALYTIC KINETICS OF SERINE PROTEASES

Michaelis-Menten Kinetics



Leonor Michaelis and Maude Menten, two pioneers in the field of enzyme kinetics. Maude Menten was an incredible Canadian woman (see Appendix I)!

Enzymes are…

... the molecules inside cells that catalyze the myriad of biological reactions that comprise life. They are remarkable catalysts that differ from man-made catalysts by the following features:

- higher rates of catalysis (10^6 –10^{12} x faster than uncatalyzed reactions) under mild conditions of pH 7, 37 ^oC, 1 atm
- greater reaction specificity of substrates and products
- capacity for regulation

In this lecture, we will ask the question: **How do enzymes work?** We will use serine proteases as the example to understand conceptually how proteins achieve their incredible functions. In particular, we will address the questions, how do enzymes achieve such phenomenal rates of catalysis, how do they exhibit such a high degree of specificity, and how do we use biochemistry to understand these phenomenon?

How fast is a 10^{10}-fold enhancement in rate?

Consider traveling from Vancouver – Halifax = ~6200 km

- walking time (4 km/hr) = 5,580,000 s or 194 days (walking 8 hours per day)
- driving time (100 km/hr) = 220,000 s or ~8 days (driving 8 hours per day)
- flying time (800 km/hr) = 19,860 s or 5.5 hours (flying distance only 4,400 km)

Driving leads to a *rate enhancement of 24 fold, while* flying leads to a *rate enhancement 280 fold*

How long would it take with a 10^{10} fold enhancement $\rightarrow$ *0.00045 s*

Enzymology and Catalytic Kinetics

Three subjects covered in this lecture:

I. General reaction kinetics

II. Michaelis-Menten enzyme kinetics

III. Thermodynamic basis for catalysis

IV. Serine proteases

I Basic concepts in chemical kinetics:

1) Reaction order

Consider the following reaction:

$$aA + bB + cC + \ldots \rightleftharpoons a'P_1 + b'P_2 \ldots$$

In this general reaction, *"a" molecules of A combine with "b" molecules of B and "c" molecules of C… to produce " a' " molecules of P_1 and " b' " molecules of P_2, etc.* <u>*The reaction order is the number of molecules that collide to make the reaction occur.*</u> The reaction order is thus a + b + c…

$$A \longrightarrow P \qquad 1^{st} \text{ order}$$

$$A + B \longrightarrow P \qquad 2^{nd} \text{ order}$$

$$A + 2B \longrightarrow P \qquad 3^{rd} \text{ order}$$

I Basic concepts in chemical kinetics:

2) Reaction rate

Consider the following reaction:

$$aA + bB + cC + \ldots \rightleftharpoons a'P_1 + b'P_2 \ldots$$

The rate of a reaction is the rate at which molecules of product are produced *or* the rate at which the reactants are used up. The rate of the reaction is proportional to the concentration of the reactants as follows:

$$Rate = k[A]^a[B]^b[C]^c \ldots$$

Where k is a kinetic rate constant which reflects how likely the particular chemical reaction will occur, regardless of the concentration of reactants. The units for reaction rate are Ms^{-1} - i.e. the number of moles of product produced per liter per second.

I Basic concepts in chemical kinetics:

2) Reaction rate

A $\longrightarrow$ **P**

$$Rate = k_1 [A]$$

- k_1 is a first order rate constant with the unit "s^{-1}"

A + B $\longrightarrow$ **P**

$$Rate = k_2 [A][B]$$

- k_2 is a second order rate constant with the unit "$M^{-1}s^{-1}$"

A + 2B $\longrightarrow$ **P**

$$Rate = k_3 [A][B]^2$$

- k_3 is a third order rate constant with the unit "$M^{-2}s^{-1}$"

I Basic concepts in chemical kinetics:

3) All reactions are bidirectional

$$Rate = k_1[A][B]^2 \qquad Rate = k_{-1}[P]$$

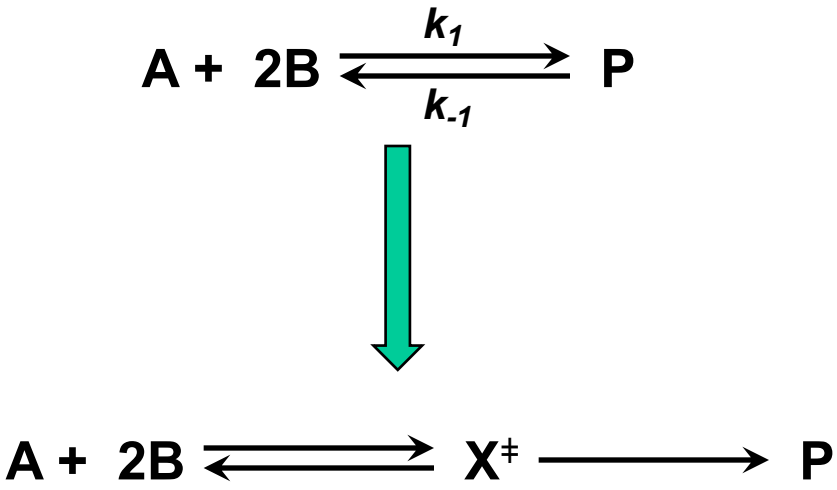
$$K_{eq}^{for} = \frac{[P]}{[A][B]^2} \qquad K_{eq}^{rev} = \frac{[A][B]^2}{[P]}$$

$$K_{eq}^{for} = 1 / K_{eq}^{rev}$$

All chemical reactions are bidirectional, so we can calculate both a forward and a reverse reaction rate governed by k_1 and k_{-1}, respectively. The equilibrium constant, K_{eq}, defines the concentration of reactants and product at equilibrium. *Rate constants are small letters, equilibrium constants are capitals*

I Basic concepts in chemical kinetics:

4) The transition state



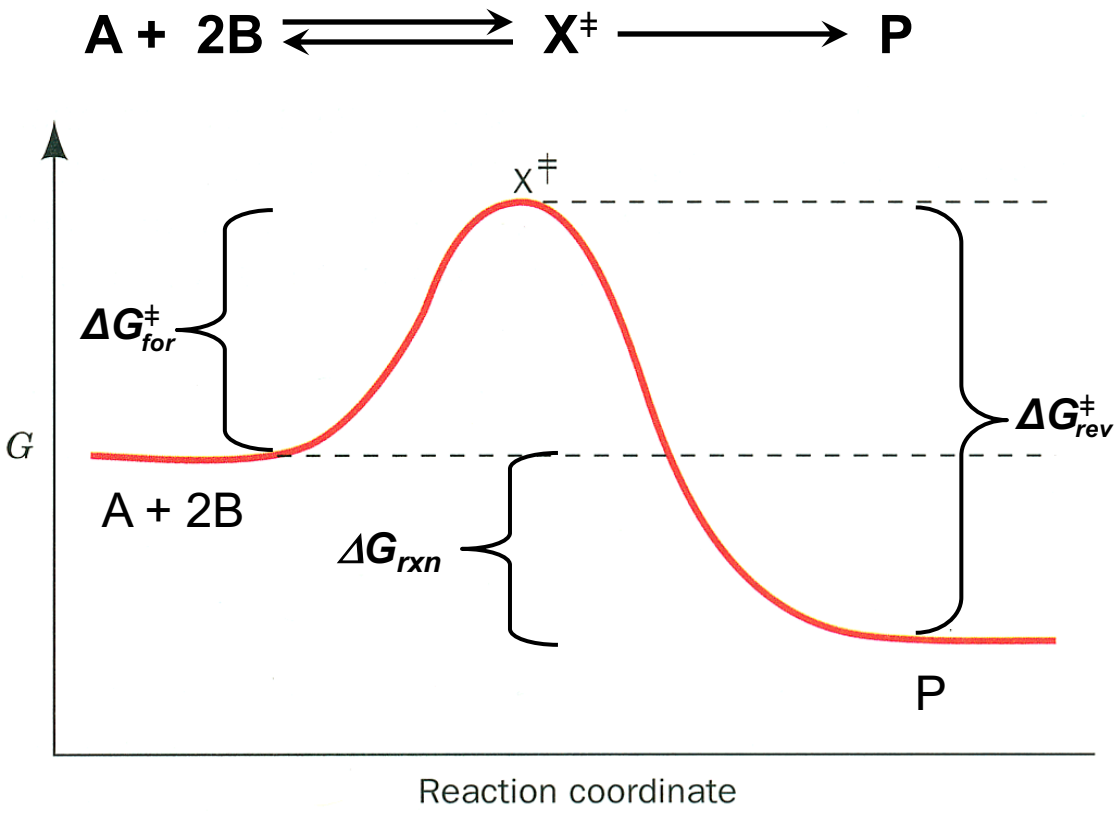
All chemical reactions go through a "transition state, $X^{\ddagger}$", the highest energy species in the path between reactants and products. The transition state is:

- unstable – it exists only transiently (for a very short time!)
- the energy barrier between reactants and products, the height of this energy barrier dictates how fast a reaction will occur.

Enzymes enhance reaction rates by lowering this activation energy barrier

I Basic concepts in chemical kinetics:

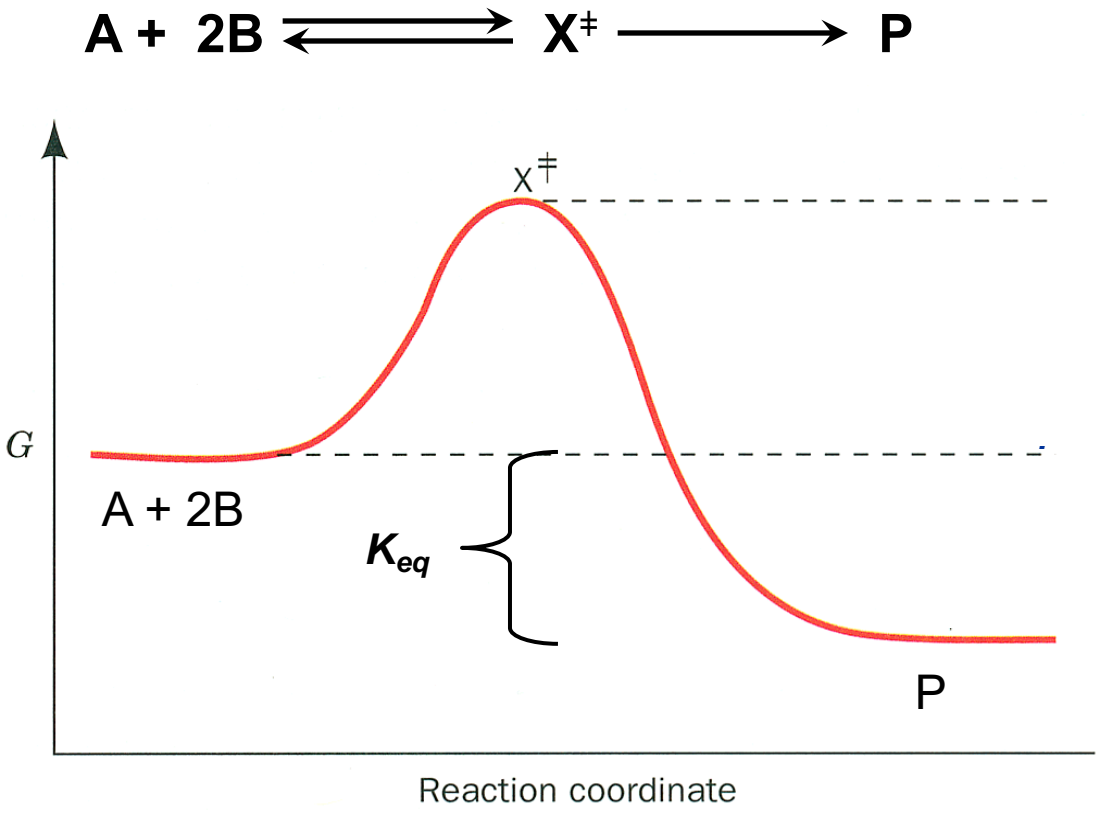
5) The reaction coordinate diagram



A reaction coordinate diagram is a plot of the Gibbs free energy vs the reaction coordinate or reaction path. The energy between the reactants and products is referred to as ΔG_{rxn}. The activation energy is the energy between reactants and the transition state, and is referred to as $\Delta G^{\ddagger}$.

I Basic concepts in chemical kinetics:

6) Reaction thermodynamics

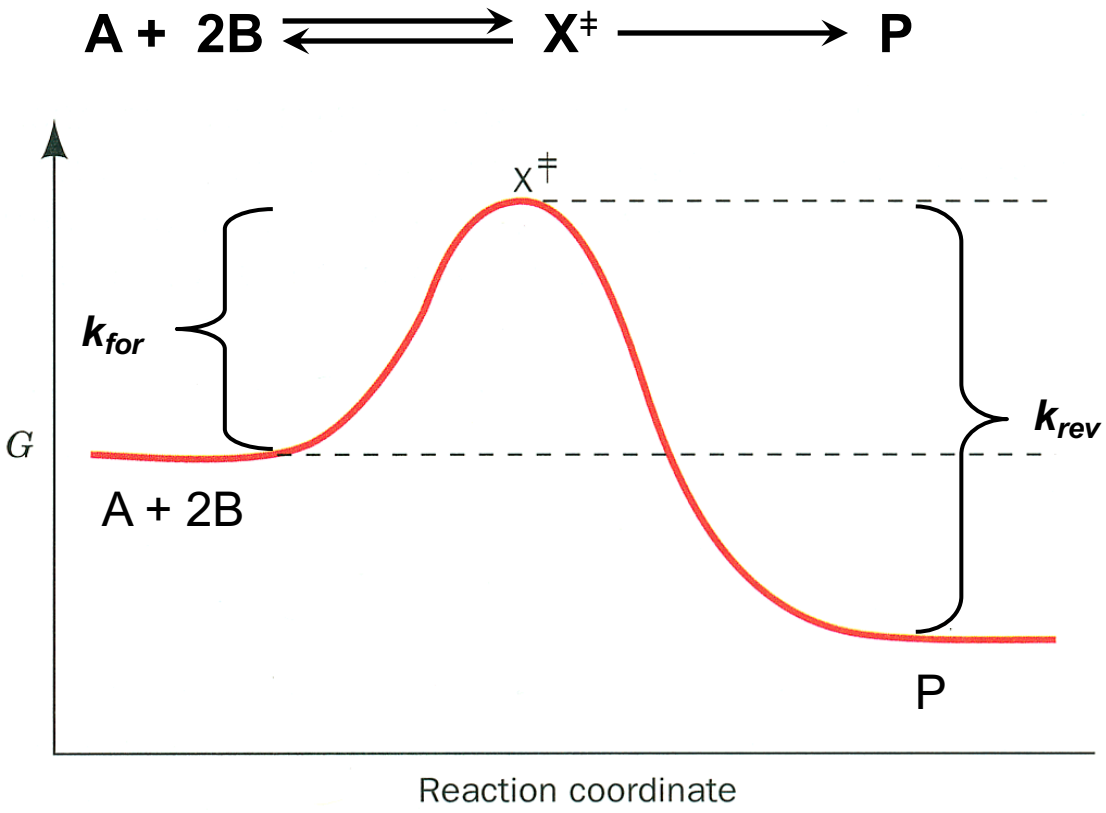


"Thermodynamics" refers to the equilibrium condition defined by the equilibrium constant, K_{eq}. K_{eq} is mathematically related to the change in Gibbs free energy between reactants and the products, ΔG_{rxn}:

$$\Delta G_{rxn} = -RTlnK_{eq}$$ (T= temperature in K, R=8.3145 $Jmol^{-1}$).

I Basic concepts in chemical kinetics:

7) Reaction kinetics

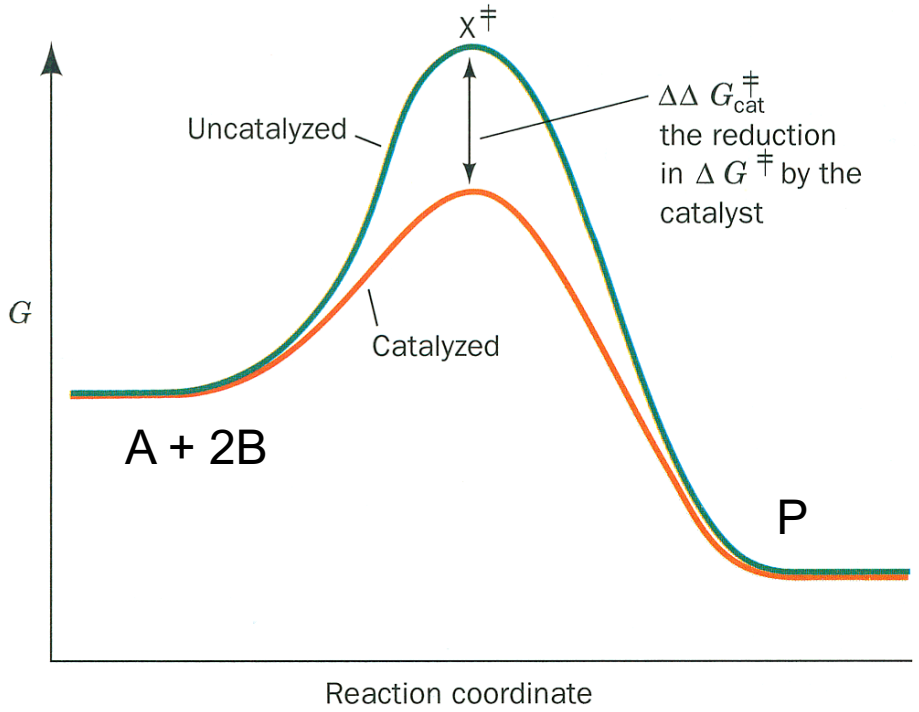


Kinetics refer to the rates that reactions approach equilibrium. The forward rate constant, k_{for} *or* k_1*)*, is mathematically related to the activation energy $\Delta G^{\ddagger}_{for}$, the energy between the reactants and transition state. The larger the activation energy $\Delta G^{\ddagger}_{for}$, the slower the reaction. The rate constant, k_{rev} (or k_{-1}) is related to the activation energy for the reverse reaction

I Basic concepts in chemical kinetics:

8) A 5.7 kJmol^{-1} drop in $\Delta G^{\ddagger}$ reaction rate leads to a 10-fold ↑ rate

$$A + 2B \rightleftharpoons X^{\ddagger} \longrightarrow P$$

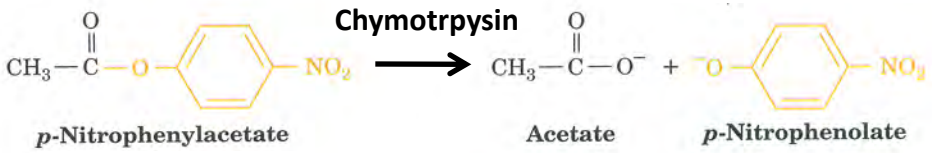


To increase the reaction rate, a catalyst must ↓ the activation energy, $\Delta G^{\ddagger}$.

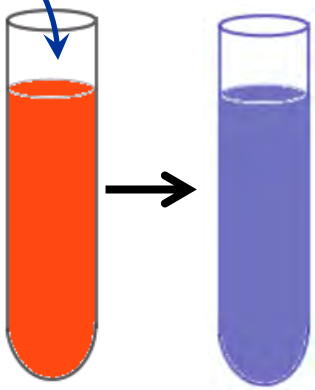
There is a mathematical relation between the ↑ rate and the ↓ in $\Delta G^{\ddagger}$. For every ↓ in $\Delta G^{\ddagger}$ of 5.7 kJmol^{-1} at 298 K, there is a 10 fold ↑ in rate.

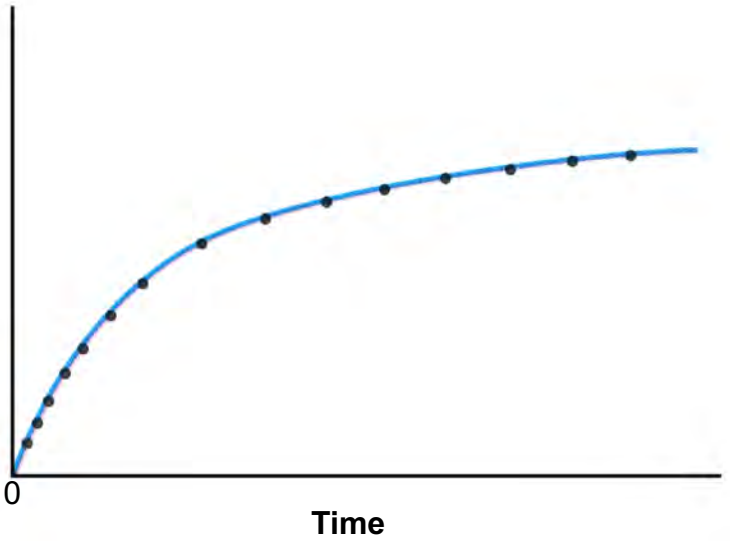
II Michaelis-Menten kinetics:

1) An enzyme catalyzed reaction has 3 phases



At time = 0, we add chymotrypsin to a solution of pNPA



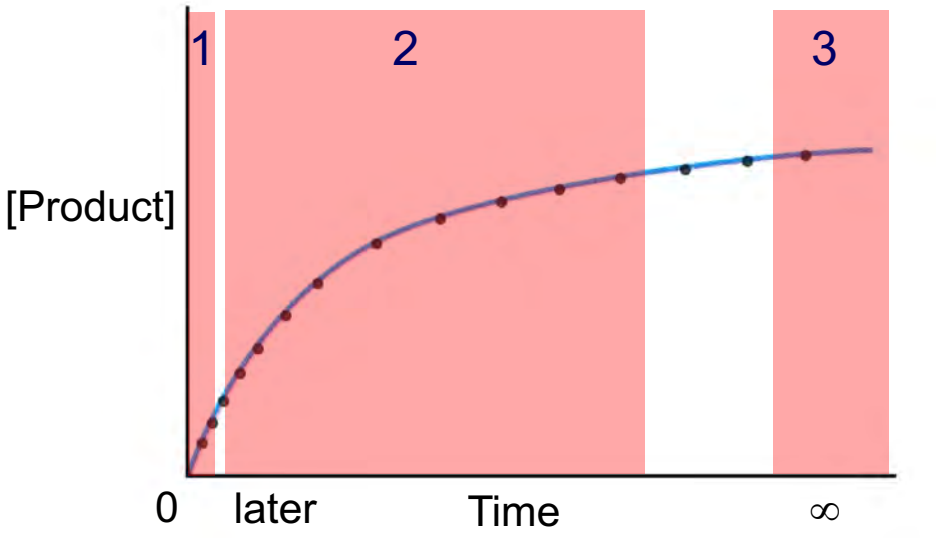


pNP absorbs at 450 nm, so we follow the rate of product (pNP) formation by measuring the A_{450}. The increase in A_{450} with time is converted directly into the increase in [pNP].

II Michaelis-Menten kinetics:

1) General enzyme catalyzed reactions have 3 phases

Enzyme + Substrate ⇌ Enzyme + Product



1) Time ≅ 0, there is a high [substrate] and no product - the initial rate of the reaction depends only on the [Enz] and [substrate]

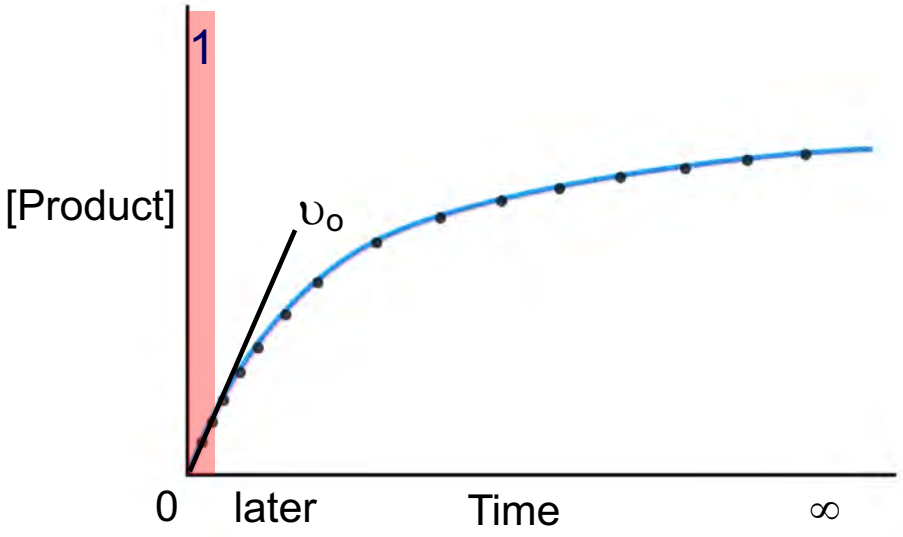
2) At a later time, there is less substrate and some product
- you start to get the reverse rxn occuring at the same time so the rsn slows down

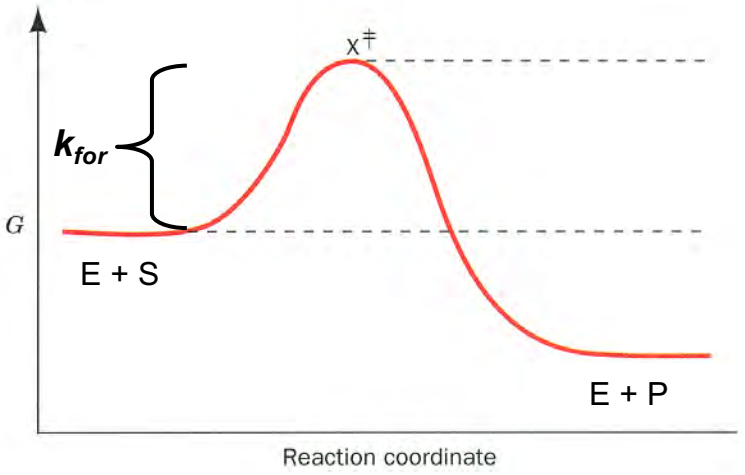
3) Time =∞, the rates of the forward and reverse reactions are equivalent
- the overall reaction rate is zero and the rxn is at equilibrium

II Michaelis-Menten kinetics:

1) General enzyme catalyzed reactions have 3 phases

Enzyme + Substrate ⇌ Enzyme + Product





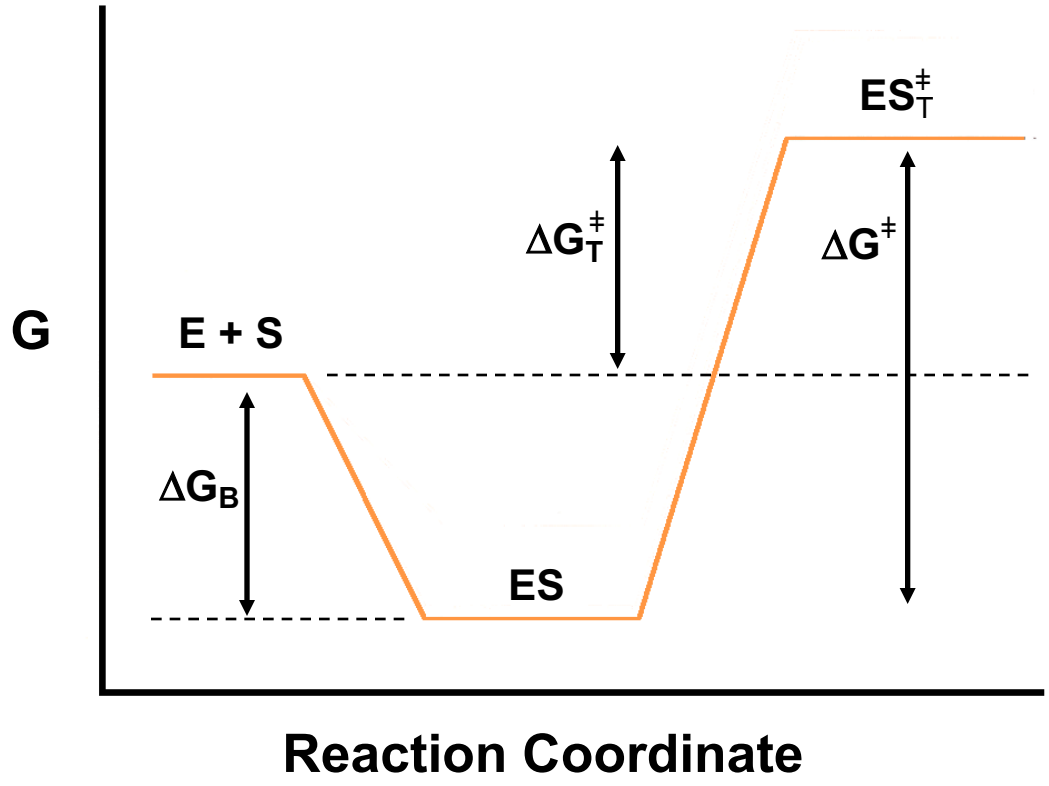
When measuring enzymatic mechanisms, we focus on only the first of the three phases because the initial reaction rate, υ_o. υ_o describes the rate in the absence of product (i.e. no back reaction), so it is directly related to k_{for}, the catalytic ability of the enzyme

But how do we interpret υ_o in terms of rate constants and ultimately the mechanisms of catalysis?

II Michaelis-Menten kinetics:

2) Adrian Brown (1902) assumed that the enzyme binds the substrate

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



Brown proposed that for catalysis to occur, the enzyme (*E*) rapidly binds the substrate (*S*) to form what we now call a "Michaelis complex", *ES*, and that *ES* goes through a transition state, $ES_T^{\ddagger}$, before decomposing to free enzyme and product (*P*).

II Michaelis-Menten kinetics:

2) Adrian Brown (1902) assumed that the enzyme binds the substrate

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

At high *[S]*, the enzyme saturates, so the reaction rate, υ_o, depends on the amount of enzyme (i.e. *[ES]*) and its catalytic ability, k_2:

$$Rate = \upsilon_o = \frac{d[P]}{dt} = k_2[ES]$$

…but given that we cannot easily measure *[ES]*, we cannot define k_2. We need to define k_2 in terms of measurable quantities.

II Michaelis-Menten kinetics:

3) The Michaelis & Menten (1913) equilibrium assumption

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

To interpret kinetic data, Michaelis and Menton assumed that the rxn $E + S \leftrightarrow ES$ reaches equilibrium (i.e that $k_{-1} >> k_2$):

$$K_{eq} = \frac{[ES]}{[E]_{free}[S]}$$

Given that: $[E]_{total} = [E]_{free} + [ES]$

the following equation can be derived*:

$$\upsilon_o = k_2[ES] = \frac{k_2[E]_{total}[S]}{K_S + [S]}$$

But in most cases, the equilibrium assumption is not correct!

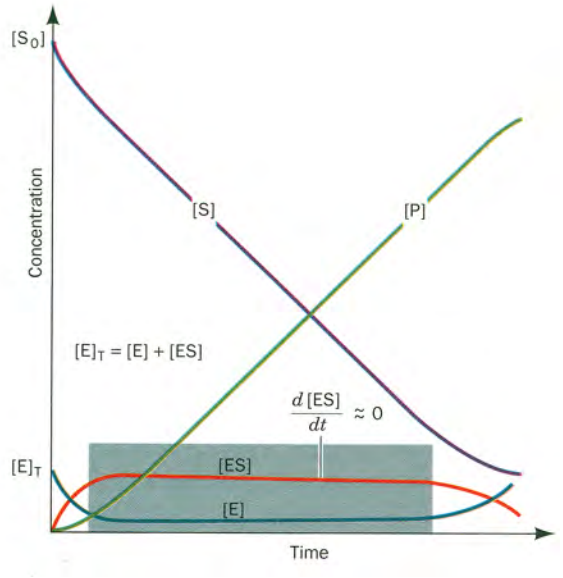
*see appendix II

II Michaelis-Menten kinetics:

4) The Briggs & Haldane (1925) steady state assumption

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

Briggs and Haldane assumed that the rxn reaches a steady state, where [ES] remains constant during the initial rate, υ_o:



In other words, they assumed that the enzyme *rapidly* binds the substrate so that the reaction reaches a steady state where the concentration of ES remains constant throughout the initial phase of the reaction (ie. υ_o).

II Michaelis-Menten kinetics:

4) The Briggs & Haldane (1925) steady state assumption

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

Briggs and Haldane assumed that the rxn reaches a steady state, where [ES] remains constant during the initial rate, υ_o:

$$\frac{d[ES]}{dt} = 0 = k_1[E]_{free}[S] - k_{-1}[ES] - k_2[ES]$$

From this assumption, we the following equation can be derived*:

$$\upsilon_o = k_2[ES] = \frac{k_2[E]_{total}[S]}{K_M + [S]}$$

In most cases, this assumption is accurate!

*see appendix III

II Michaelis-Menten kinetics:

Equilibrium versus steady state assumption

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

Equilibrium	Steady State
$\upsilon_o = \frac{k_2[E]_{Total}[S]}{K_S + [S]}$	$\upsilon_o = \frac{k_2[E]_{Total}[S]}{K_M + [S]}$
$K_S = \frac{1}{K_{eq}} = K_D$	$K_M = \frac{k_{-1} + k_2}{k_1}$

Both assumptions lead to similar equations. The pioneering work of Michaelis-Menten led to both derivations - this is why the *steady state assumption* leads to an equation typically referred to as the Michaelis-Menten equation – even though it was not derived by Michaelis-Menten.

II Michaelis-Menten kinetics:

Equilibrium versus steady state assumption

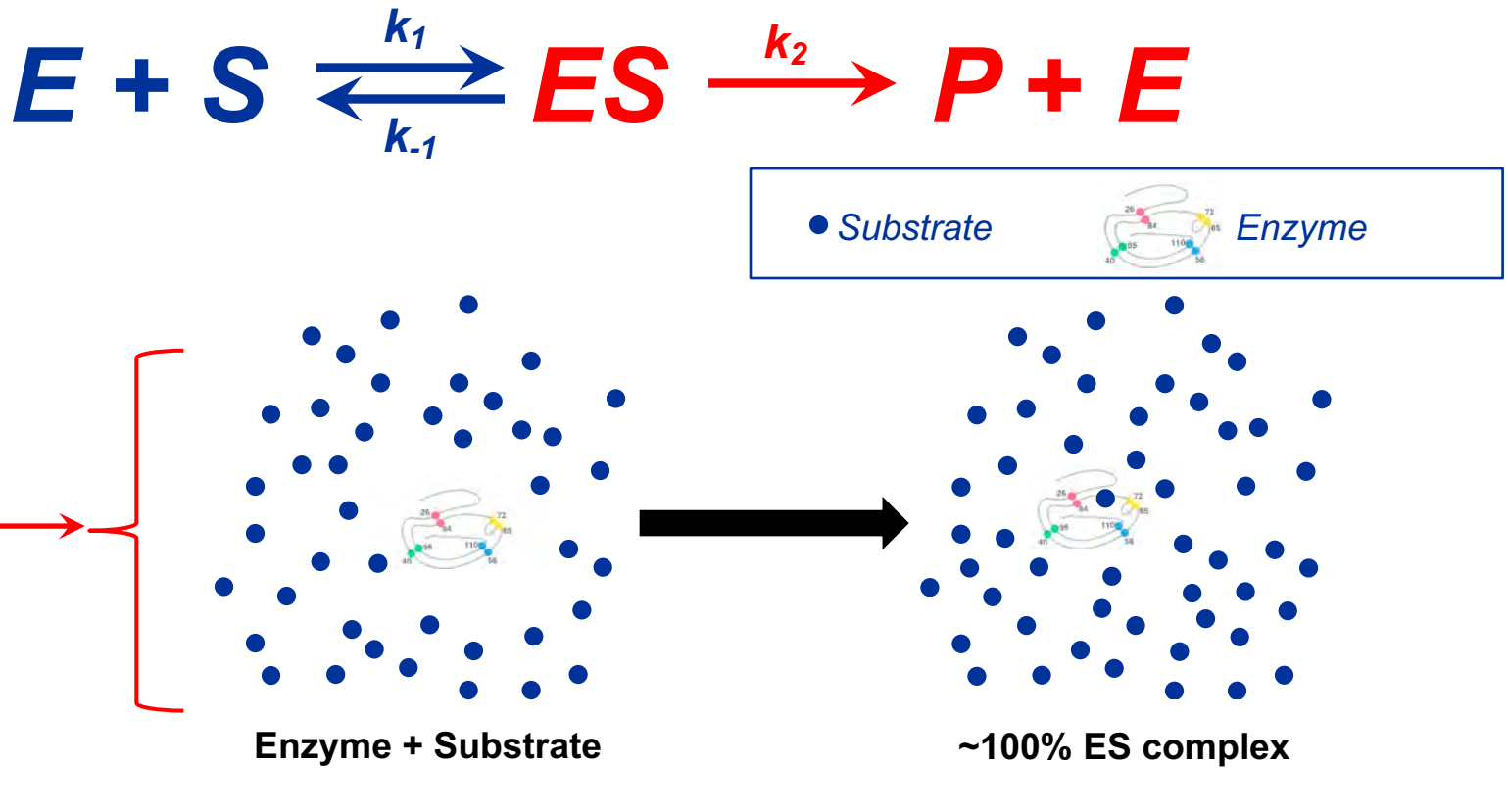
$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} P + E$$

Equilibrium	Steady State
$\upsilon_o = \frac{k_2[E]_{Total}[S]}{K_S + [S]}$	$\upsilon_o = \frac{k_2[E]_{Total}[S]}{K_M + [S]}$
$K_S = \frac{1}{K_{eq}} = K_D$	$K_M = \frac{k_{-1} + k_2}{k_1}$

To understand what these equations are telling us about catalysis, we need to consider three cases: 1) *[S]* >> K_M, 2) *[S]* = K_M, and 3) *[S]* << K_M.

II Michaelis-Menten kinetics:

Case I: [S] >> K_M



When [S] is very high ([S]>>>K_M), the enzyme saturates with substrate. In other words, the enzyme spends ~100% of its time as *ES* - as soon as the product is produced and the free enzyme regenerated, there is so much substrate that the regenerated free enzyme immediately re-binds substrate.

II Michaelis-Menten kinetics:

Case I: [S] >> K_M

$$\upsilon_o = \frac{k_2[E]_{Total}[S]}{K_M + [S]}$$

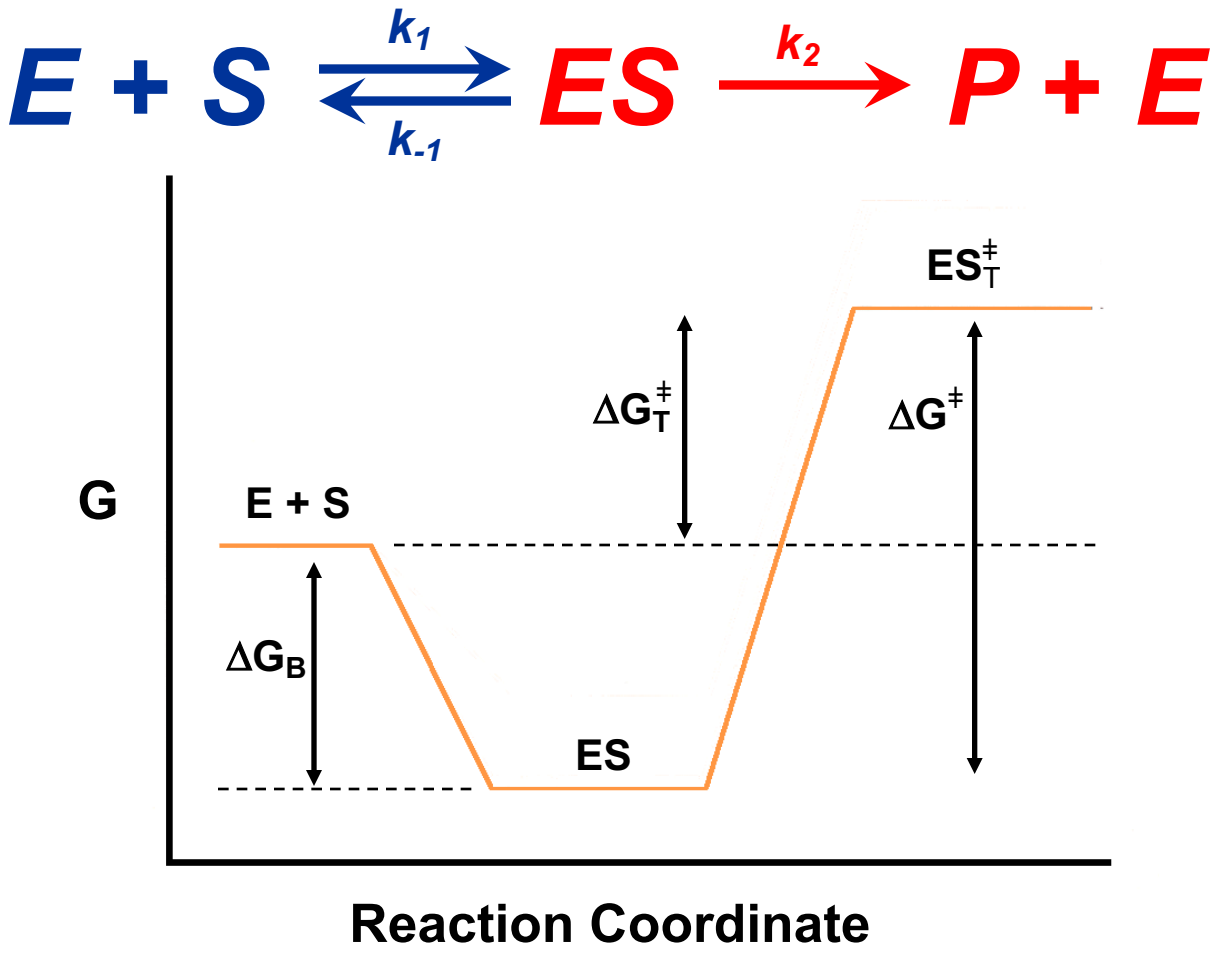
When the *[S]>>K_M*, the enzyme is saturated with substrate and the reaction reaches its maximal velocity, V_{max}:

$$\upsilon_o = V_{\max} = \frac{k_2[E]_{Total}[S]}{K_M + [S]} \approx k_2[E]_{Total} = k_{cat}[E]_{Total}$$

In this situation, we "remove" the binding component of catalysis and the reaction rate depends purely on the catalytic ability of the enzyme. For this reason, we refer to k_2 as k_{cat}.

II Michaelis-Menten kinetics:

Case I: [S] >> K_M



When [S] is very high ([S]>>>K_M), the enzyme is saturated (i.e. predominantly in ES), so the reaction rate depends on the transition from ES to $ES^{\ddagger}_{T}$, or k_2/k_{cat}.

II Michaelis-Menten kinetics:

Case I: [S] >> K_M

$$\upsilon_O = V_{\max} = k_2[E]_{Total} = k_{cat}[E]_{Total}$$

When the concentration of substrate is high and drives the enzyme into the *ES* form, the energy from the binding of the substrate to the enzyme has no effect on the reaction rate – the reaction is dependent only on k_2 or k_{cat}, which is referred to as the "catalytic constant" or the "turnover number.

The turnover number tells us the number of reactions that each active site catalyzes per unit time and has units of s^{-1}. k_{cat} tells us about the pure catalytic ability of the enzyme – how fast it can make a reaction occur once the substrate is bound to the enzyme. k_{cat} is directly related to the energy between *ES* and the transition state, $ES^{\ddagger}_{T}$.

II Michaelis-Menten kinetics:

Case II: [S] = K_M

$$\upsilon_o = \frac{k_{cat}[E]_{Total}[S]}{K_M + [S]} = \frac{V_{\max}[S]}{K_M + [S]}$$

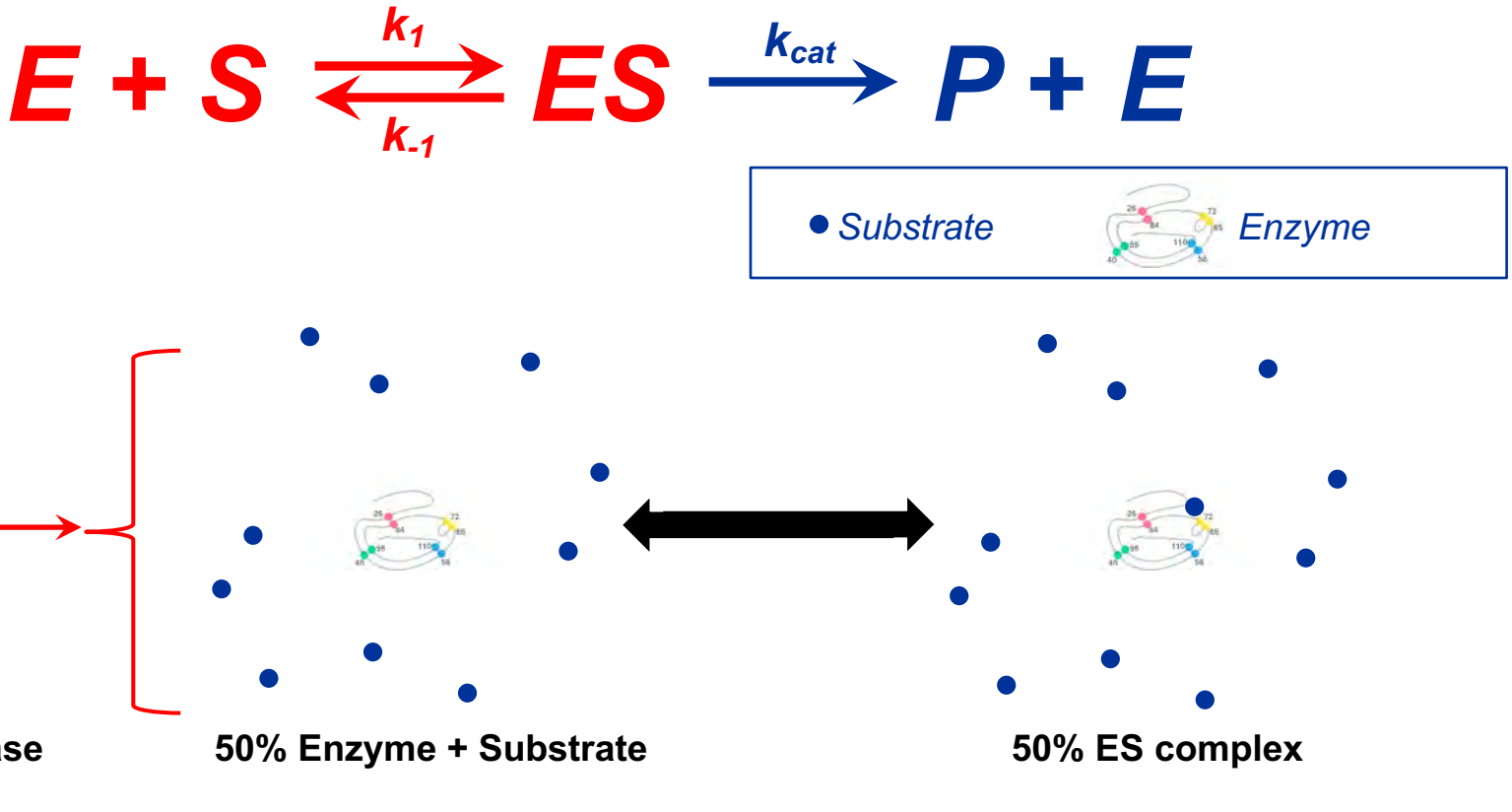
K_M has the units of M (mol per liter). When [S] = K_M:

$$\upsilon_o = \frac{V_{\max}K_M}{K_M + K_M} = \frac{V_{\max}}{2}$$

So the reaction is at half maximal velocity.

II Michaelis-Menten kinetics:

Case II: [S] = K_M



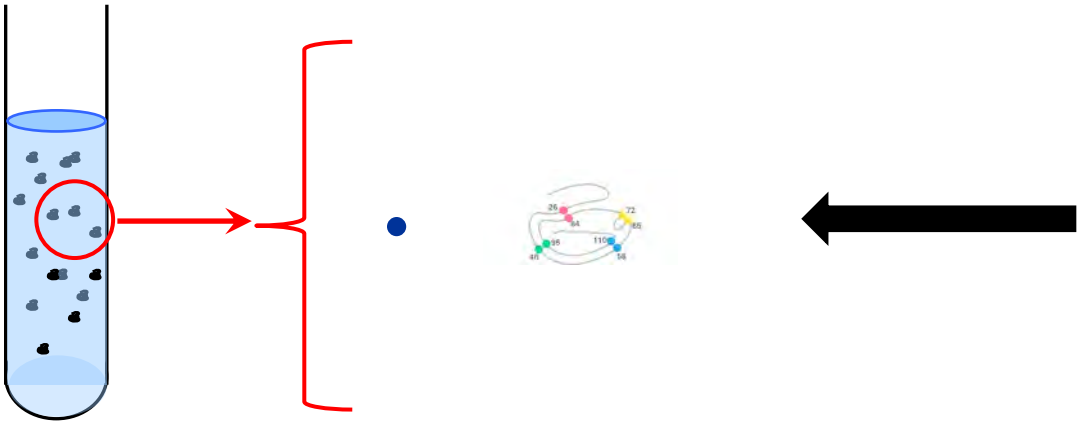
When [S] = K_M, the equilibrium between free and bound enzyme is such that ~50% of the enzyme binds substrate and ~50% is free.

K_M is thus similar to K_D, and tells us about the affinity of the enzyme for the substrate.

II Michaelis-Menten kinetics:

Case III: [S] << K_M

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



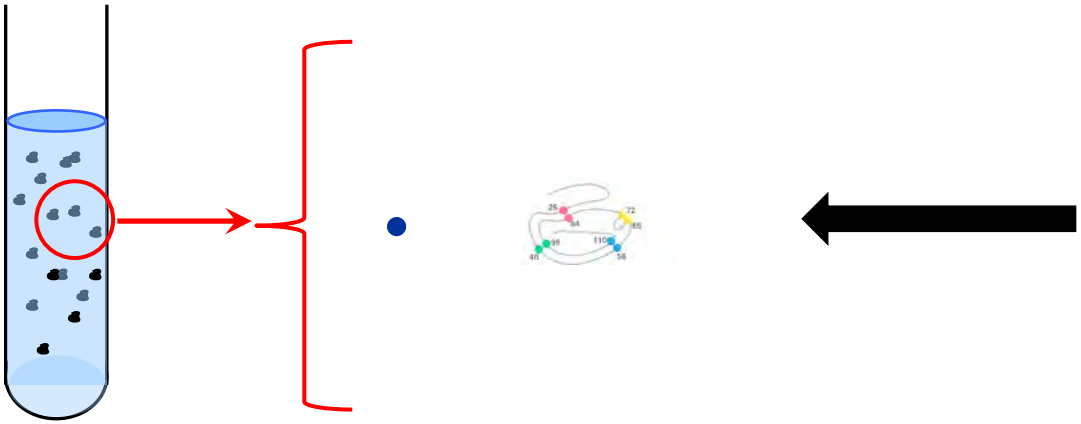
Ribonuclease + RNA | **~100% Enzyme + Substrate** | **ES complex**

When [S] is very low ([S]<<<K_M), the rate of the reaction depends not only on the catalytic ability of the enzyme, k_{cat}, but also on how often the enzyme and substrate collide leading to a productive *ES* complex – the latter depending on affinity of E for S – i.e. the K_D.

II Michaelis-Menten kinetics:

Case III: [S] << K_M

$$E + S \xrightarrow{k_{cat}/K_M} P + E$$



Ribonuclease + RNA | **~100% Enzyme + Substrate** | **ES complex**

When [S] is very low ([S]<<<K_M), the rate of the reaction depends not only on the catalytic ability of the enzyme, k_{cat}, but also on how often the enzyme and substrate collide leading to a productive *ES* complex – the latter depending on affinity of E for S – i.e. the K_D.

II Michaelis-Menten kinetics:

Case III: [S] << K_M

$$E + S \xrightarrow{k_{cat}/K_M} P + E$$

$$\upsilon_o = \frac{k_{cat}[E]_{Total}[S]}{K_M + [S]}$$

$$\upsilon_o = \frac{k_{cat}[E]_{total}[S]}{K_M + [S]} \approx \frac{k_{cat}}{K_M}[E]_{total}[S]$$

k_{cat}/K_M is referred to as the specificity constant. The units of k_{cat}/K_M are $M^{-1}s^{-1}$: i.e. they are the expected units for a bi-molecular reaction!

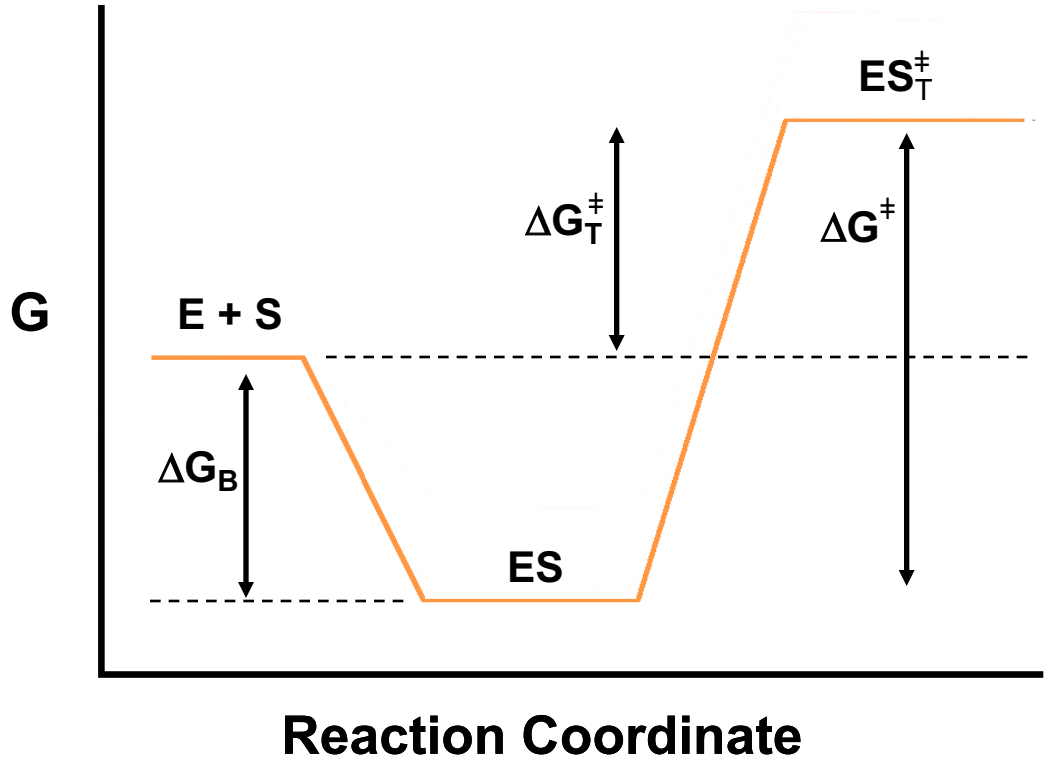
At low [S], the reaction depends on how often the enzyme and substrate collide (i.e. $[E]_{total}[S]$), the affinity of the enzyme for the substrate (i.e. K_M - the chances that a collision will lead to formation of ES), and the catalytic ability of the enzyme (k_{cat}).

K_{cat}/K_M is directly related to the energy between the reactants and the transition state $ES^{\ddagger}_T$.

II Michaelis-Menten kinetics:

4) The transition state and reaction thermodynamics

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} ES_T^{\ddagger} \longrightarrow P + E$$

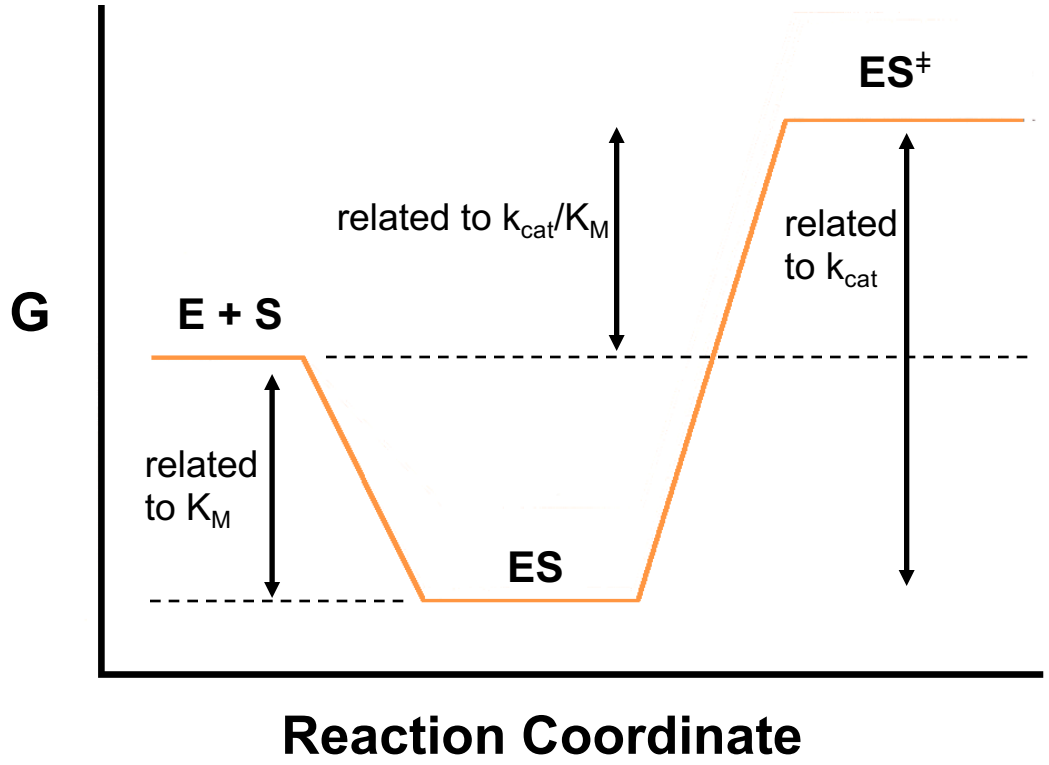


The reaction coordinate diagram shows the energetic contributions of binding (ΔG_B) and catalysis ($\Delta G^{\ddagger}$). The term $\Delta G_T^{\ddagger}$ is a measure of how both binding and catalysis contribute to the overall catalytic ability of the enzyme.

II Michaelis-Menten kinetics:

4) The transition state and reaction thermodynamics

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} ES_T^{\ddagger} \longrightarrow P + E$$



$$\Delta G_B \cong RT\ln K_M$$

$$\Delta G^{\ddagger} = RT\ln(k_B T/h) - RT\ln(k_{cat}/K_M)$$

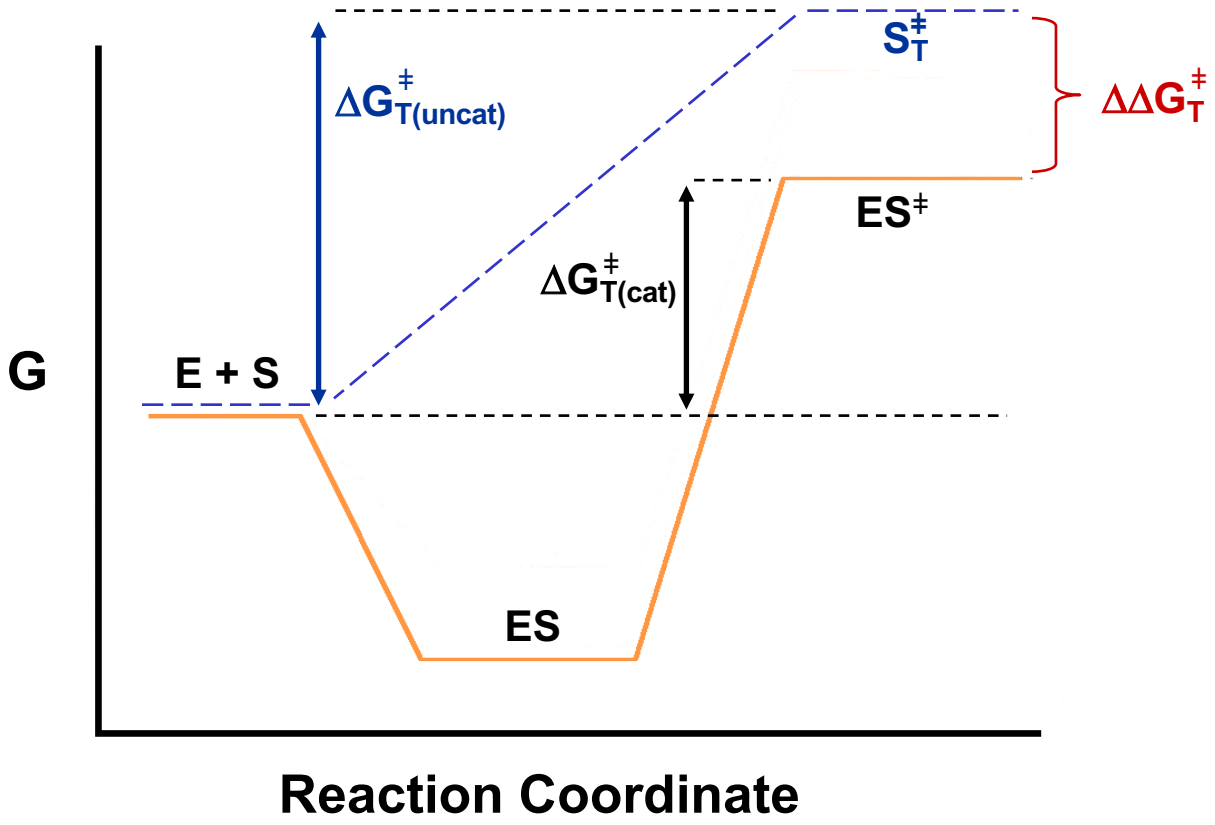
$$\Delta G^{\ddagger} \cong RT\ln(k_B T/h) - RT\ln(k_{cat})$$

$$\Delta G_T^{\ddagger} = \Delta G_B + \Delta G^{\ddagger}$$

Each of the energy differences between states in the reaction coordinate diagram is related to a kinetic parameter. So by defining our kinetic parameters, we get insight into the different aspects of catalysis.

II Michaelis-Menten kinetics:

4) The transition state and reaction thermodynamics



We are interested in the mechanisms of catalysis – how the enzyme lowers the activation energy – the energy difference between $S_T^{\ddagger}$ and $ES_T^{\ddagger}$:

$$\Delta\Delta G_T^{\ddagger} = \Delta G_{T(cat)}^{\ddagger} - \Delta G_{T(uncat)}^{\ddagger}$$

The enzyme uses binding energy and interactions with the transition state to catalyze the rxn.

II Michaelis-Menten kinetics:

Kinetic parameters, K_M

TABLE 13-1. THE VALUES OF K_M, K_{CAT}, AND K_{CAT}/K_M FOR SOME ENZYMES AND SUBSTRATES

Enzyme	Substrate	K_M (M)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}\ s^{-1}$)
Acetylcholinesterase	Acetylcholine	9.5×10^{-5}	1.4×10^{4}	1.5×10^{8}
Carbonic anhydrase	CO_2	1.2×10^{-2}	1.0×10^{6}	8.3×10^{7}
	HCO_3^-	2.6×10^{-2}	4.0×10^{5}	1.5×10^{7}
Catalase	H_2O_2	2.5×10^{-2}	1.0×10^{7}	4.0×10^{8}
Chymotrypsin	*N*-Acetylglycine ethyl ester	4.4×10^{-1}	5.1×10^{-2}	1.2×10^{-1}
	N-Acetylvaline ethyl ester	8.8×10^{-2}	1.7×10^{-1}	1.9
	N-Acetyltyrosine ethyl ester	6.6×10^{-4}	1.9×10^{2}	2.9×10^{5}
Fumarase	Fumarate	5.0×10^{-6}	8.0×10^{2}	1.6×10^{8}
	Malate	2.5×10^{-5}	9.0×10^{2}	3.6×10^{7}
Urease	Urea	2.5×10^{-2}	1.0×10^{4}	4.0×10^{5}

K_M tells us about the affinity of the enzyme for the substrate – K_M is analogous to K_D.

K_M must have a values such that the enzyme can bind substrate under physiological conditions – so acetylcholinesterase binds its substrate with a much higher affinity than catalase binds H_2O_2.

II Michaelis-Menten kinetics:

Kinetic parameters, k_{cat}

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Urease	Urea	2.5×10^{-2}	1.0×10^{4}	4.0×10^{5}

k_{cat} tells us about the pure catalytic ability of the enzyme. In other words, once substrate is bound, how fast does the enzyme catalyze the reaction.

Catalase is a very fast enzyme – each molecule catalyzes 10^7 molecules of H_2O_2 per second, whereas each fumarase enzyme can only catalyze ~10^3 molecules of substrate per second.

II Michaelis-Menten kinetics:

Kinetic parameters, k_{cat}/K_M

TABLE 13-1. THE VALUES OF K_M, K_{CAT}, AND K_{CAT}/K_M FOR SOME ENZYMES AND SUBSTRATES

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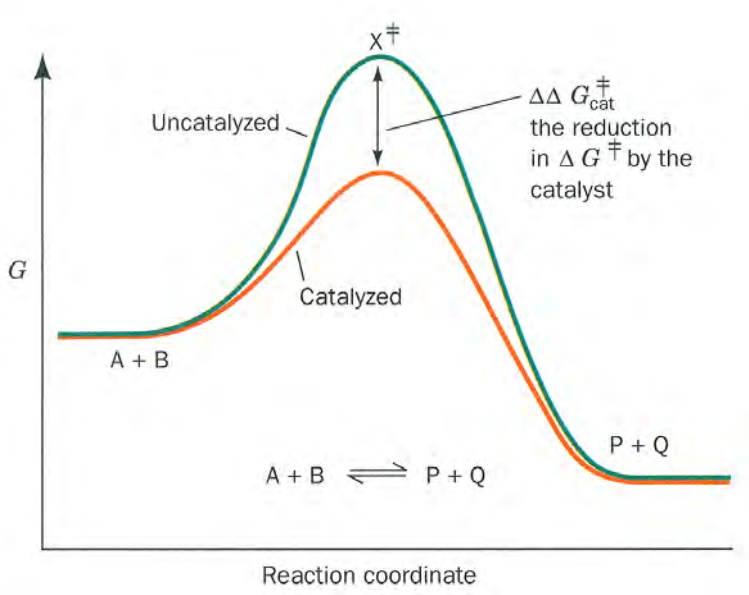
The specificity constant, k_{cat}/K_M gives us a sense of overall catalytic efficiency taking into account both pure catalytic capability and ability to bind substrate effectively in solution.

Note that the diffusion limit is 10^8-10^9 $M^{-1}s^{-1}$ tells us how often a collision will actually occur in solution. Acetylcholinesterase, catalase, and fumerase are all extremely efficient with there specificity constants approaching the diffusion controlled limit. *This means that almost every time a collision occurs, there is catalysis!*

III Thermodynamic basis of catalysis:

Catalysts, including enzymes, increase reaction rates by lowering the activation energy for the reaction – i.e. lowering the energy of the transition state. *There is a mathematical relationship between "rate of enhancement" and the "drop in activation energy". Let's develop this relationship using a standard chemical reaction:*

$$A + B \rightleftharpoons X^{\ddagger} \longrightarrow P + Q$$



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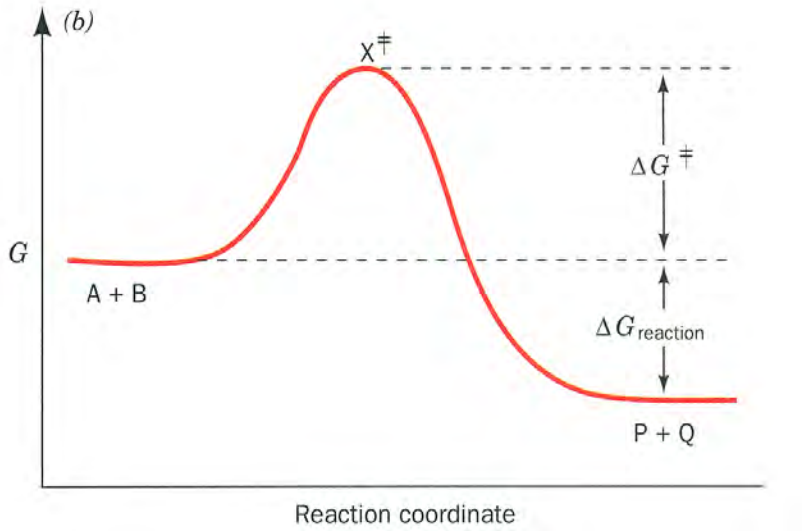
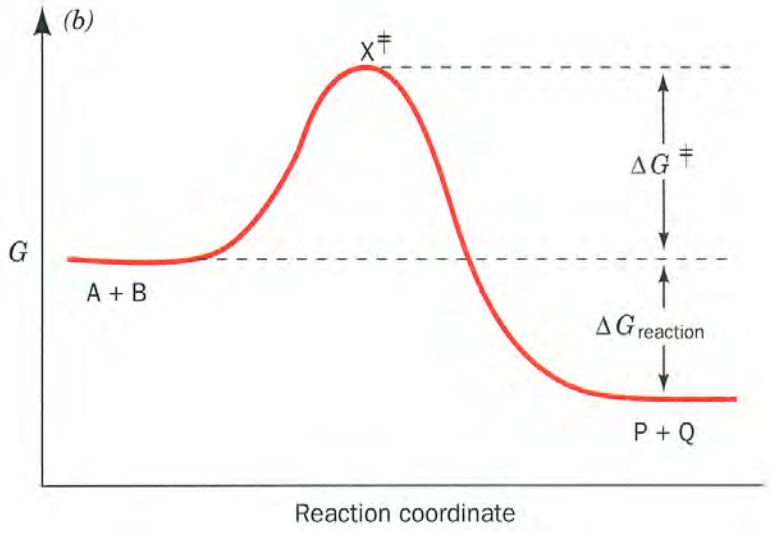


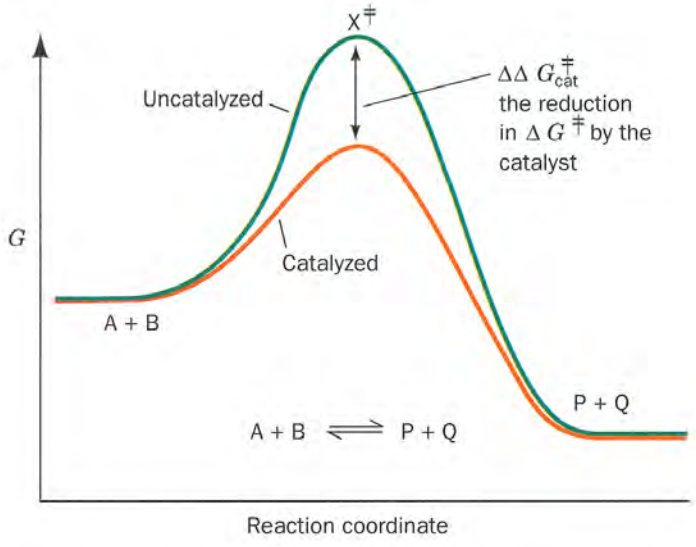
$$Rate = k' e^{\frac{-\Delta G^{\neq}}{RT}} [A][B]$$

The rate of the reaction depends on the concentration of A and B, as well as the ***activation energy*** *– the energy difference between A, B and the transition state $X^{\ddagger}$ ($\Delta G^{\ddagger}$). Catalysis occurs when the enzyme lowers this activation energy ($\Delta G^{\ddagger}$).*

III Thermodynamic basis of catalysis:

$$A + B \rightleftharpoons X^{\ddagger} \longrightarrow P + Q$$





$$Rate = k' e^{\frac{-\Delta G^{\neq}}{RT}} [A][B]$$

$$Rate_{uncat} = k' e^{\frac{-\Delta G^{\neq}_{uncat}}{RT}} [A][B]$$

$$Rate_{cat} = k' e^{\frac{-\Delta G^{\neq}_{cat}}{RT}} [A][B]$$

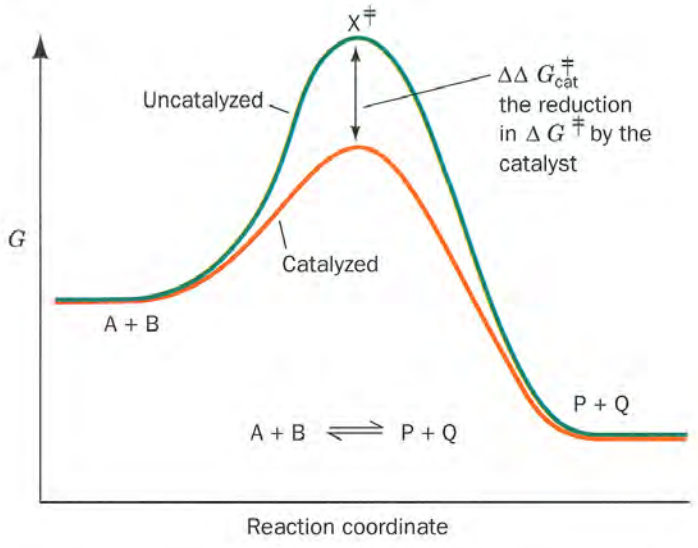
The rate of the reaction depends on the concentration of A and B, as well as the ***activation energy*** *– the energy difference between A, B and the transition state $X^{\ddagger}$ ($\Delta G^{\ddagger}$). Catalysis occurs when the enzyme lowers this activation energy ($\Delta G^{\ddagger}$).*

III Thermodynamic basis of catalysis:

$$A + B \rightleftharpoons X^{\ddagger} \longrightarrow P + Q$$

$$Rate_{uncat} = k' e^{\frac{-\Delta G^{\neq}_{uncat}}{RT}} [A][B] \quad (1)$$

$$Rate_{cat} = k' e^{\frac{-\Delta G^{\neq}_{cat}}{RT}} [A][B] \quad (2)$$



If at a given [A], [B], the catalyzed rxn is 10^{10} fold faster, then:

$$Rate_{cat} = 10^{10} Rate_{uncat} \quad (3)$$

Sub (1) and (2) into (3)

$$\therefore k' e^{\frac{-\Delta G^{\neq}_{cat}}{RT}} [A][B] = 10^{10} k' e^{\frac{-\Delta G^{\neq}_{uncat}}{RT}} [A][B]$$

continued…

[A], [B], k' cancel:

$$e^{\frac{-\Delta G^{\neq}_{cat}}{RT}} = 10^{10} e^{\frac{-\Delta G^{\neq}_{uncat}}{RT}}$$

Rearrange:

$$10^{10} = \frac{e^{\frac{-\Delta G^{\neq}_{cat}}{RT}}}{e^{\frac{-\Delta G^{\neq}_{uncat}}{RT}}}$$

Take the ln:

$$\therefore \ln(10^{10}) = \ln\left(\frac{e^{\frac{-\Delta G^{\neq}_{cat}}{RT}}}{e^{\frac{-\Delta G^{\neq}_{uncat}}{RT}}}\right) = \ln\left(e^{\frac{-\Delta G^{\neq}_{cat}}{RT} - \frac{-\Delta G^{\neq}_{uncat}}{RT}}\right)$$

Rearrange:

$$\ln(10^{10}) = \frac{-(\Delta G^{\neq}_{cat} - \Delta G^{\neq}_{uncat})}{RT}$$

$$\Delta\Delta G^{\neq}_{cat} = \Delta G^{\neq}_{cat} - \Delta G^{\neq}_{uncat} = -RT\ln(10^{10})$$

III Thermodynamic basis of catalysis:

$$\Delta\Delta G^{\neq}_{cat} = \Delta G^{\neq}_{cat} - \Delta G^{\neq}_{uncat} = -RT\ln(10^{10})$$

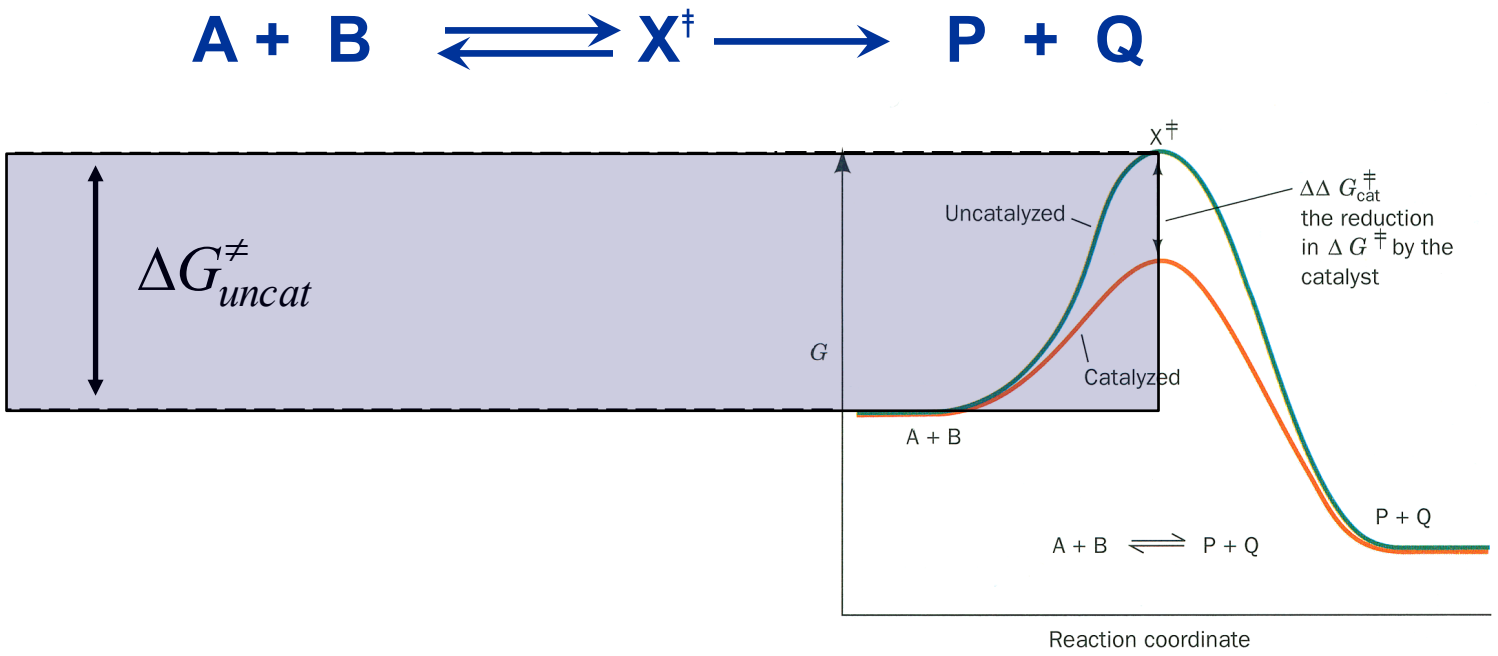
The above equation tells us that there is a direct mathematical relationship between the rate enhancement provided by a catalyst, and the amount by which the activation energy is lowered. $\Delta\Delta G^{\ddagger}_{cat}$ is the "symbol" used to denote the difference in activation energy between a catalyzed and an uncatalyzed reaction. In this case, the catalyzed reaction is 10^{10} times faster, which corresponds to a:

$$\Delta\Delta G^{\ddagger}_{cat} = -57.1 \text{ kJmol}^{-1}$$

As we shall see, for every 5.7 kJmol^{-1} that the activation energy is lowered, there is a 10-fold increase in reaction rate (25 ^{o}C).

III Thermodynamic basis of catalysis

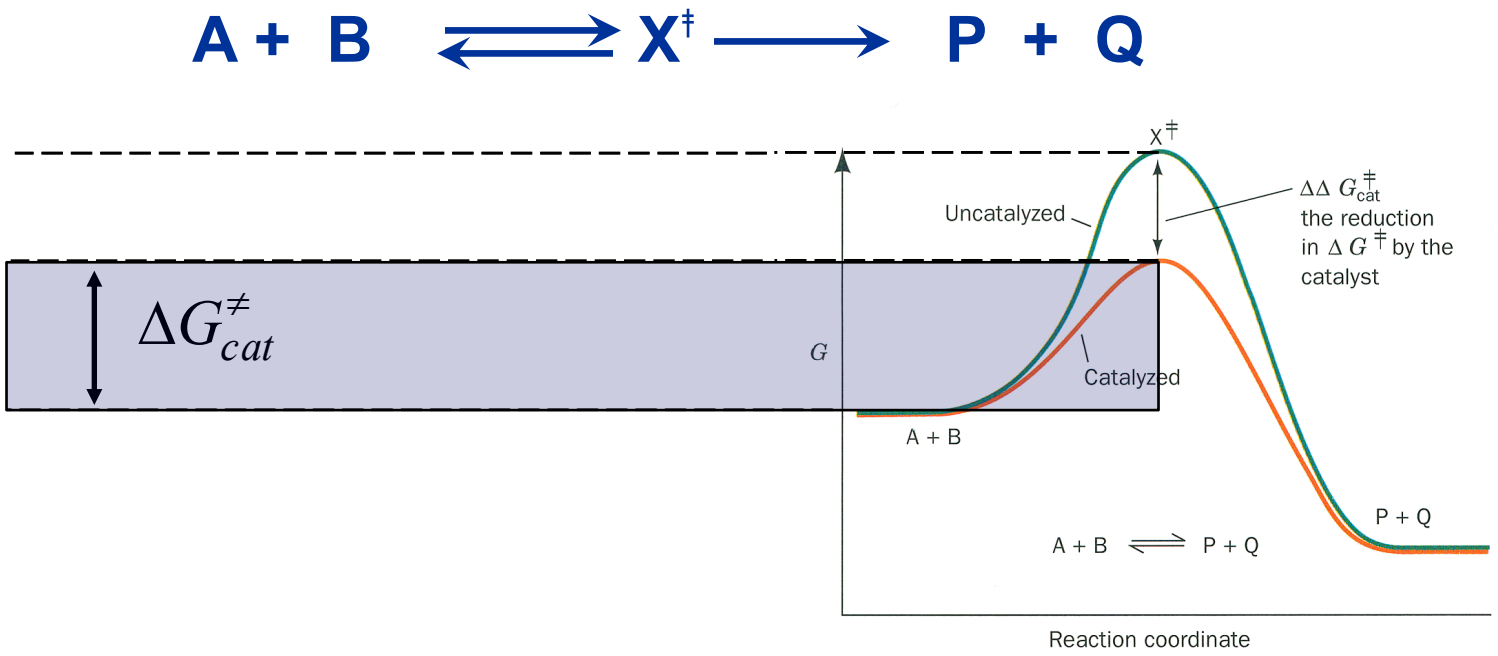
1) $\Delta G^{\ddagger}_{uncat}$



$\Delta G^{\ddagger}_{uncat}$ is the activation energy for the uncatalyzed chemical reaction.

III Thermodynamic basis of catalysis

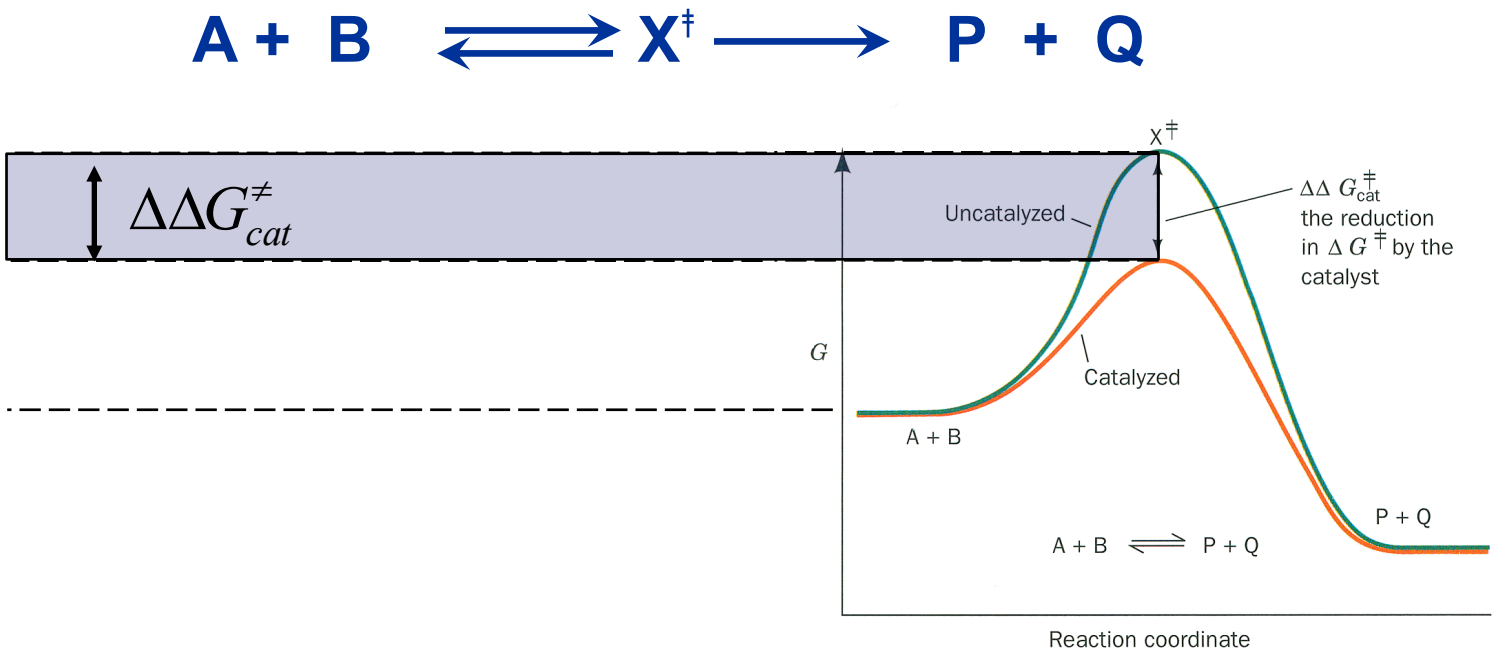
2) $\Delta G^{\ddagger}_{cat}$



$\Delta G^{\ddagger}_{cat}$ is the activation energy for the catalyzed chemical reaction.

III Thermodynamic basis of catalysis

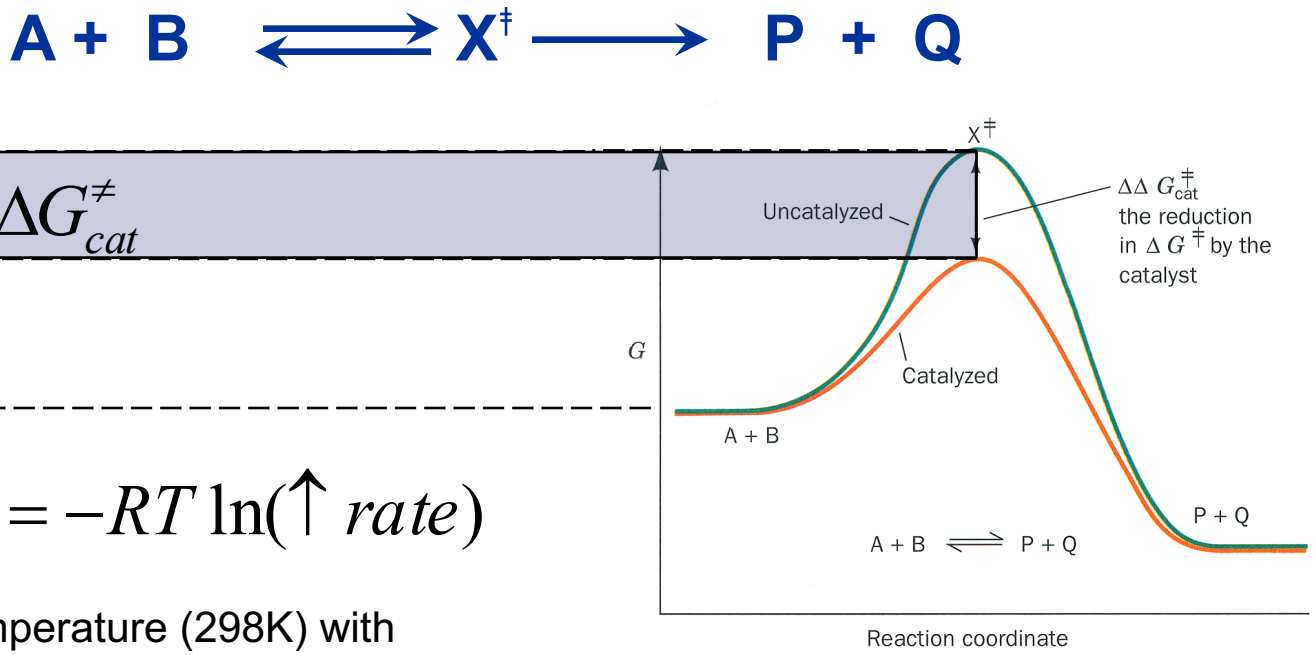
3) $\Delta\Delta G^{\ddagger}_{cat}$



$\Delta\Delta G^{\ddagger}_{cat}$ is the difference in the activation energy between the catalyzed and uncatalyzed chemical reaction. The units of $\Delta\Delta G^{\ddagger}_{cat}$ are $Jmol^{-1}$.

III Thermodynamic basis of catalysis

3) Relationship between $\Delta\Delta G^{\ddagger}_{cat}$ and rate enhancement



$$\Delta\Delta G^{\neq}_{cat} = -RT\ln(\uparrow rate)$$

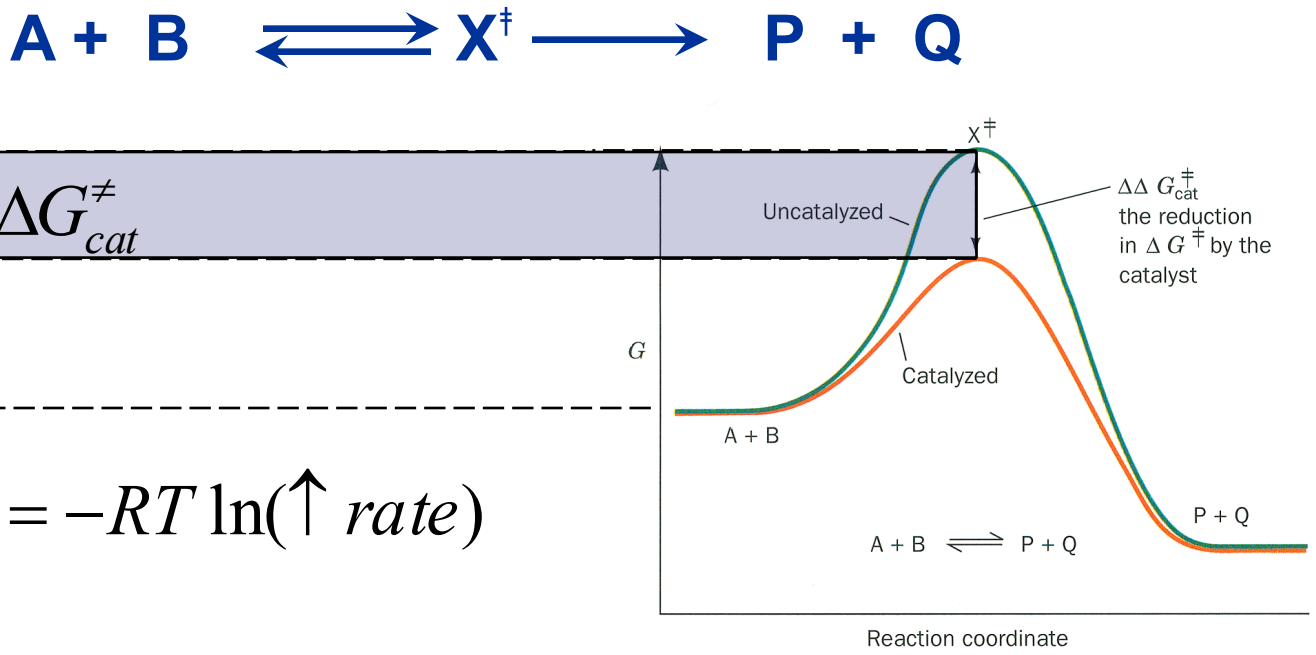
At room temperature (298K) with
R (gas constant) = 8.314 $JK^{-1}mol^{-1}$

For a 10^{10}-fold rate enahancement: $\Delta\Delta G^{\neq}_{cat} = -57 kJmol^{-1}$

To get a 10^{10} fold faster rate, the catalyst must lower the activation energy by 57 $kJmol^{-1}$!

III Thermodynamic basis of catalysis

3) Relationship between $\Delta\Delta G^{\ddagger}_{cat}$ and rate enhancement



$$\Delta\Delta G^{\neq}_{cat} = -RT\ln(\uparrow rate)$$

For a 10 fold ↑ rate Activation energy ↓ 1 x 5.7 kJmol $^{-1}$

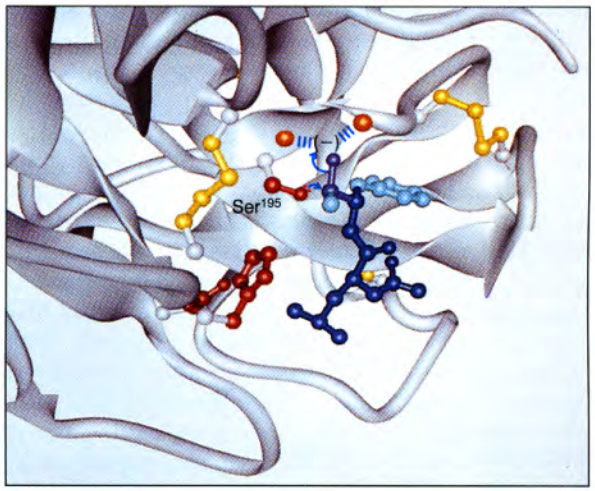
For a 10^2 fold ↑ rate Activation energy ↓ by 2 x 5.7 kJmol^{-1} = 11.4 kJmol $^{-1}$

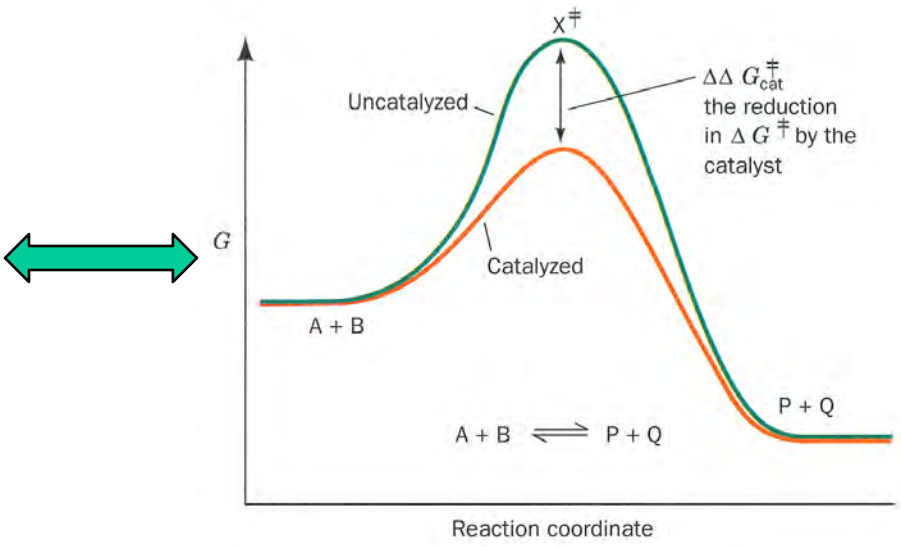
For a 10^3 fold ↑ rate Activation energy ↓ by 3 x 5.7 kJmol^{-1} = 17.1 kJmol $^{-1}$

For a 10^4 fold ↑ rate Activation energy ↓ by 4 x 5.7 kJmol^{-1} = 22.8 kJmol $^{-1}$

III Thermodynamic basis of catalysis

3) Structure, thermodynamics and mechanism of catalysis

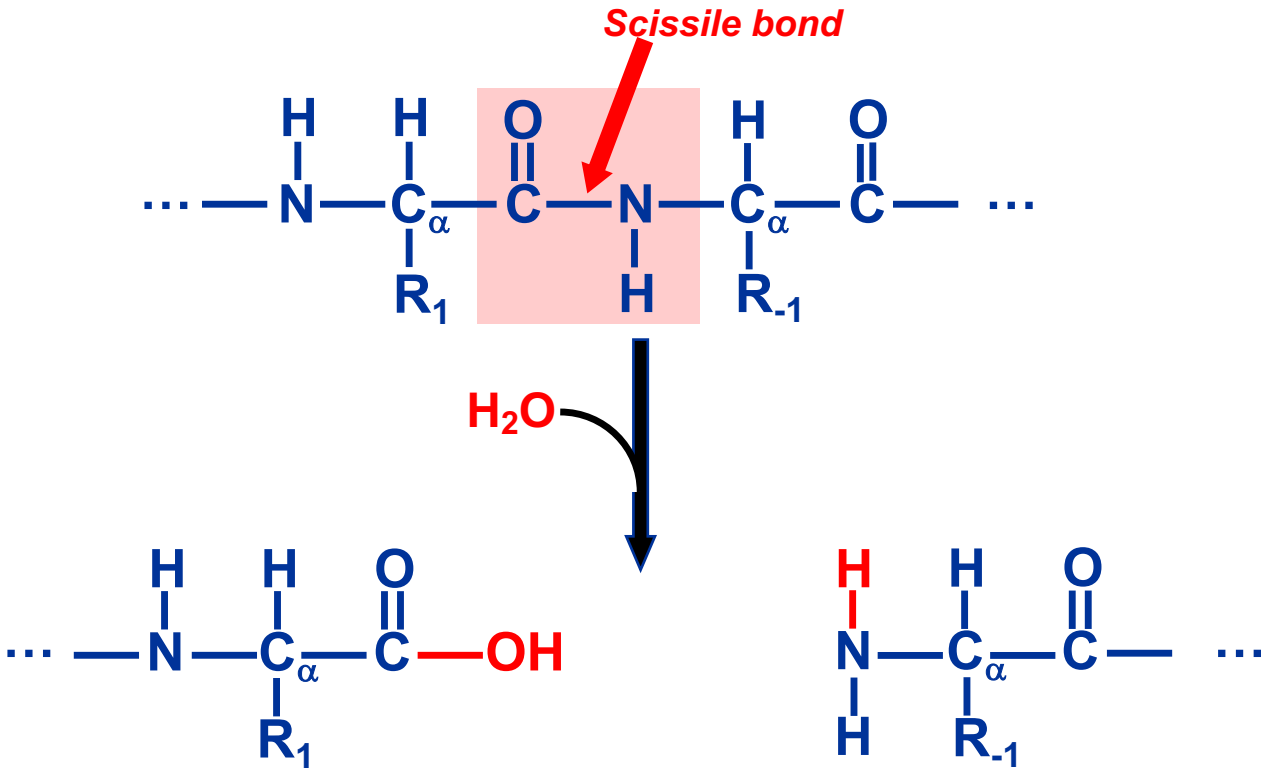




The mathematical link between “rate enhancement” and $\Delta\Delta G^{\ddagger}_{T}$ is important, because it defines how much energy the enzyme provides through enzyme-substrate interactions to stabilize the transition state and thus cause catalysis. We can then look at structure, estimate how much energy should be derived from specific interactions (H-bonds, etc.), and gain more quantitative insight into reaction mechanisms. As you will see, with protein engineering, we can dissect the mechanisms of enzyme function in detail.

IV Serine proteases are…

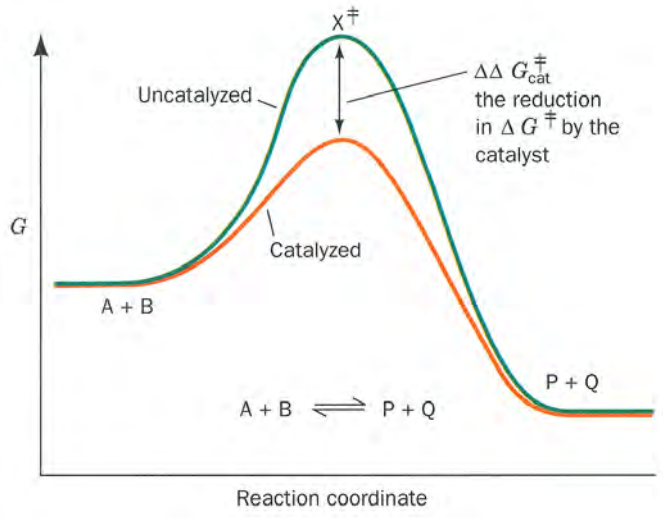
…a family of digestive enzymes that catalyze the hydrolysis of peptide bonds



Serine proteases increase hydrolysis rates by ~10^{10}-*fold*. <u>How do they do this?</u>

Catalysts enhance rates by lowering the activation energy

Catalysts, including enzymes, increase reaction rates by lowering the activation energy barrier between reactants and products.



There is a direct mathematical relationship between the rate enhancement provided by a catalyst, and the amount by which the catalyst lowers the activation energy. $\Delta\Delta G^{\ddagger}_{cat}$ is the "symbol" used to denote the change in activation energy between a catalyzed and an uncatalyzed reaction:

$$\Delta\Delta G^{\neq}_{cat} = -RT\ln(\uparrow rate)$$

In the case of a serine protease, the catalyzed reaction is 10^{10} times faster, which corresponds to a:

$$\Delta\Delta G^{\neq}_{cat} = \Delta G^{\neq}_{cat} - \Delta G^{\neq}_{uncat} = -RT\ln(10^{10})$$

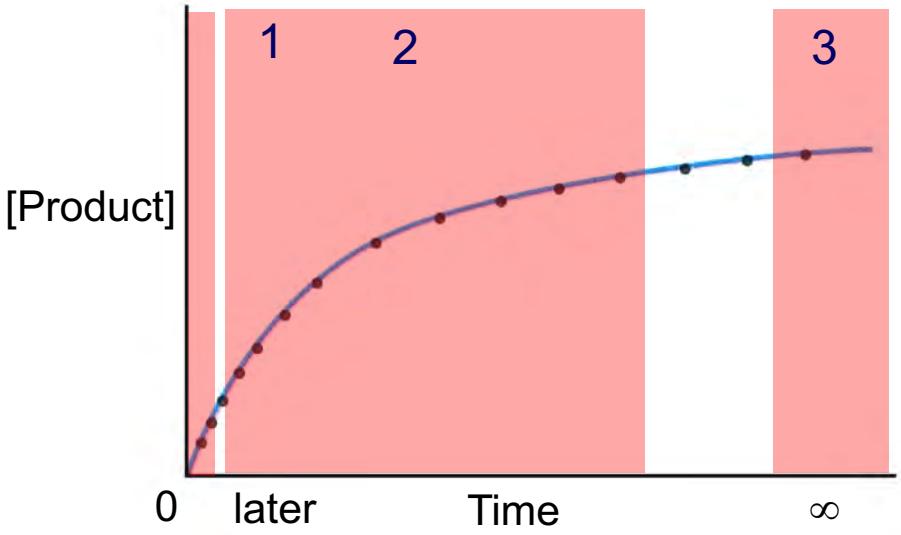
$$\Delta\Delta G^{\ddagger}_{cat} = -57.1\ \text{kJmol}^{-1}$$

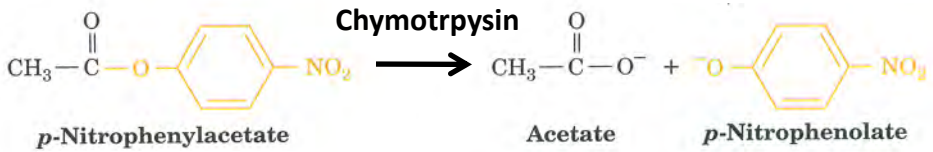
Note that for every 5.7 kJmol^{-1} that the activation energy is lowered, there is a 10-fold increase in reaction rate (25 ^{o}C).

Towards the mechanism underlying catalysis by serine proteases

1) The assay for enzymatic function

Enzyme + Substrate ⇌ Enzyme + Product





(pNPA) (pNP)

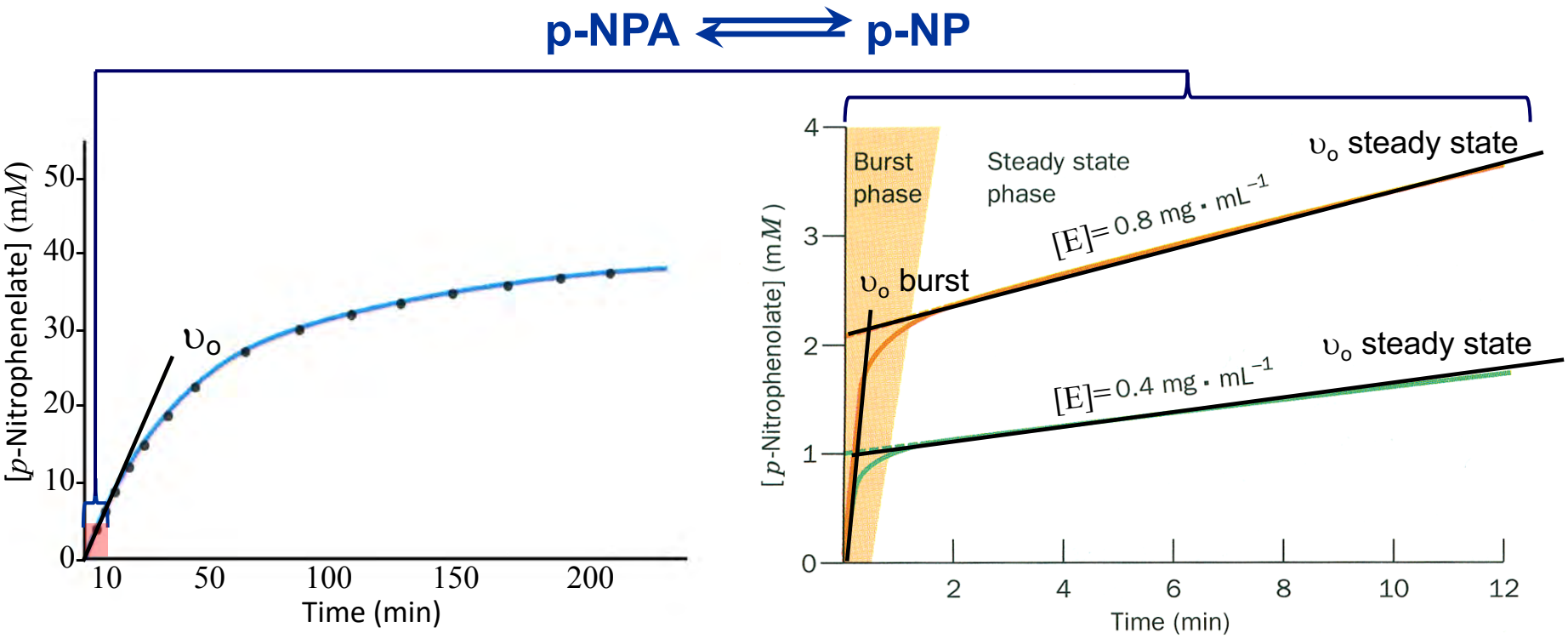
1) Time ≅ 0, there is a high [substrate] and no product - the initial rate of the reaction depends only on the [Enz] and [substrate]

2) At a later time, there is less substrate and some product
   - you start to get the reverse rxn occuring at the same time so the rsn slows down

3) Time =∞, the rates of the forward and reverse reactions are equivalent
   - the overall reaction rate is zero and the rxn is at equilibrium

Towards the mechanism underlying catalysis by serine proteases

1) The assay for enzymatic function reveals biphasic kinetics



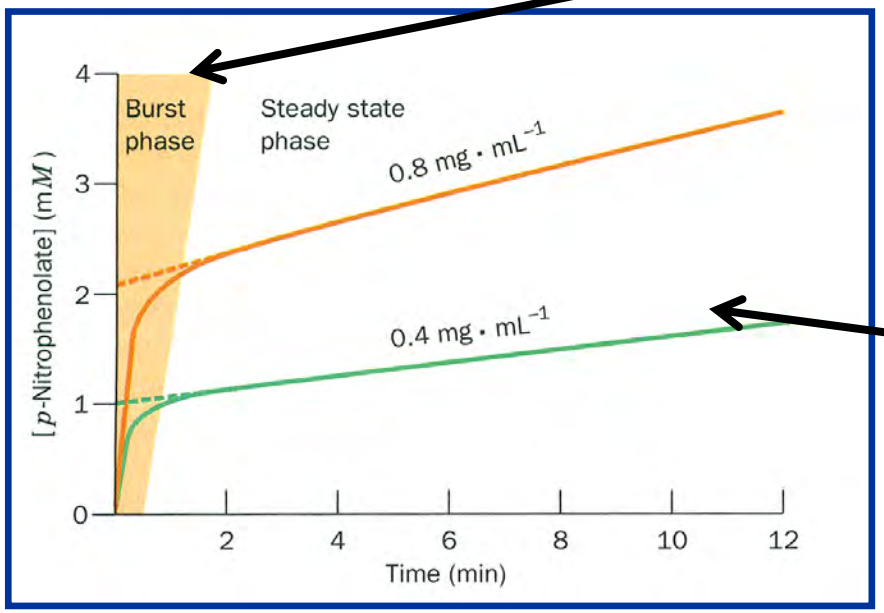
A typical enzyme catalyzed rxn is linear at the start (i.e. υ_o). At the start of a serine protease catalyzed reaction (small red box on left expanded on the right), the reaction is not linear – it is biphasic with an initial burst of activity and then a linear component corresponding to υ_o. The biphasic rxn occurs at [enzyme] = 0.4 & 0.8 mgml^{-1}. *This biphasic behavior tells us that the reaction has 2 steps.*

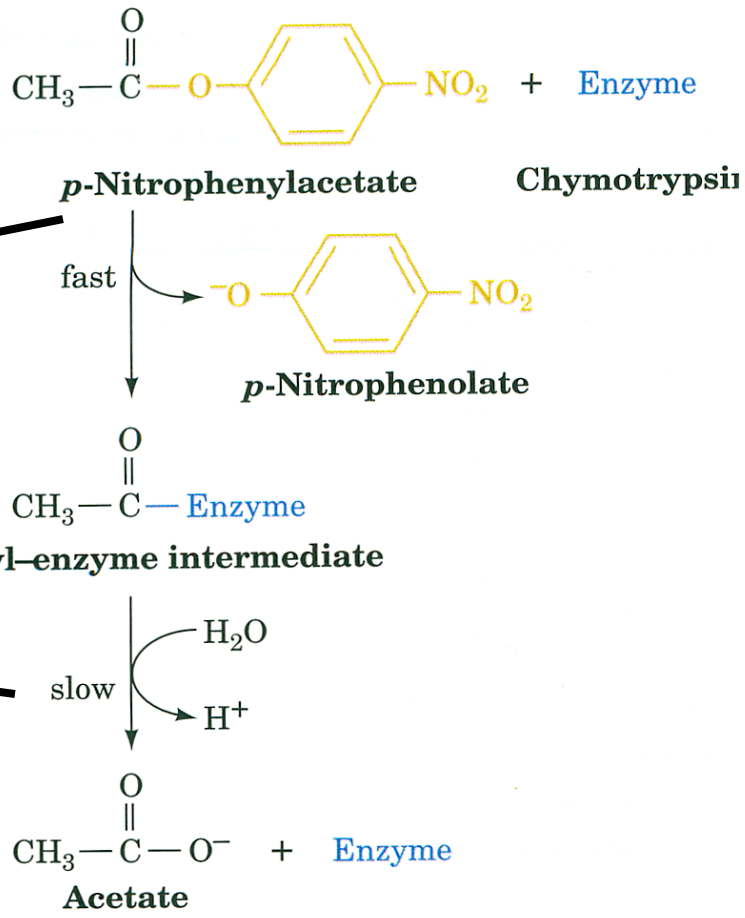
Towards the mechanism underlying catalysis by serine proteases

1) The assay for enzymatic function shows there are 2 steps

Step 1) formation of acyl enzyme intermediate and the release of p-NP - *fast*

Step 2) the hydrolysis of the acyl-enzyme intermediate leading to the formation of free enzyme and acetate - *slow*





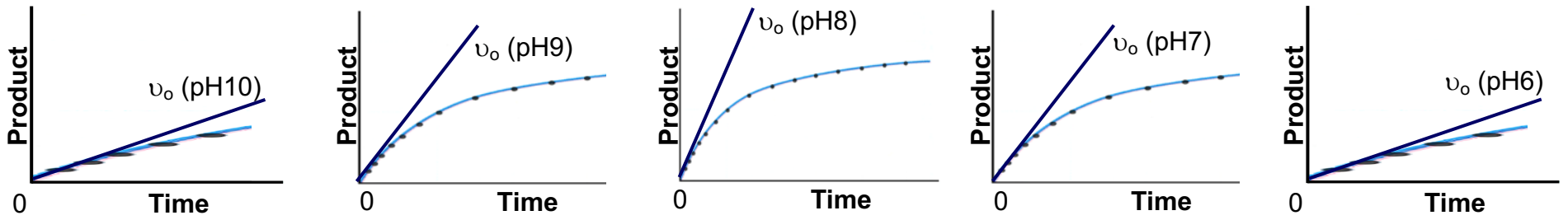
Towards the mechanism underlying catalysis by serine proteases

Clue #1: *Enzyme catalyzed peptide bond hydrolysis is a two step reaction that occurs through a covalent intermediate*

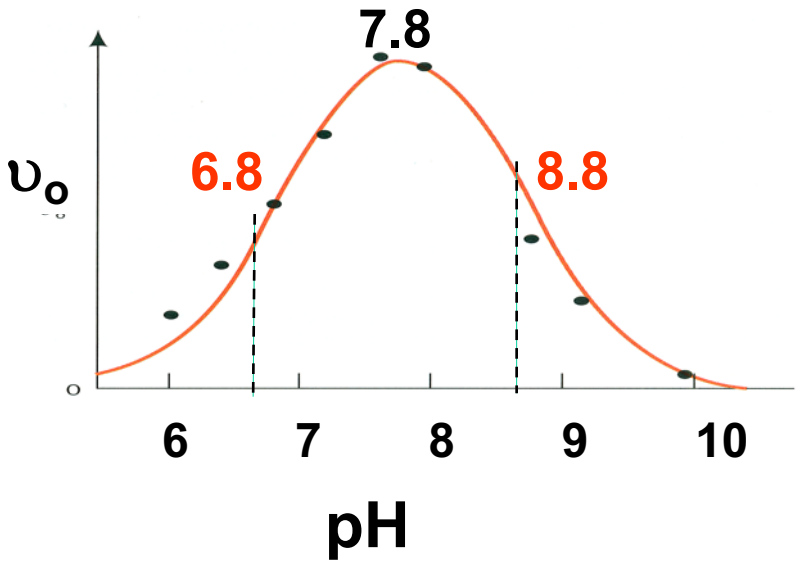
- *What group on the protein forms the covalent bond with the substrate?*
- *Can the formation of a covalent intermediate lower the activation by 57.1 kJmol^{-1} leading to 10^{10} fold increase in rate?*

Towards the mechanism underlying catalysis by serine proteases

2) Insight from the pH dependence of catalysis



A typical experiment is to measure the rate of an enzyme catalyzed reaction at various pH values. The initial rates are then plotted versus pH.



A pH dependence tells us that specific chemical groups in the protein (**usually side chains**) must adopt a specific protonation state for catalysis to occur. By curve-fitting the above dependence we can determine the pK_a of the residues involved. *The pH activity profile of an enzyme can provide important clues to the mechanisms of catalysis!*

1. *One residue: pKa ~ 6.8, deprotonated (which?)*
2. *One residue: pKa ~ 8.8, protonated (which?)*

Towards the mechanism underlying catalysis by serine proteases

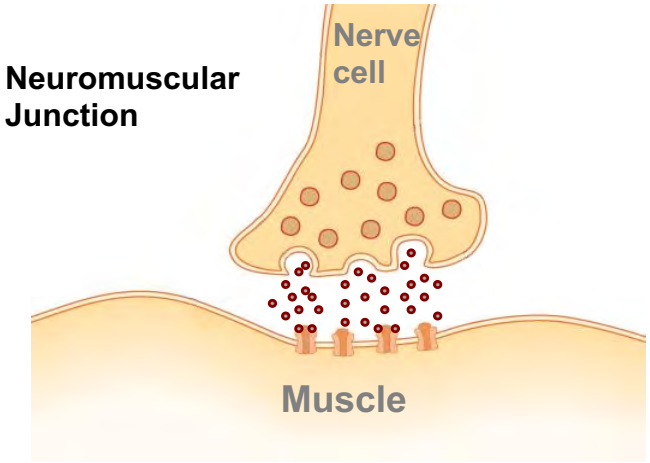
Clue #1: *Enzyme catalyzed peptide bond hydrolysis is a two step reaction that occurs through a covalent intermediate*

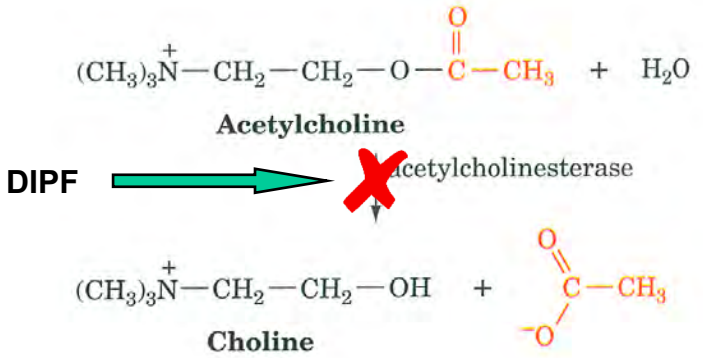
Clue #2: *The enzyme requires a deprotonated His and another residue – possibly the N-terminus - to be protonated?*

- *Could both a His and the N-terminus be involved in catalysis. Could either covalently link with the substrate? What is the role of both?*

Towards the mechanism underlying catalysis by serine proteases

3) Protease inhibitors in mustard gas used in WW1





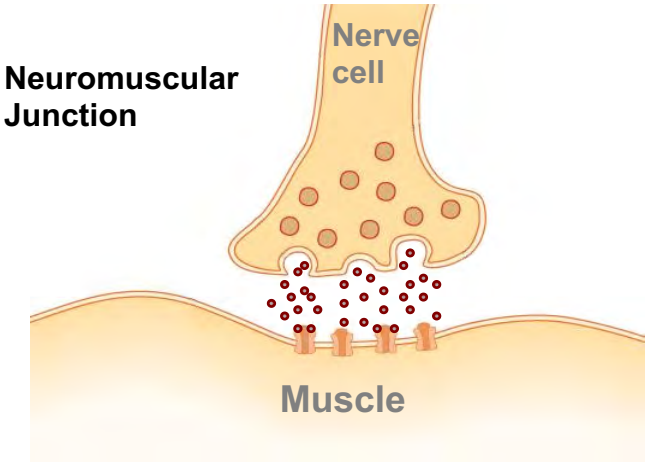
Communication at the neuromuscular junction is mediated by the neurotransmitter, acetylcholine (ACh). ACh is released from the nerve and binds to the surface of the muscle cell leading to muscle contraction. ACh is hydrolyzed by the enzyme acetylcholinesterase to allow the muscle to relax.

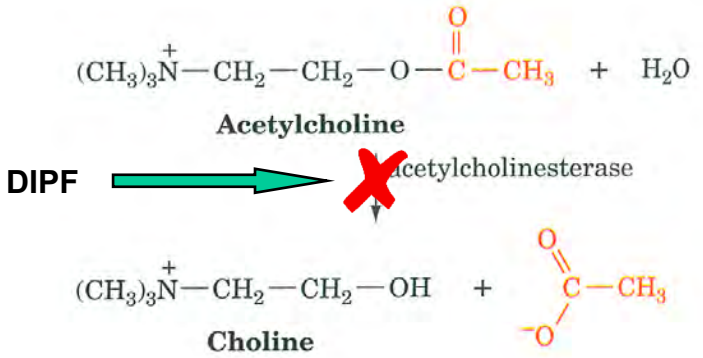
ACh esterase catalyzes ester bond hydrolysis the same way that serine proteases catalyze peptide/ester bond hydrolysis.

A compound called DIPF in mustard gas irreversibly inactivates both acetylcholine esterase and (serine proteases) to prevent relaxation of diaphragm ∴ DIPF prevents breathing!

Towards the mechanism underlying catalysis by serine proteases

3) Protease inhibitors in mustard gas used in WW1





Given that DIPF irreversibly inactivates ACh esterase/serine proteases, It was hypothesized that DIPF reacts covalently with the protein. We know from Clue #1 that there is a highly reactive group that forms a covalent bond with the peptide during hydrolysis. Could DIPF be reacting covalently with this highly reactive group?

Hypothesis:

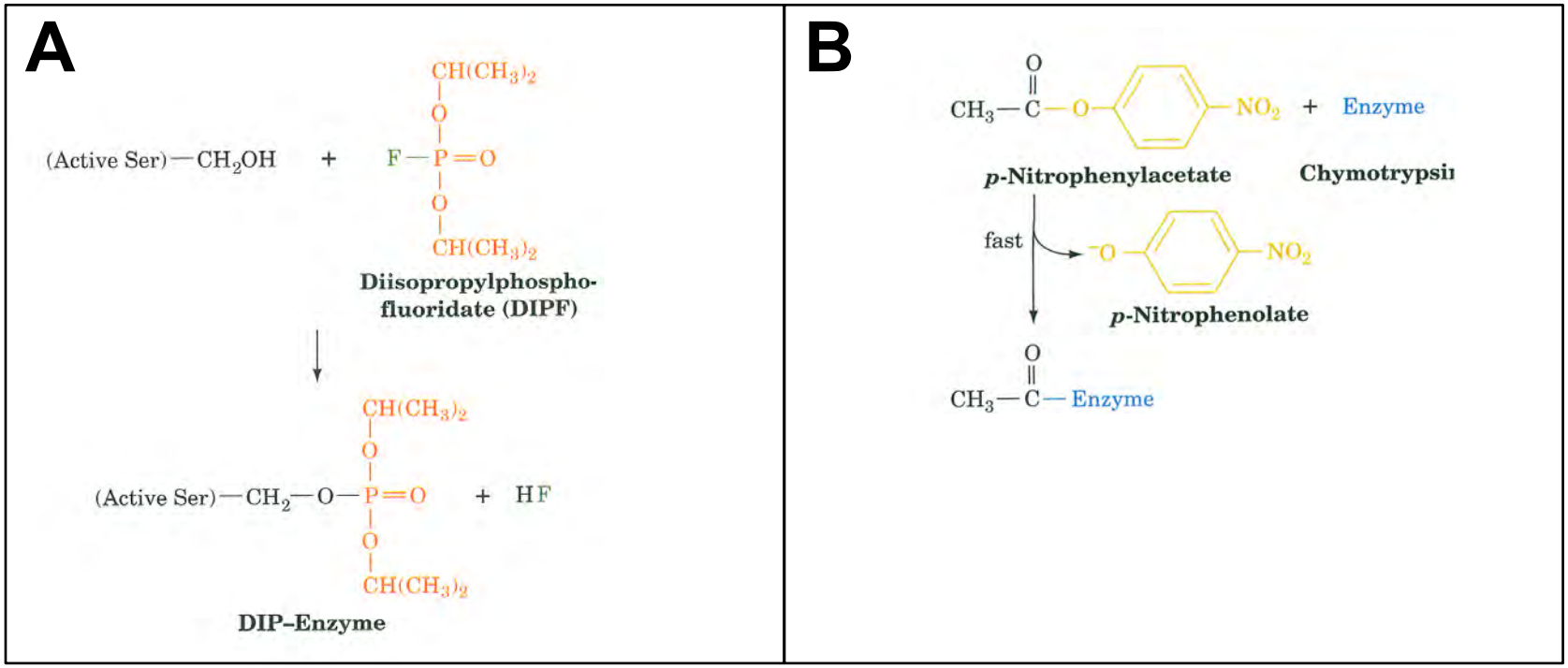
1) DIPF forms a covalent bond with a reactive side chain

2) The reactive side chain forms the acyl-enzyme intermediate during catalysis

How can we identify this residue?

Towards the mechanism underlying catalysis by serine proteases

3) Ser195 is the reactive residue involved in covalent catalysis



The covalent reaction of Ser195 with DIPF (*A*) is similar to the reaction between the enzyme and both pNPA and a peptide bond (B). Ser-195 appears to be the side chain that forms the acyl-enzyme intermediate. *But why does only Ser-195 react with DIPF?*

Towards the mechanism underlying catalysis by serine proteases

Clue #1: *Enzyme catalyzed peptide bond hydrolysis is a two step reaction that occurs through a covalent intermediate*

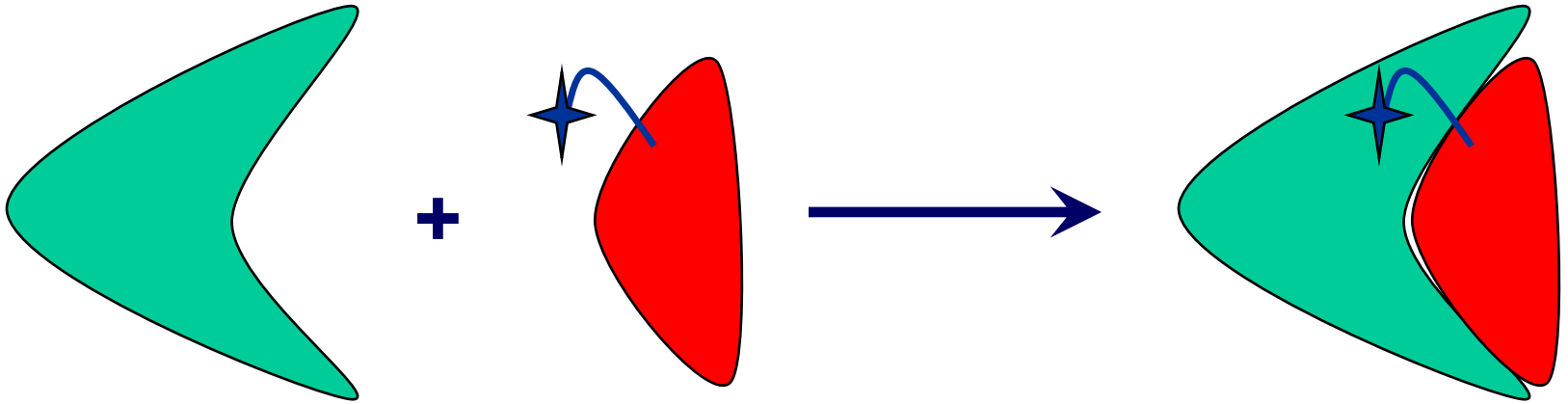
Clue #2: *The enzyme requires a deprotonated His and another residue – possibly the N-terminus - to be protonated?*

Clue #3*: Ser-195 is the reactive residue that forms a covalent bond with the substrate*

- *Why is Ser-195 so much more reactive than other serine residues in the protein?*

Towards the mechanism underlying catalysis by serine proteases

4) Affinity labelling identifies other residues in the “active sites”



Enzyme

Substrate with a reactive group

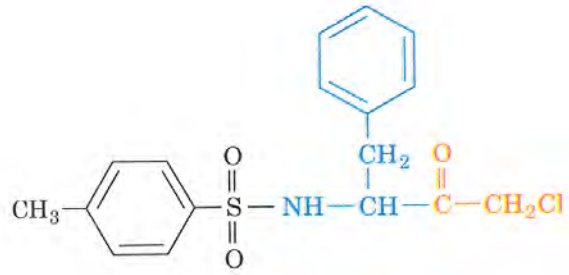
ES Complex

A substrate that binds to the active site of the enzyme is synthesized with a highly reactive group that can form a covalent bond with “close by” side chains. When this substrate binds to the active site, it forms a covalent bond this close by residue in the active site. By peptide mapping, one can identify this and other residues in the active site.

Towards the mechanism underlying catalysis by serine proteases

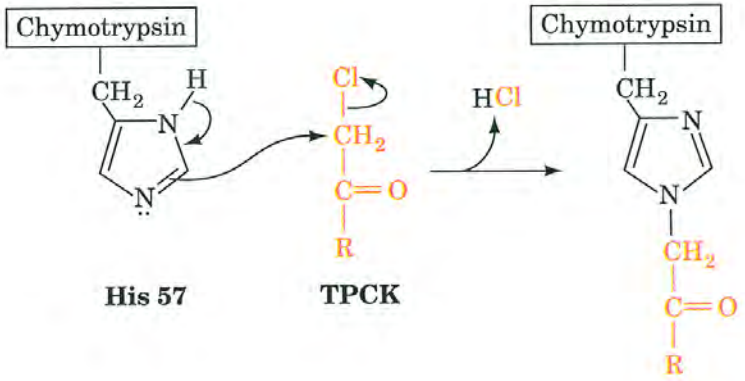
4) TPCK affinity labels His57

A



Tosyl-L-phenylalanine chloromethyl ketone

B



TPCK (*A*) is a competitive inhibitor of chymotrypsin – so it binds to the active site. TPCK reacts covalently with His 57 (*B*) suggesting that His 57 is located in or close to the active site. The observation of a His residue close to the active site of chymotrypsin correlates with the previous observation that a residue with a pKa of 6.8 (likely a His) is involved in catalysis.

Towards the mechanism underlying catalysis by serine proteases

Clue #1: *Enzyme catalyzed peptide bond hydrolysis is a two step reaction that occurs through a covalent intermediate*

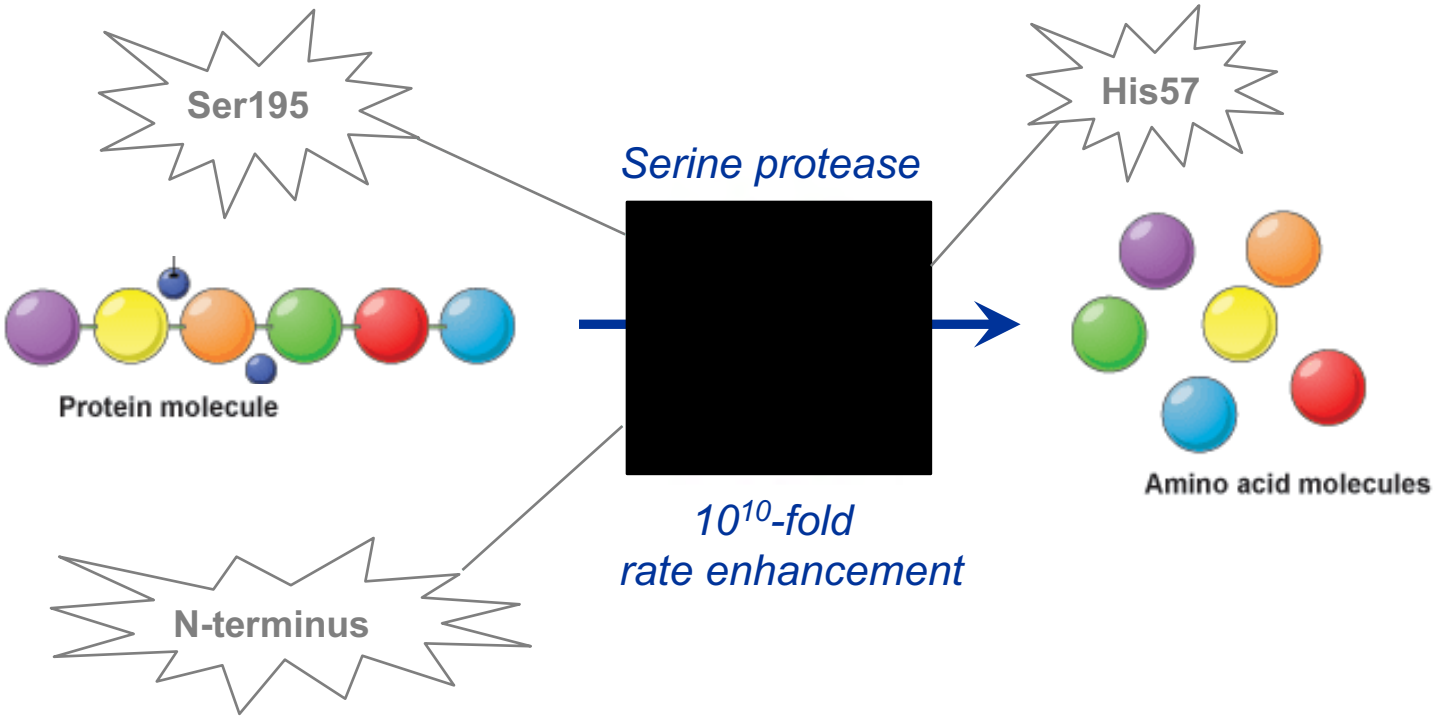
Clue #2: *The enzyme requires a deprotonated His and another residue – possibly the N-terminus - to be protonated?*

Clue #3*: Ser-195 is likely the reactive residue that forms a covalent bond with the substrate*

Clue #4: *A highly reactive His-57 is located in the active site and may play a role in catalysis*

- *Why is His-57 so much more reactive than other His residues in the protein?*
- *Could His-57 be involved making Ser-195 highly reactive?*

Towards the mechanism underlying catalysis by serine proteases

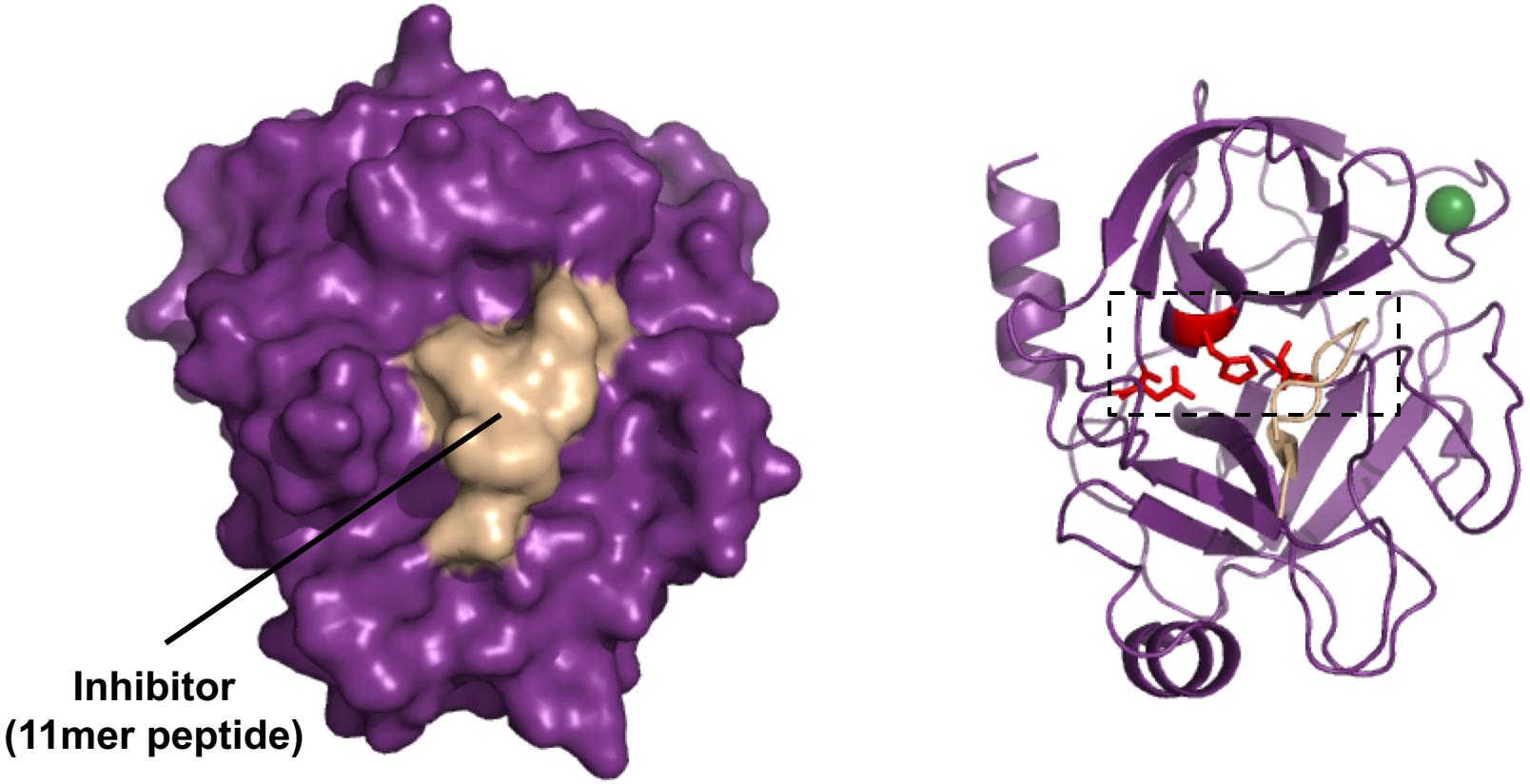


Now, can we propose a mechanisms that explains how a serine protease lower the activation energy of the transition state by 57.1 kJmol^{-1} thus leading to a 10^{10}-fold rate enhancement in peptide bond hydrolysis?

Without a structure, it is extremely difficult to gain mechanistic insight!

Towards the mechanism underlying catalysis by serine proteases

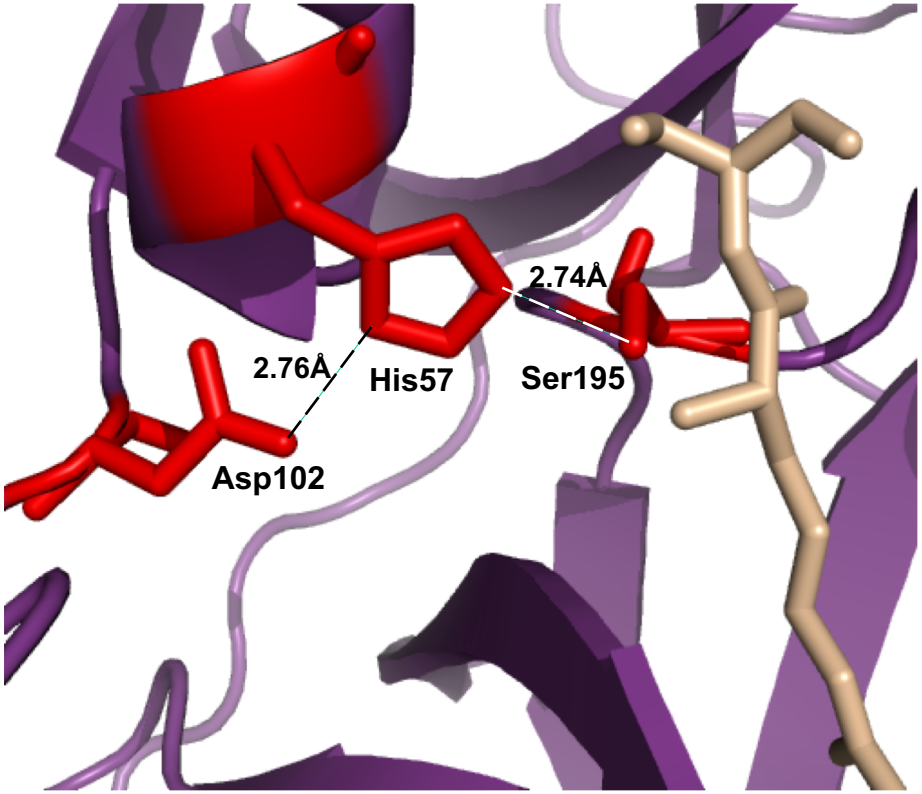
5) Crystal structure of trypsin



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

Towards the mechanism underlying catalysis by serine proteases

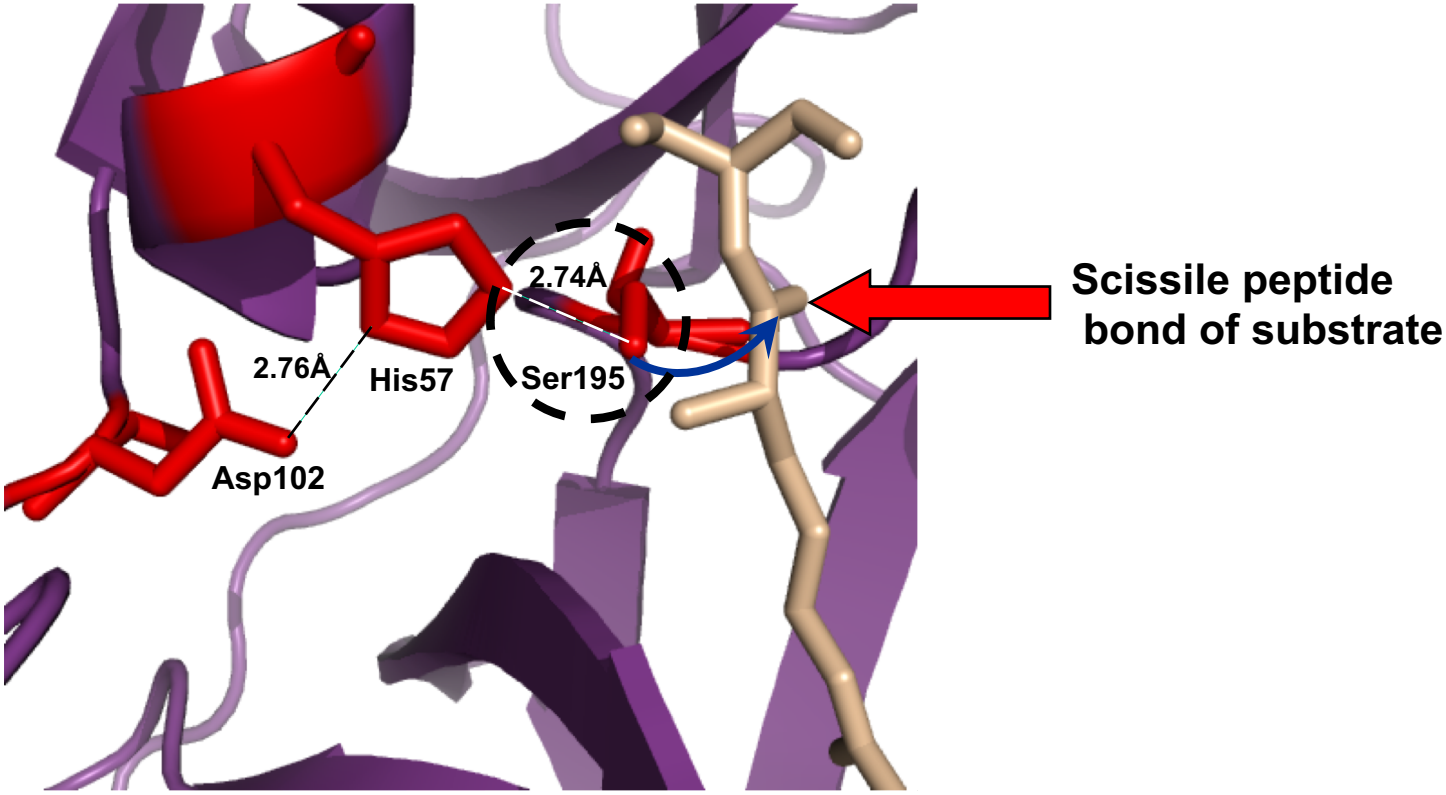
5) The catalytic triad and roles of Ser195, His57 and Asp102



The active site reveals three residues that are key to peptide bond hydrolysis – *Asp102, His57,* and *Ser195* – the catalytic triad. The catalytic triad is found in all serine proteases and plays a key role in catalysis.

Towards the mechanism underlying catalysis by serine proteases

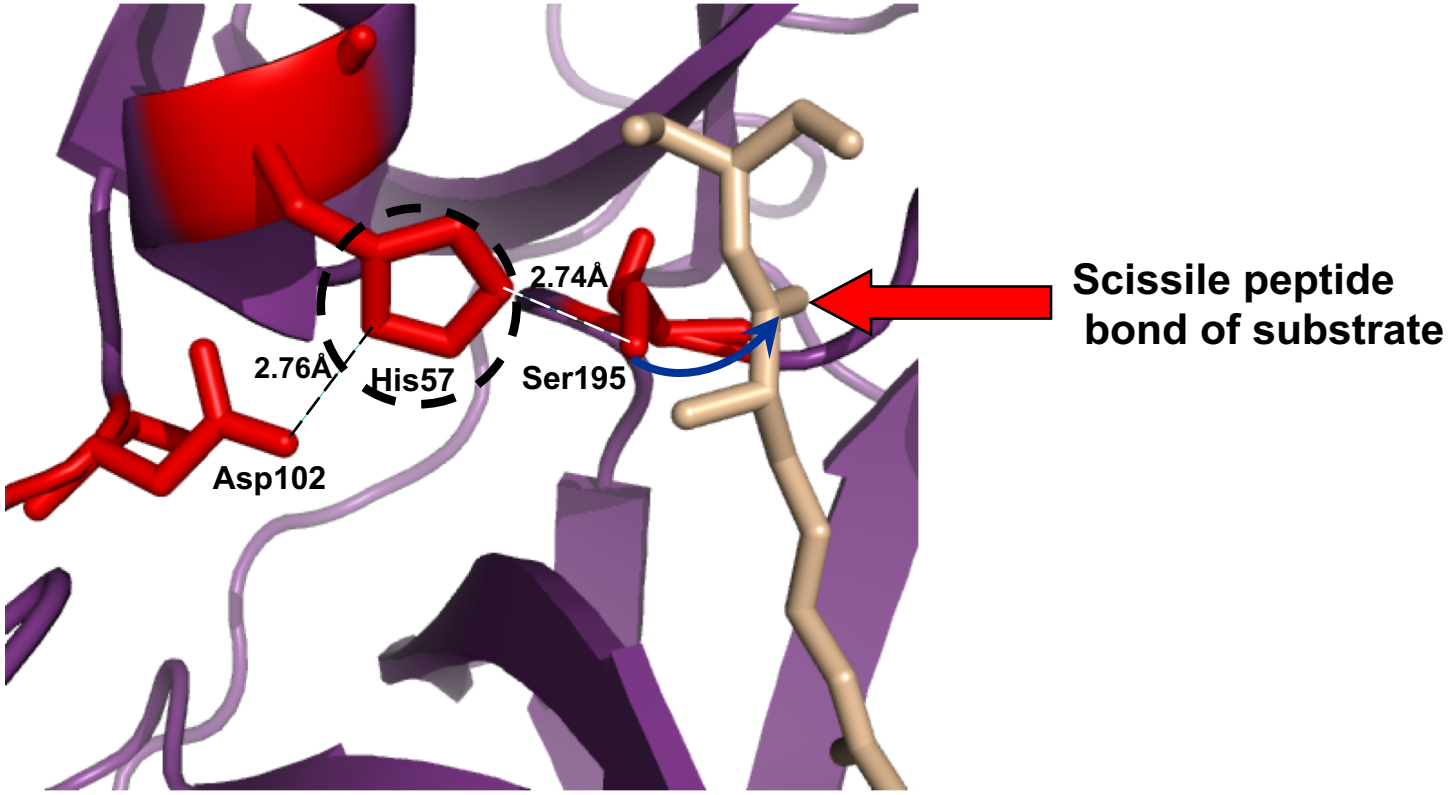
5) The catalytic triad and roles of Ser195, His57 and Asp102



Consistent with the biochemical data, Ser195, is present and perfectly positioned for nucleophilic attack on the substrate – consistent with both the 2-step mechanism (Clue #1) and DIPF labelling (Clue #3).

Towards the mechanism underlying catalysis by serine proteases

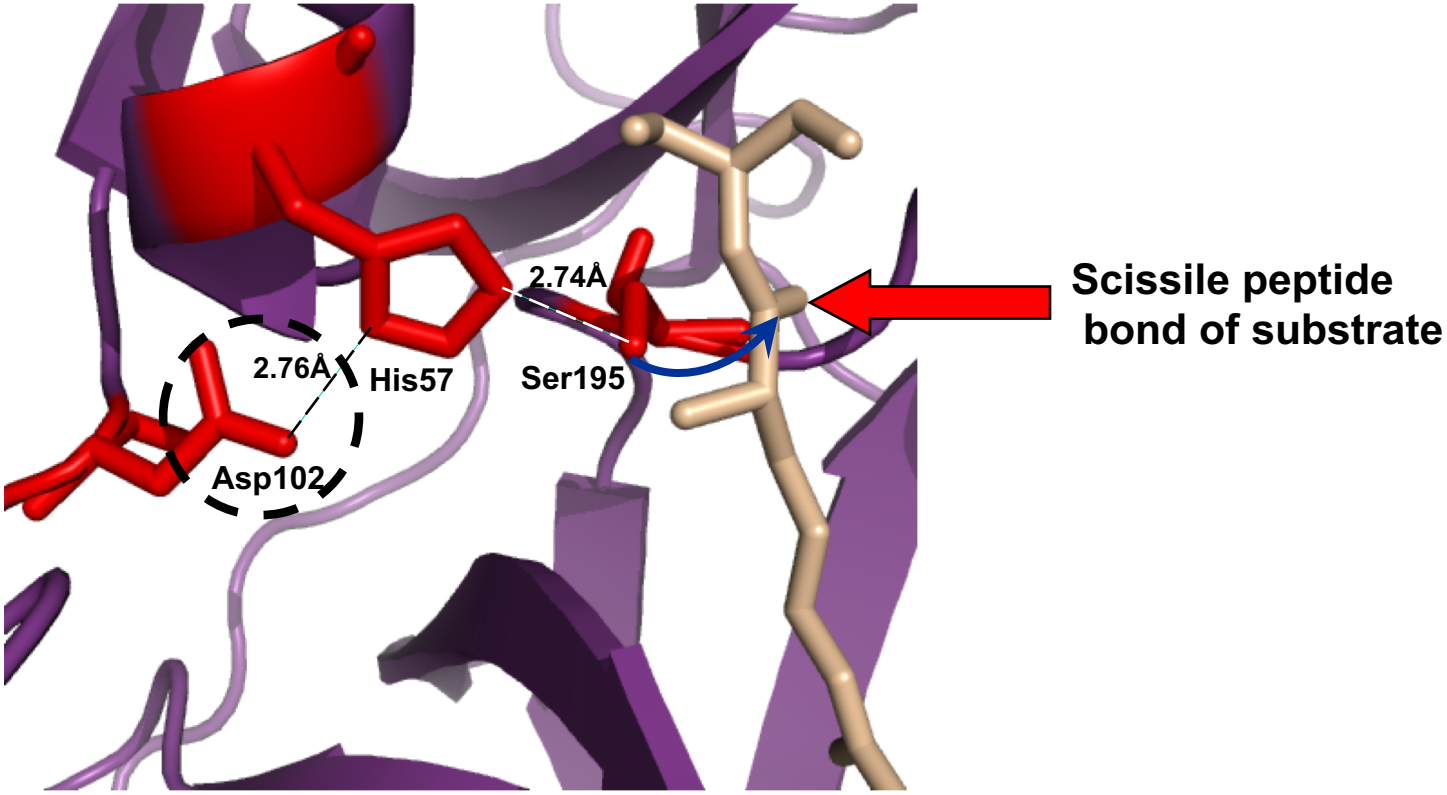
5) The catalytic triad and roles of Ser195, His57 and Asp102



Consistent with the biochemical data, His57, is in the active site (consistent with TPCK labelling, Clue #4). His57 forms a hydrogen bond with Ser195 and thus likely activates Ser195. To act as a hydrogen bond acceptor to the Ser195 hydroxyl, His57 must be deprotonated - consistent with the pH activation profile (Clue #2).

Towards the mechanism underlying catalysis by serine proteases

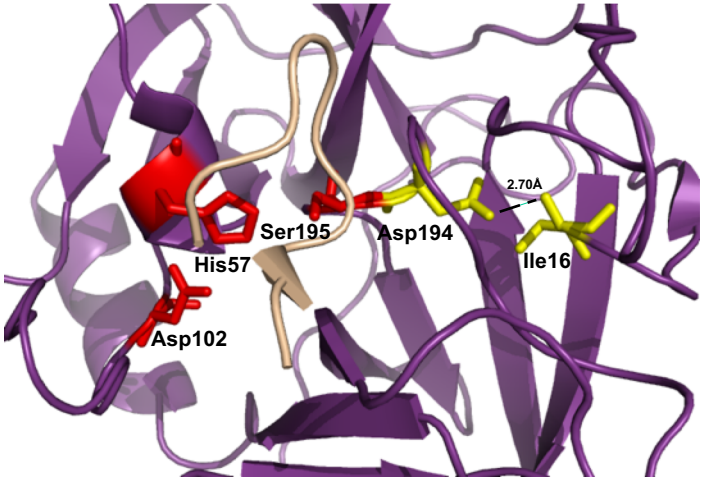
5) The catalytic triad and roles of Ser195, His57 and Asp102

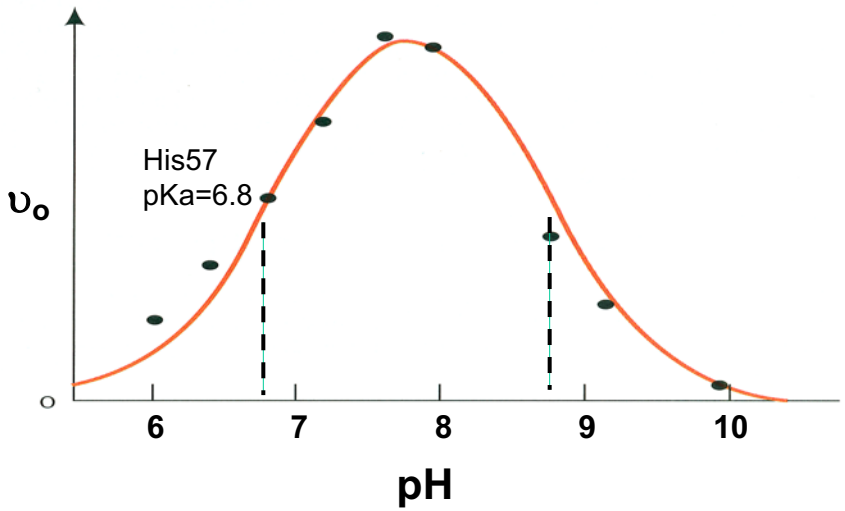


The structure also shows why His57 is more reactive to TPCK (Clue #4)– it forms a very strong polarizing hydrogen bond with Asp102.

Towards the mechanism underlying catalysis by serine proteases

5) The structure explains the role of the N-terminus





The pH dependence data suggested that the N-terminus must be protonated for the enzyme to be active.

Towards the mechanism underlying catalysis by serine proteases

Clue #1: *Enzyme catalyzed peptide bond hydrolysis is a two step reaction that occurs through a covalent intermediate.*

Clue #2: *The enzyme requires a deprotonated His and another residue – possibly the N-terminus - to be protonated?*

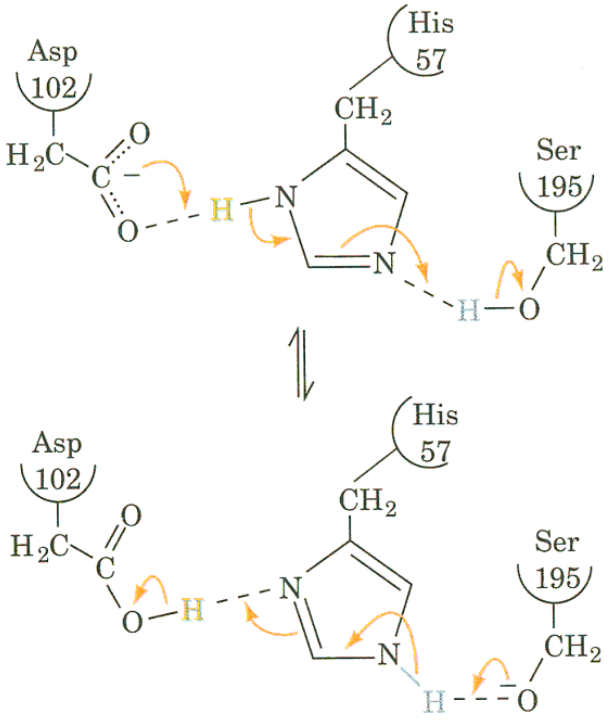
Clue #3*: Ser-195 is likely the reactive residue that forms a covalent bond with the substrate.*

Clue #4: *A highly reactive His-57 is located in the active site and may play a role in catalysis.*

Clue #5: *Crystal structure reveals the catalytic triad.*

The reaction mechanism

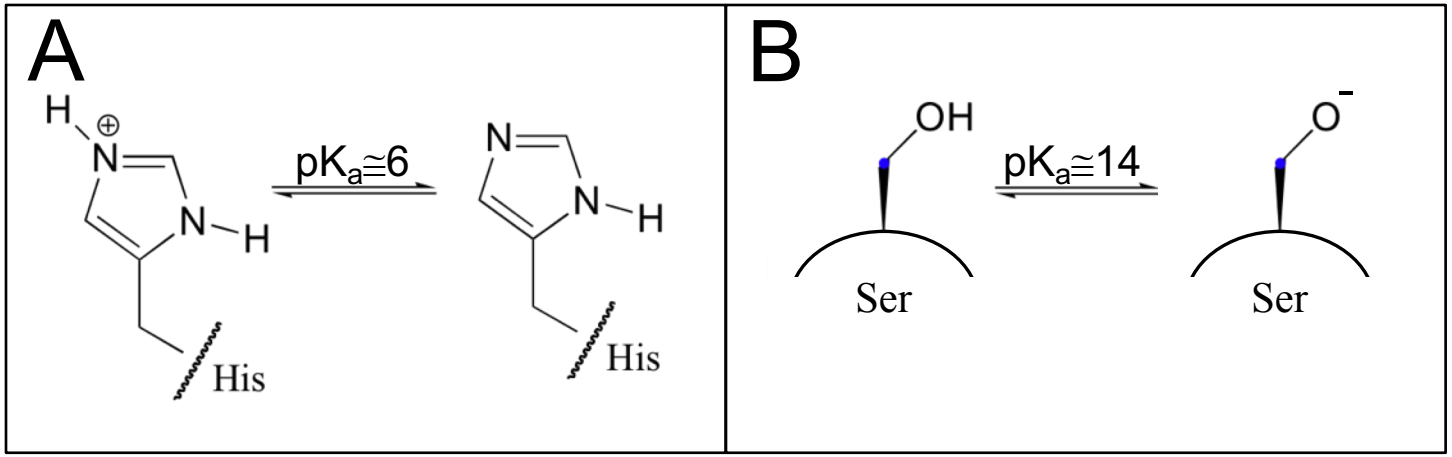
1) Hypothesis #1- the charge relay system



An early hypothesis suggested that Asp102 abstracts a proton from His57, which in turn abstracts a proton from the Ser195 hydroxyl to create a highly reactive C-O^- alkoxide anion. This hypothesis could explain the highly reactive nature of the Ser195 side chain. *Does this hypothesis seem reasonable?*

The reaction mechanism

1) Hypothesis #1- the charge relay system



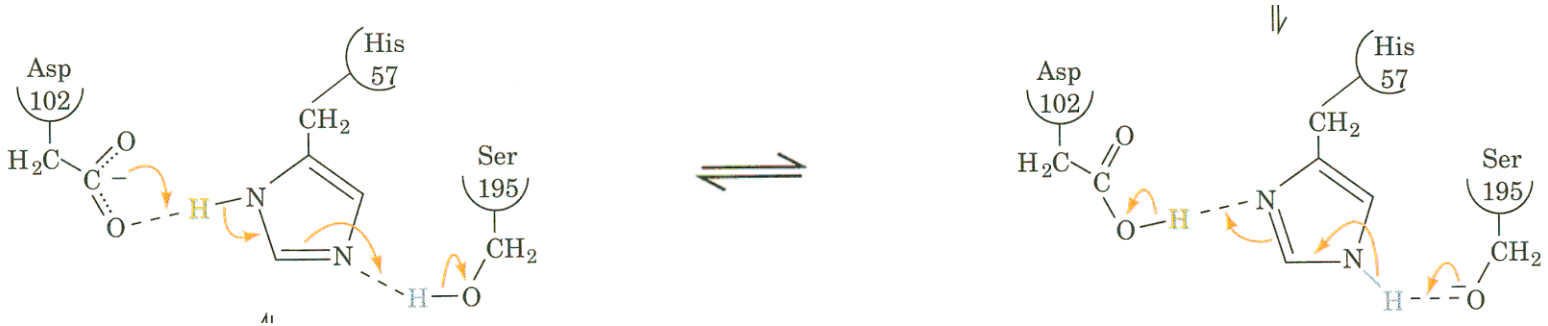
pK_a is the pH at which the side chain is 50% protonated and 50% deprotonated.

The pKa is a measure of the relative ease by which the side chain gives up its proton – so His with a moderately low pK_a ($pK_a \cong 6$)(*A*) deprotonates much more easily than Ser ($pK_a \cong 14$)(*B*) – i.e. a higher pH ($\downarrow[H^+]$) is required for Ser to deprotonate. Asp also gives up its proton more easily than His.

Note that the given pK_a values are for side chains in aqueous solution. The local environment in a protein can favor one form over the other and shift the pK_a by several pH units.

The reaction mechanism

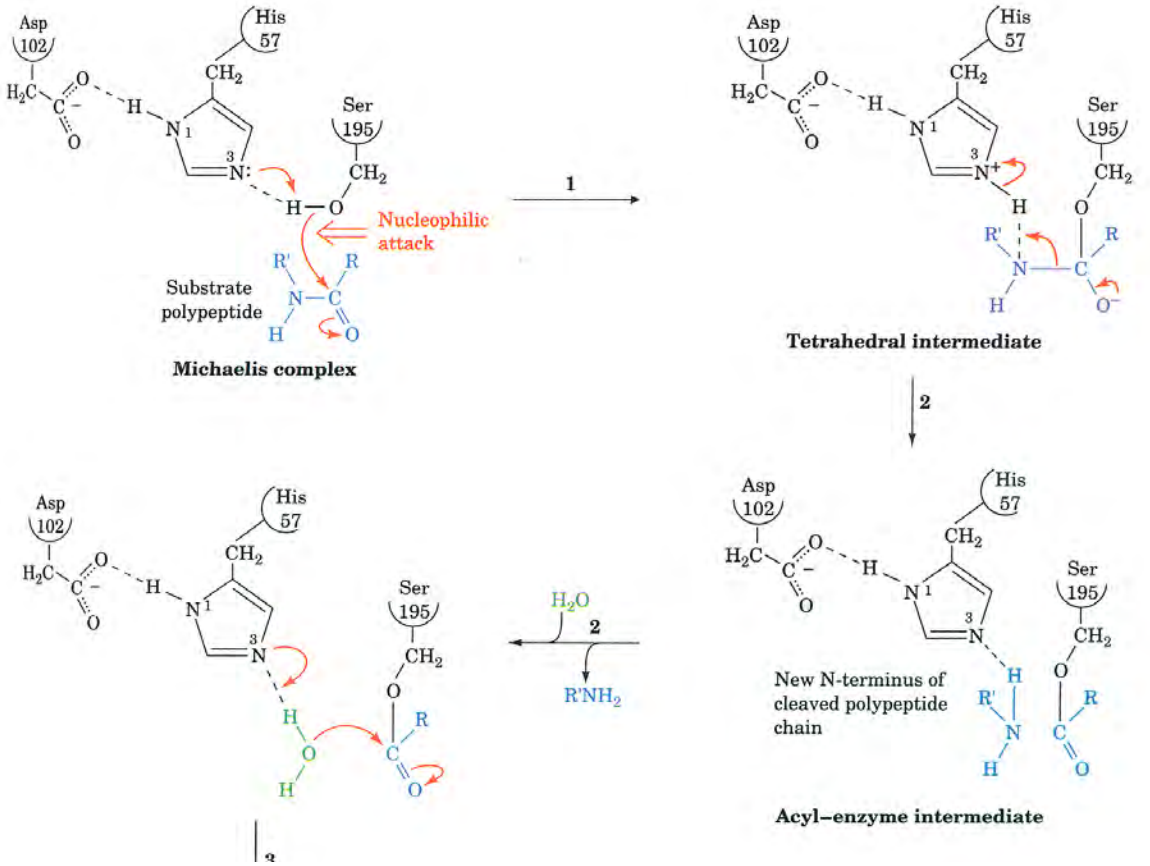
1) Who will win this battle over the proton?



The pKa of the Ser-OH is ≅14, while the pKa of His ≅ 6 (pKa of Asp≅4). The ability of His57 to pull the proton away from Ser195 is not possible. But Asp102 & His57 can polarize the OH bond to make it reactive.

The reaction mechanism

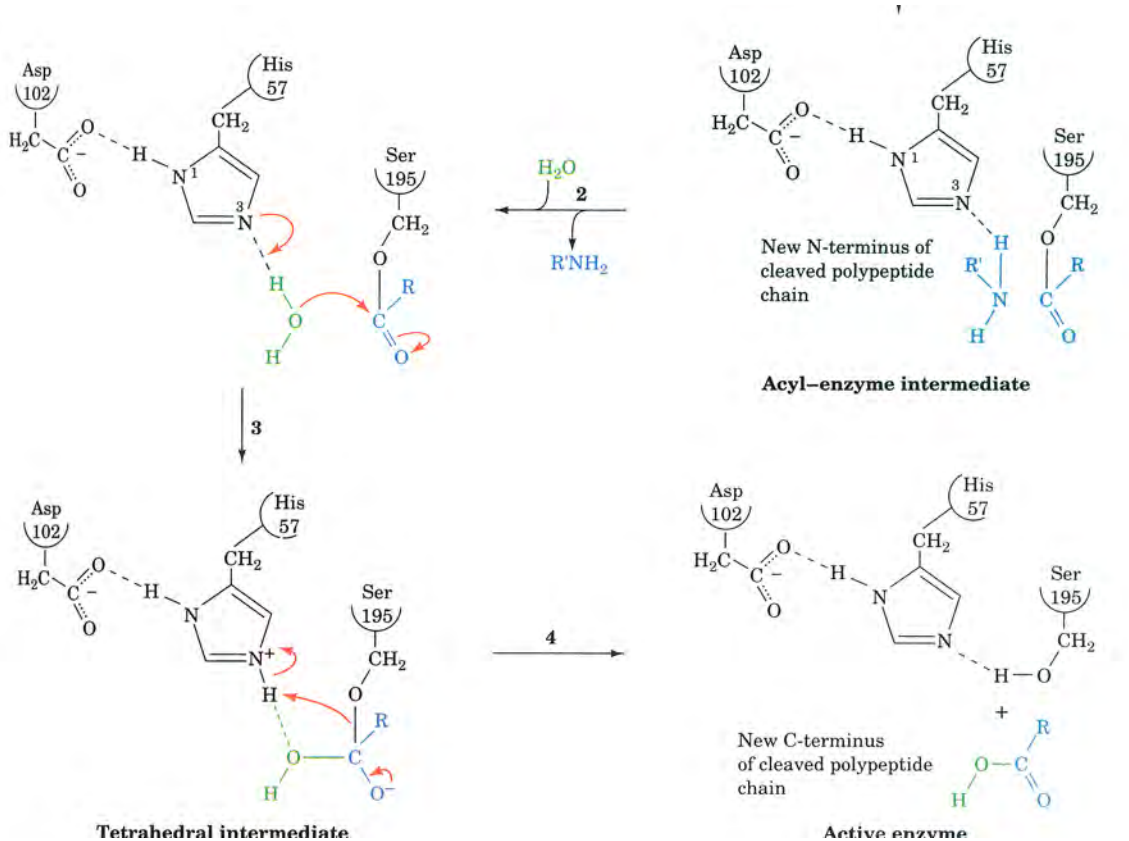
2) Hypothesis #2: The polarized hydroxyl hypothesis



$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \longrightarrow ES_T^{\ddagger} \longrightarrow P + E$$

The reaction mechanism

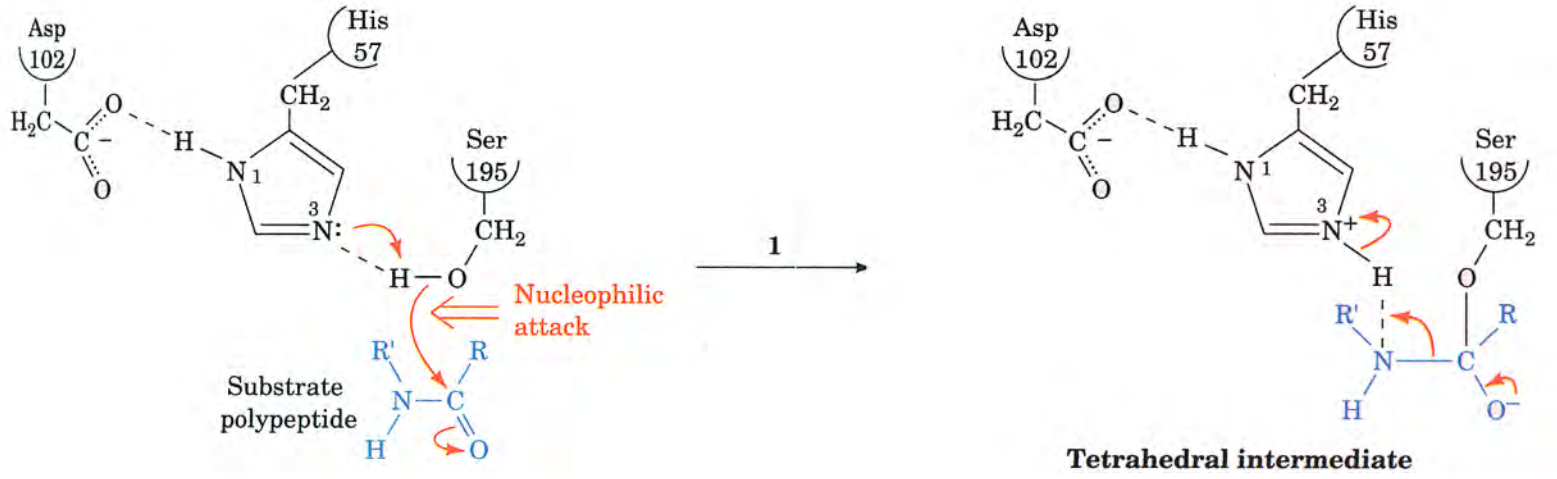
2) Hypothesis #2: The polarized hydroxyl hypothesis



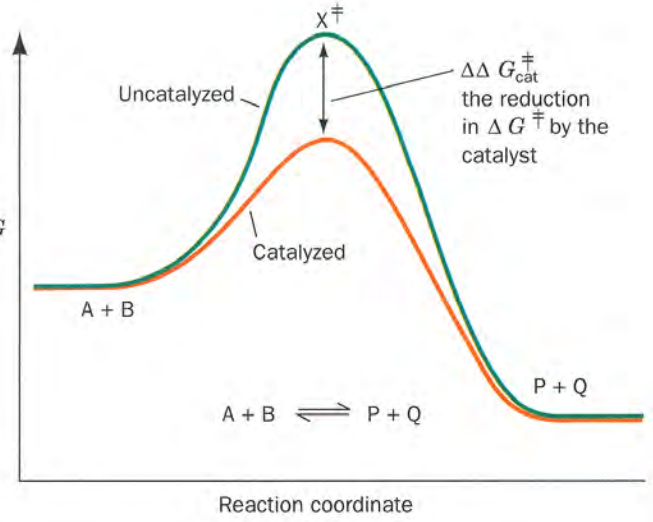
$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \longrightarrow ES_T^{\ddagger} \longrightarrow P + E$$

The reaction mechanism

2) Does the acyl-enzyme ↓ activation energy by 57 kJmol^{-1}?



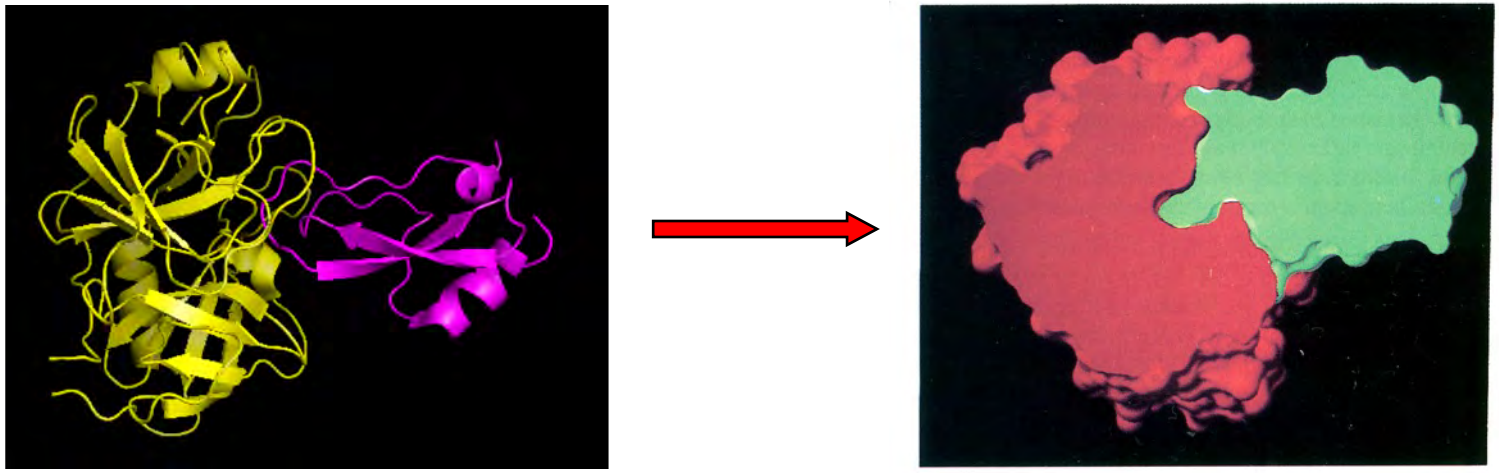
The high energy carboxy anion (C-O$^-$), which forms during peptide bond hydrolysis, is the transition state. So, to increase the reaction rate, the enzyme must lower the energy of carboxy anion. It appears that the carboxy anion is stabilized by forming a covalent bond with the SerOH – a process called covalent catalysis.



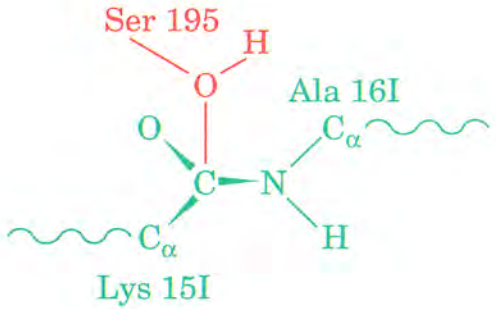
But how do we know if formation of this covalent bond is sufficient to lower the energy of the carboxy-anion by 57.1 kJmol^{-1}? In other words, is this mechanism the entire story?

Clues to the mechanism underlying catalysis by serine proteases

6) The structure trypsin bound to BPTI



The interaction between trypsin (yellow) and the **bovine pancreatic trypsin inhibitor (BPTI**, purple) is one of the strongest known protein-protein interactions (K_D in the femtomolar range (10^{-15} M)).



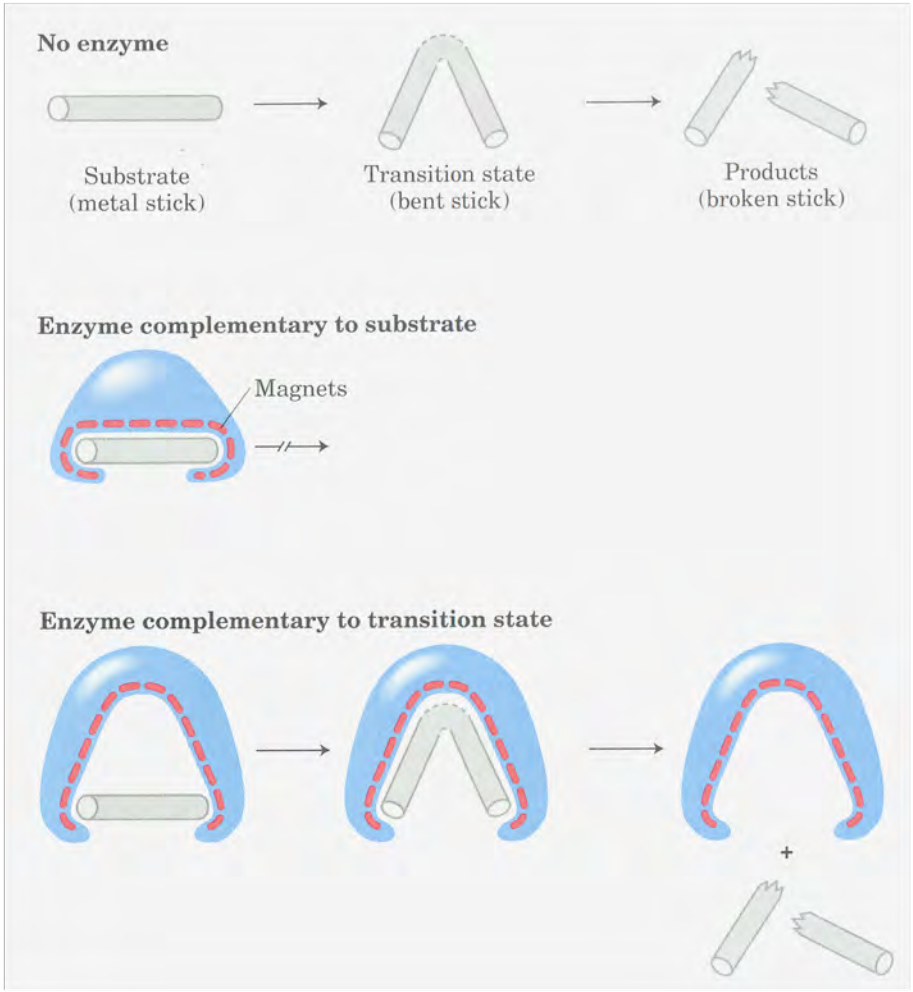
A cross section of the trypsin (red)/BPTI (green) complex shows the tight van der Waals contacts. Surprisingly, the BPTI Lys15 to Ala 16 peptide bond (the one to be cleaved) is distorted close to a tetrahedral geometry and the Ser195 oxygen has formed a covalent bond with the Lys backbone C=O. *The trypsin/BPTI complex is locked in the transition state!*

The fact that 1) the trypsin/BPTI interaction is extremely tight , and that 2) it mimics the transition state suggested that the enzyme has evolved to bind preferentially (i.e. with highest affinity) with substrate transition state – i.e. physical interactions are maximized in the transition state. This is now viewed as a common feature of all enzymes!

Transition state stabilization

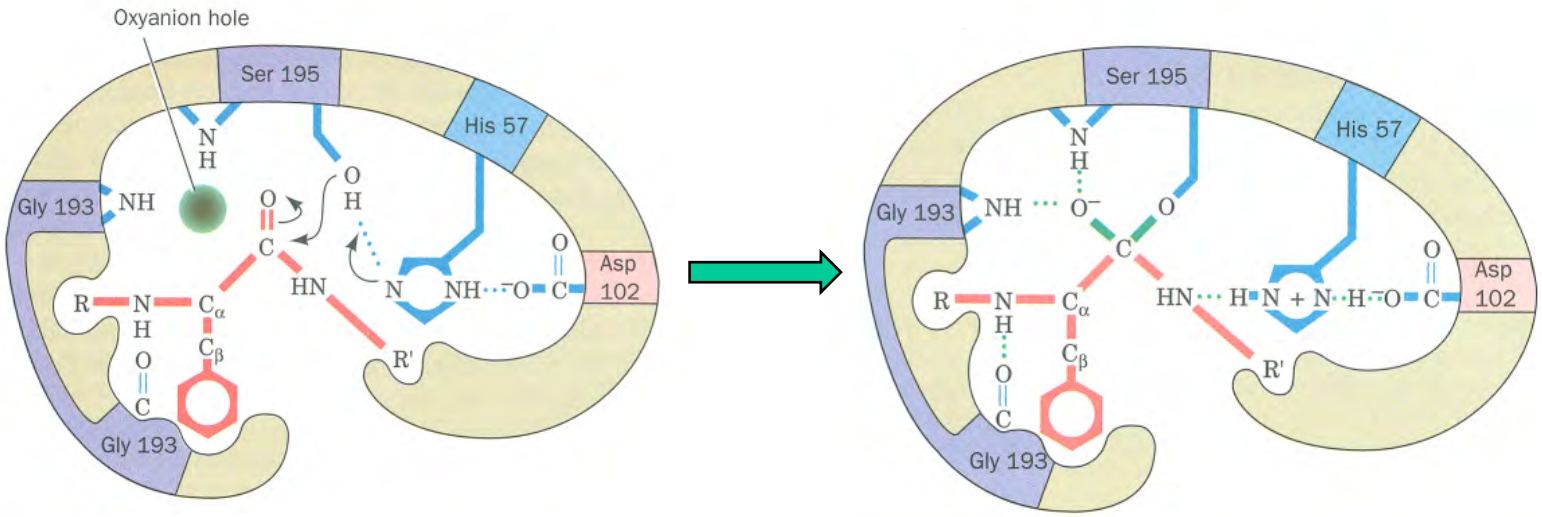
Consider an enzyme that catalyzes the breakage of a metal stick. An enzyme that maximizes interactions with the substrate in its natural form would stabilize the substrate and would not be a good catalyst. An enzyme that maximizes interactions with the transition state lowers maximally the energy of the transition state and is thus a good catalyst!

So are there new interactions that form between trypsin and its substrate in the transition state?



Transition state stabilization in the BTPI complex

Mechanism underlying catalysis by serine proteases?



Enzyme-Substrate (Michaelis) Complex

Enzyme-Substrate Transition State

The structure and biochemical data suggest that serine proteases catalyze peptide bond hydrolysis via two main specific mechanisms: 1) they form a covalent bond with the substrate and 2) they form hydrogen bonds between the backbone N-H of Ser195/Gly193 and the oxyanion. Both are thought to stabilize the transition state and thus enhance the reaction rate.

But, we know that serine enhance the reaction rate by 10^{10}-fold. Can these interactions account for this rate enhancement?

Towards the mechanism underlying catalysis by serine proteases

Clue #1: *Enzyme catalyzed peptide bond hydrolysis is a two step reaction that occurs through a covalent intermediate.*

Clue #2: *The enzyme requires a deprotonated His and another residue – possibly the N-terminus - to be protonated?*

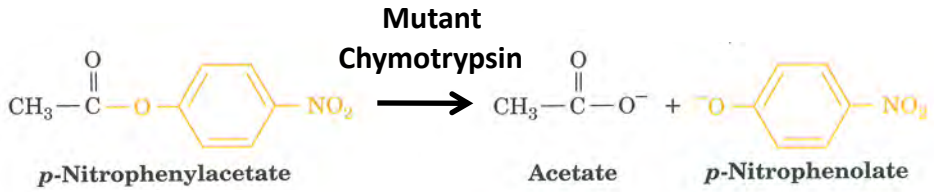
Clue #3*: Ser-195 is likely the reactive residue that forms a covalent bond with the substrate.*

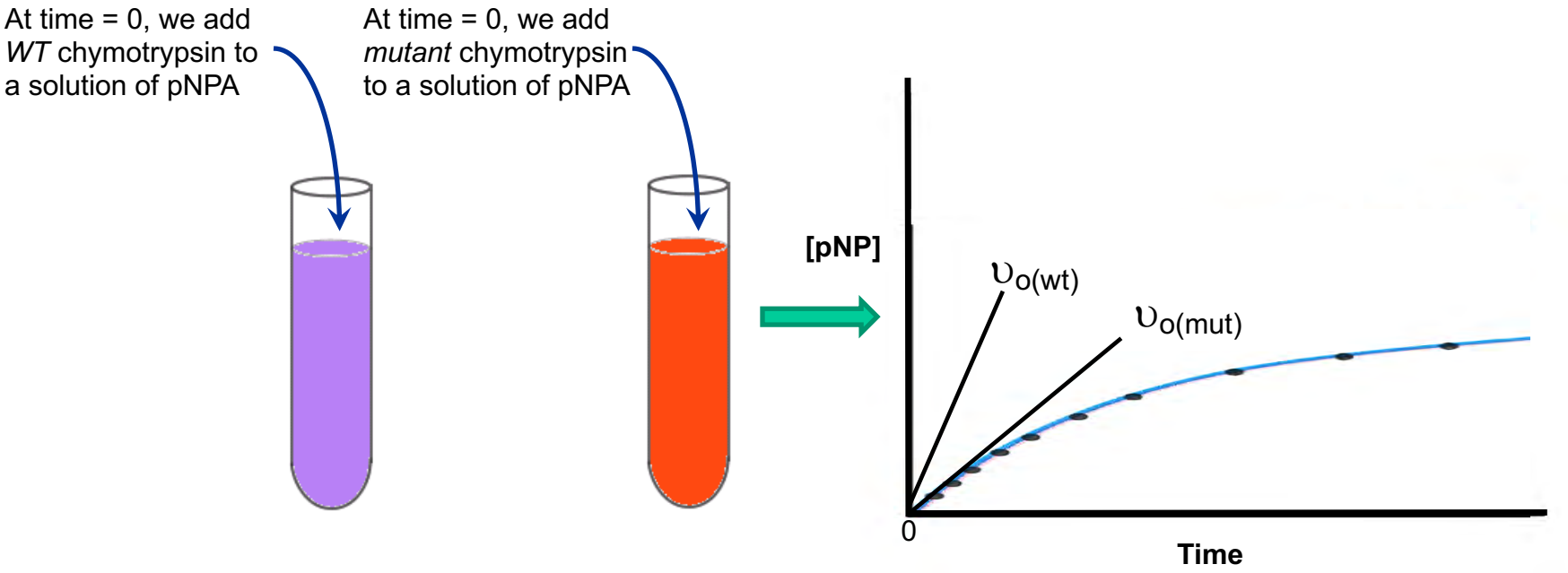
Clue #4: *A highly reactive His-57 is located in the active site and may play a role in catalysis.*

Clue #5: *Crystal structure reveals the catalytic triad.*

Clue #6: *Enzymes stabilize the transition-states.*

Measure the rate of catalysis for the mutant enzyme





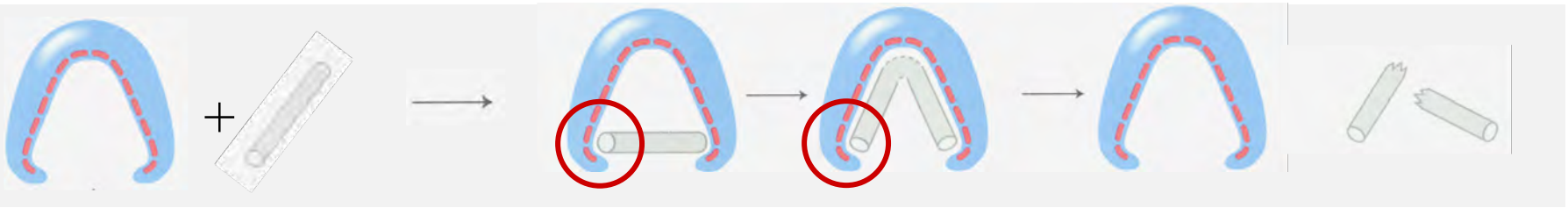
Interpret the measured υ_o in terms of the kinetic parameters k_{cat}, K_M, and k_{cat}/K_M for the mutant enzyme.

Measure the rate of catalysis for the mutant enzyme

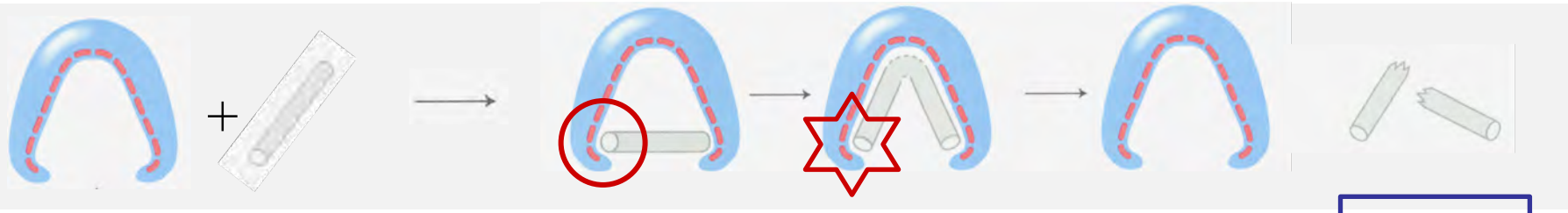
$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} ES_T^{\ddagger} \longrightarrow E + P$$

Case

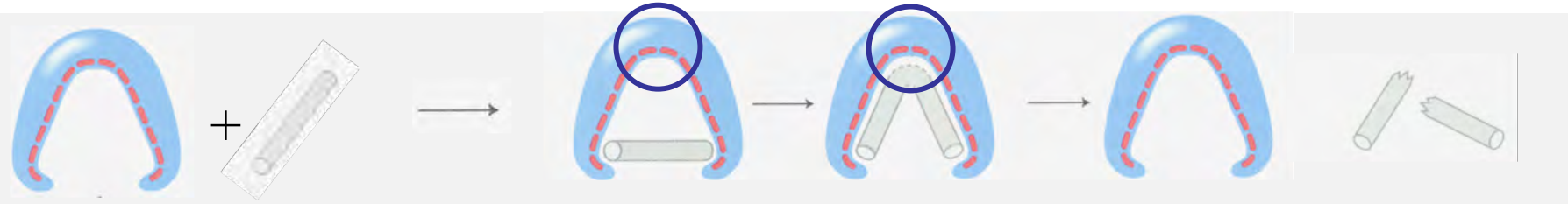
1



2

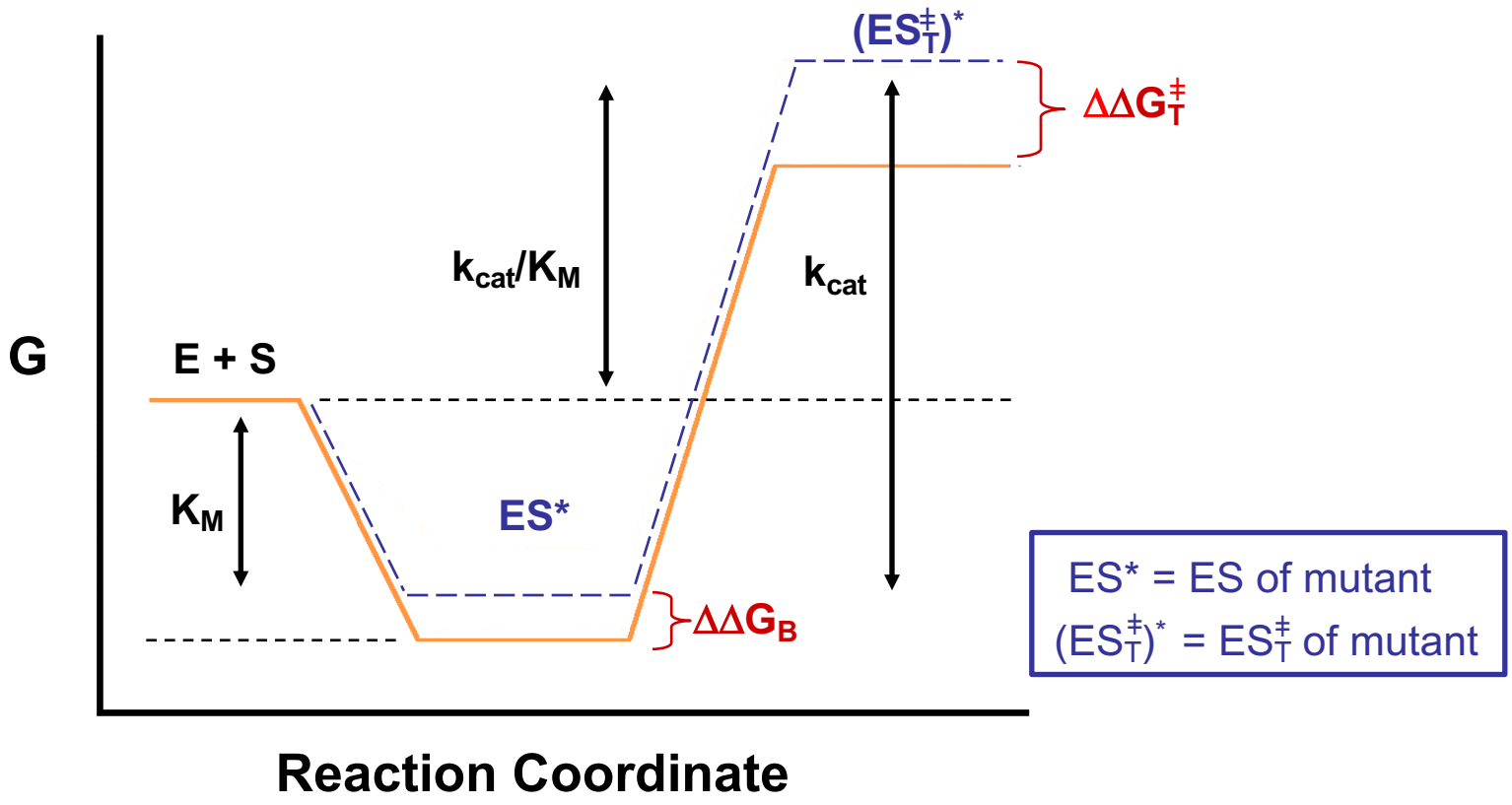


3



Interpretation of mutations and their effects on enzyme kinetics

We can also calculate $\Delta\Delta G_B$ and $\Delta\Delta G_T^{\ddagger}$



The $\Delta\Delta G_B$ and $\Delta\Delta G_T^{\ddagger}$ values tell us how much energy is derived from the residue interacting with the substrate in both the ES and $ES_T^{\ddagger}$ complexes. By adding up these values for different residues, we can piece together how the enzyme catalyzes the reaction.

Testing the mechanism underlying catalysis by serine proteases

Role of the catalytic triad and the oxyanion hole

Mutation	K_m	k_{cat}	k_{cat}/K_m	$\Delta\Delta G_T^{\ddagger}$
S195A	~no change	$\Downarrow 2x10^6$	$\Downarrow 2x10^6$	
H57A	~no change	$\Downarrow 2x10^6$	$\Downarrow 2x10^6$	-35.9
D102A	~no change	$\Downarrow 10^4$	$\Downarrow 10^4$	
N193T	~no change	$\Downarrow 2x10^3$	$\Downarrow 2x10^3$	-18.8

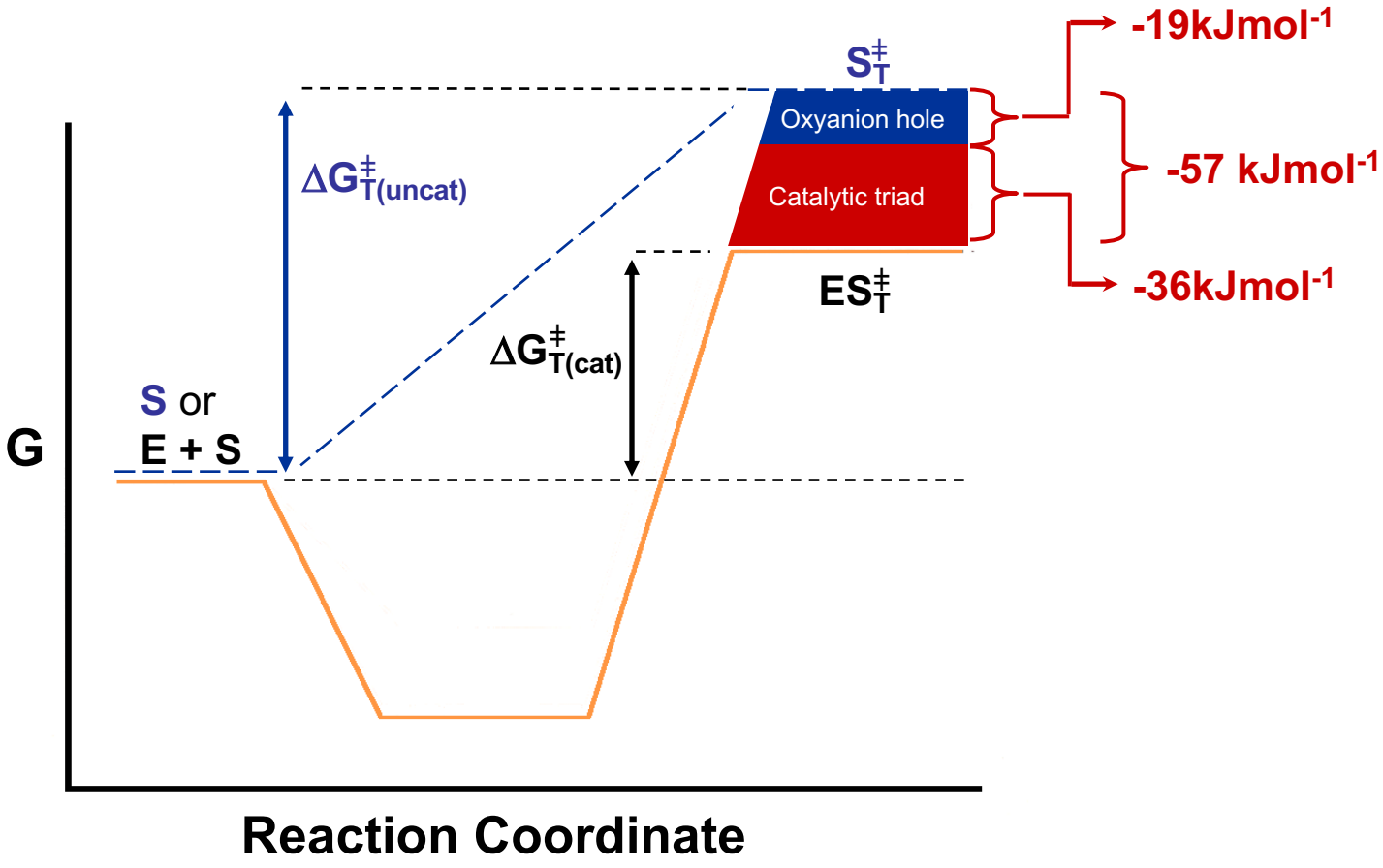
$$\Delta\Delta G_T^{\neq} = RT \ln \frac{1}{2x10^3} = -19 kJmol^{-1}$$

In subtilisn, the N193T (oxyanion hole stabilization) mutant is a poorer catalyst by $2x10^3$ fold. This shows that, as predicted, the carbanion formed in the transition state does move into the oxyanion hole to be stabilized by H-bonding.

Does this mean that our model accounts for the catalytic power of the enzyme?

Testing the mechanism underlying catalysis by serine proteases

1) The catalytic triad + the oxyanion hole



Serine proteases increase reaction rates 10^{10}-fold by lowering the activation energy of the transition state (blue) by 57.1 kJmol^{-1}. The catalytic triad contributes 35.9 kJmol^{-1} of this stabilization energy – or roughly 60% of the total catalytic power. The oxyanion hole accounts for 19 kJmol^{-1}. Together the triad and oxyanion hole account for the entire catalytic power!

Appendix I Maude Lenora Menten

Maud Leonora Menten (March 20, 1879 – July 26, 1960, Ontario) was a Canadian medical scientist who made significant contributions to enzyme kinetics and biochemistry. Her name is associated with the famous Michaelis-Menten equation in biochemistry.

Maud Menten was born in Port Lambton, Ontario and studied medicine at the University of Toronto (B.A. 1904, M.B. 1907, M.D. 1911). She was among the first women in Canada to earn a medical doctorate. She completed her thesis work at University of Chicago. At that time women were not allowed to do research in Canada, so she decided to do research in other countries such as the United States and Germany.

In 1912 she moved to Berlin where she worked with Leonor Michaelis, obtaining a Ph.D. in 1916. Menten worked as a pathologist at the University of Pittsburgh (1923–1950) and as a research fellow at the British Columbia Medical Research Institute (1951–1953).
Her most famous work was on enzyme kinetics together with Michaelis, based on earlier findings of Victor Henri. This resulted in the Michaelis-Menten equations. Menten also invented the azo-dye coupling reaction for alkaline phosphatase, which is still used in histochemistry. She characterised bacterial toxins from *B. paratyphosus*, *Streptococcus scarlatina* and *Salmanella ssp.*) and conducted the first electrophoresis separation of proteins in 1944. She worked on the properties of hemoglobin, regulation of blood sugar levels, and kidney function. Despite suffering from arthritis she was also an accomplished musician and painter; there were several exhibitions of her paintings. (Wikipidia.com)

Appendix II

The equilibrium assumption:

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

Michaelis and Menton assumed that the rxn E + S ↔ES reaches equilibrium (i.e that $k_{-1} >>> k_2$):

$$K_{eq} = \frac{[ES]}{[E]_{free}[S]} \quad (1)$$

given that: $$[E]_{total} = [E]_{free} + [ES] \quad (2)$$

We can define $[E]_{free}$ as:

$$[E]_{free} = [E]_{total} - [ES] \quad (3)$$

Sub equation 3 in equation 1:

$$K_{eq} = \frac{[ES]}{[E]_{total}[S] - [ES][S]}$$

Appendix II

The equilibrium assumption:

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

Rearrange to give:

$$K_{eq}([E]_{total}[S] - [ES][S]) = [ES]$$

$$\therefore K_{eq}[E]_{total}[S] = [ES] + K_{eq}[ES][S]$$

$$\therefore K_{eq}[E]_{total}[S] = [ES](1 + K_{eq}[S])$$

$$\therefore [ES] = \frac{K_{eq}[E]_{total}[S]}{1 + K_{eq}[S]}$$

$$\therefore [ES] = \frac{[E]_{total}[S]}{\frac{1}{K_{eq}} + [S]} = \frac{[E]_{total}[S]}{K_D + [S]} \quad (4)$$

Appendix II

The equilibrium assumption:

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

Sub equation 4 into:

$$\upsilon_o = k_{cat}[ES] = \frac{k_{cat}[E]_{total}[S]}{K_D + [S]}$$

Let $K_S = K_D\,(1/K_{eq})$ to give:

$$\boxed{\upsilon_o = \frac{k_{cat}[E]_{total}[S]}{K_S + [S]}}$$

Appendix III

The steady state assumption:

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

Mathematically, the steady state assumption is the following:

$$\frac{d[ES]}{dt} = 0 = k_1[E]_{free}[S] - k_{-1}[ES] - k_{cat}[ES] \quad (1)$$

We also know that the total enzyme concentration is:

$$[E]_T = [E]_{free} + [ES] \quad (2)$$

If we rearrange equation 2 solving for $[E]_{free}$, and then sub into equation 1, we get:

Appendix III

The steady state assumption:

$$0 = k_1([E]_{total} - [ES])[S] - k_{-1}[ES] - k_{cat}[ES]$$

Solve for *[ES]*:

$$k_1[E]_{total}[S] = k_1[ES][S] + k_{-1}[ES] + k_{cat}[ES]$$

Rearrange:

$$k_1[E]_{total}[S] = (k_1[S] + k_{-1} + k_{cat})[ES]$$

Appendix III

The steady state assumption:

$$[ES] = \frac{k_1[E]_{total}[S]}{k_1[S] + k_{-1} + k_{cat}}$$

Multiply top and bottom by $1/k_1$:

$$[ES] = \frac{[E]_{total}[S]}{[S] + \frac{k_{-1} + k_{cat}}{k_1}} \qquad (3)$$

Appendix III

The steady state assumption:

$$[ES] = \frac{[E]_{total}[S]}{[S] + \frac{k_{-1} + k_{cat}}{k_1}} \quad (3)$$

$$Rate = \upsilon_o = k_{cat}[ES] \quad (4)$$

Sub equation 4 into equation 3:

$$\upsilon_o = \frac{k_{cat}[E]_{Total}[S]}{\frac{k_{-1} + k_{cat}}{k_1} + [S]}$$

$$\upsilon_o = \frac{k_2[E]_{Total}[S]}{K_M + [S]}$$