Chapter 4 : Enzymatic regulation (Allostery)

Introduction

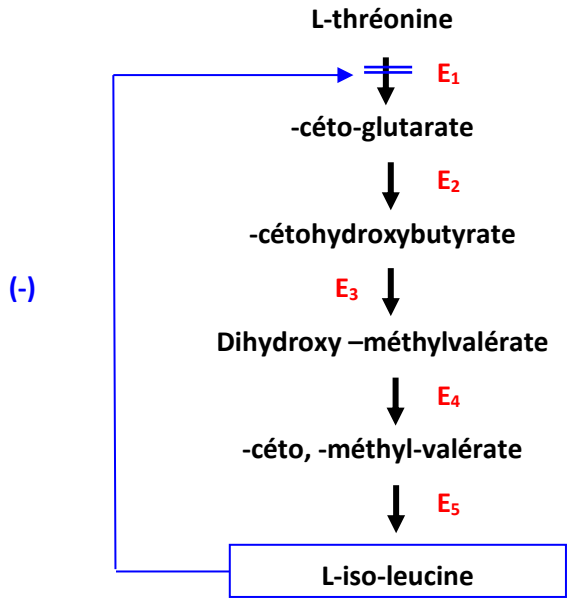
Many enzymes do not follow classical **Michaelis-Menten kinetics**; the curve representing the velocity (v) as a function of substrate concentration ([S]) is not hyperbolic but **sigmoidal** (S-shaped). These enzymes play a crucial role in regulating metabolic processes; they are known as **key regulatory enzymes**. This type of enzyme possesses multiple binding sites: one for the substrate and one or more others for co-factors (known as **effectors** or **modulators**).

The term **"allosteric"** was introduced for these enzymes by Jacques Monod and his collaborators in 1965 (*allostery* comes from the Greek *allos* meaning "**other**" and *stereon* meaning "**space**").

1. Self-regulation properties of enzymatic systems

Several multienzyme systems possess the ability of **self-regulation**. In most cases, the final product of a reaction sequence can **inhibit the first enzyme** in that pathway. This type of regulation is known as **feedback inhibition**, a key concept in metabolic control.

Example: Conversion of **L-threonine** to **isoleucine** occurs in 5 steps:



This classic example illustrates **Feedback Inhibition** (also known as end-product inhibition). In this metabolic pathway:

1. The starting substrate is **L-threonine**.

2. The first enzyme (**E1)** in the sequence is **threonine dehydratase**.
3. After five enzymatic steps, the final product, **L-isoleucine**, is synthesized.
4. When the concentration of isoleucine reaches a sufficient level, it acts as an **allosteric inhibitor** of the first enzyme (threonine dehydratase).

This inhibition is atypical, as it is neither competitive nor uncompetitive with respect to the substrate (L-threonine). Instead, L-isoleucine acts through a **feedback inhibition mechanism** (also known as **retro-inhibition**).

As in the previous example, it is the **first enzyme of "self-regulated enzymatic systems"** that is inhibited by the final product of the pathway. The enzyme is therefore a **regulatory or allosteric enzyme**. The inhibitor corresponds to an **effector or modulator** (in this case, a negative one).

2. Properties and classification of regulatory enzymes

2.1. Properties

Regulatory enzymes possess specific characteristics that distinguish them from simple metabolic enzymes:

- **Size:** Regulatory enzymes are very **large and complex** molecules. Due to their high molecular weight and intricate structures, they are often difficult to purify.
- **Structure:** All known regulatory enzymes are **multimeric**, meaning they are composed of several polypeptide chains, known as **subunits**.
- **Stability:** Some of these enzymes exhibit **unusual thermal stability**, they may be unstable at **0°C** (cold-labile) but more stable at **room temperature**.

2.2. Classification

According to the model proposed by **Monod, Wyman and Changeux (1965)**, regulatory enzymes are classified into three distinct groups based on the nature of their effectors:

- **Homotropic enzymes:** In this group, the **substrate molecule itself** acts as the modulator. Typically, the binding of one substrate molecule to a subunit increases the enzyme's activity (positive cooperativity), facilitating the binding of subsequent substrate molecules.
- **Heterotropic enzymes:** These enzymes are **stimulated or inhibited** by an **effector (modulator) that is different** from the substrate. The effector binds to a specific regulatory site, distinct from the active site.
- **Homo-heterotropic enzymes:** In this group, the enzymes are regulated by **both the substrate and other molecules**. They exhibit a combination of homotropic and heterotropic effects.

Example: Threonine Deaminase

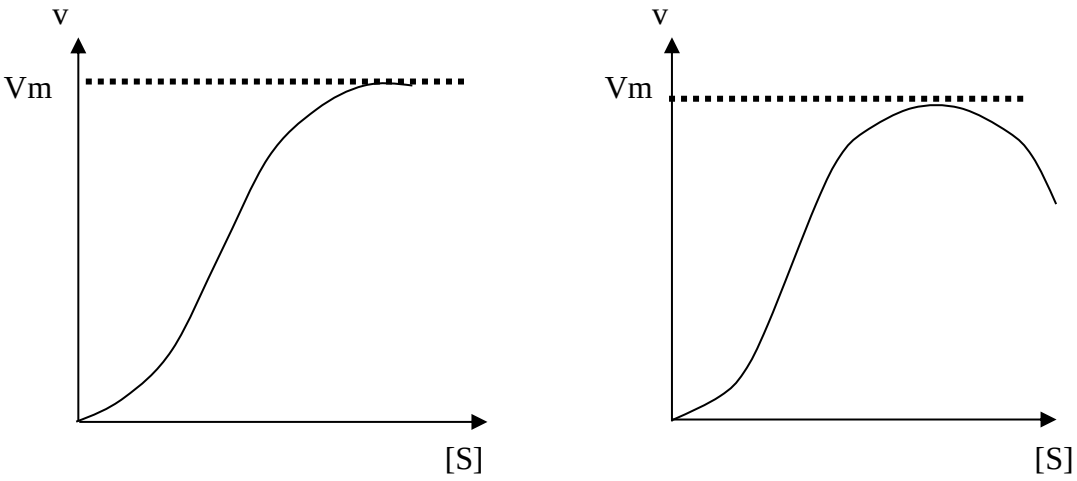
Threonine deaminase is a classic example of a **heterotropic enzyme**. It possesses two distinct types of binding sites:

- **One site for the substrate:** L-threonine (L-Thr).
- **One site for the effector:** L-isoleucine (L-Ile), which acts as a negative modulator.

3. Kinetics of regulatory enzymes

3.1. Curve Shapes

The velocity curves as a function of substrate concentration (v = f([S]) for regulatory enzymes exhibit a **sigmoidal** (S-shaped) profile. This shape reflects **cooperativity**, where the binding of one molecule influences the binding of subsequent molecules. The specific profile of these curves varies depending on whether the enzyme is **homotropic** or **heterotropic**:

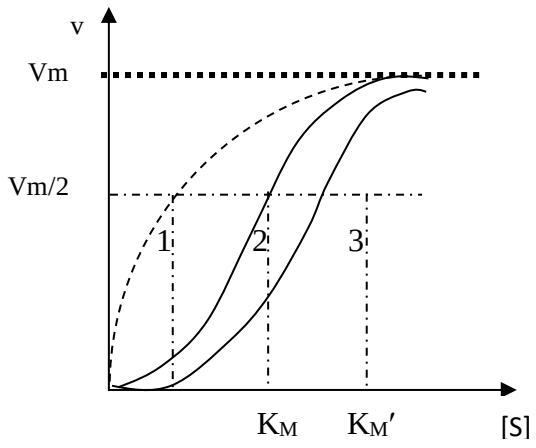


(1) **Heterotropic Enzymes** (2) **Homotropic Enzymes**

3.2. Kinetic parameters and action of modulators

Considering the kinetics of **threonine deaminase** in the absence and in the presence of **isoleucine (Ile)**. The following curves are obtained:

1- Kinetics of a Michaelis-Menten enzyme.
2- Kinetics of threonine deaminase in the absence of isoleucine
3- Kinetics of threonine deaminase in the presence of isoleucine (KM′>KM)



3.3. Desensitization

The ability of regulatory enzymes to be activated or inhibited by specific modulators may disappear without altering their catalytic activity. This process can occur after treatment of the enzyme with a **chemical or thermal agent**, leading to structural modifications specifically at the **effector (allosteric) sit**. The enzyme is then said to be **desensitized**, loses its ability to respond to regulatory signals but remains capable of converting substrate into product. In many cases, this desensitization is **irreversible**.

In certain instances, the desensitization can also result from a **genetic mutation**; this leads to the synthesis of an enzyme that has lost its sensitivity to the effector. It can therefore be concluded that the **amino acid sequence** determines not only the catalytic activity but also the enzyme's ability to act as a **"pacemaker" or regulatory enzyme**.

3.4. Cooperativity

Allosteric enzymes are typically **oligomeric proteins** (proteins made of multiple subunits) that possess an **axis of symmetry**. The number of subunits in these enzymes is usually an **even number** (e.g., dimers, tetramers, or hexamers). Cooperativity describes the interaction between these subunits and can be either positive or negative.

The **cooperativity** describes the functional interaction between these subunits. When a substrate or ligand binds to one subunit, it induces a conformational change that is transmitted to the other subunits, affecting their affinity for the substrate. This interaction can be either positive or negative :

- **Positive cooperativity:** This occurs when the initial binding of a ligand **accelerates** or facilitates subsequent binding events.
- **Negative cooperativity:** This occurs when the initial binding of a ligand **decelerates** or hinders subsequent binding.

3.5. The Hill Number (nH)

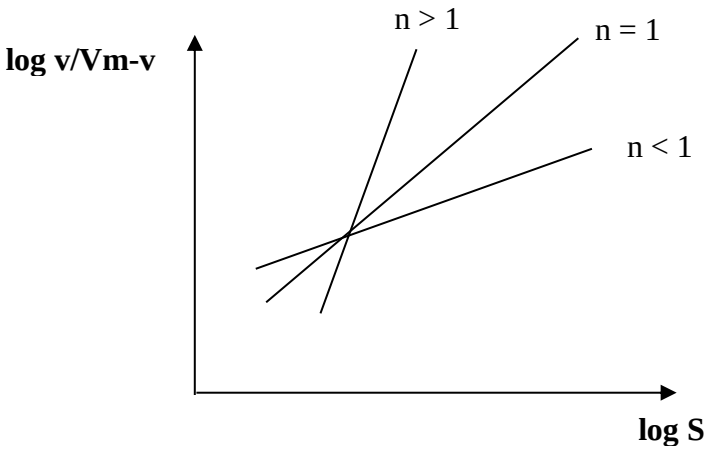
The **Hill number** (or Hill coefficient) expresses the degree of **cooperativity** between the binding sites of an allosteric enzyme or receptor. It provides a numerical value that describes how the binding of one ligand influence the binding of subsequent ligands. It is calculated using the **Hill transformation**, which is written as follows:

$$\boldsymbol{log \frac{v}{Vm - v} = nH.log\, S - log\, KM}$$

This relationship is derived from the **Hill Equation**, which was established in 1910 to explain the binding behavior of **hemoglobin**. It expresses the fraction of occupied sites (or the saturation percentage) as a function of substrate concentration. In the specific case of hemoglobin, the substrate is oxygen, and concentration is measured as partial pressure (pO_2).

$$\mathbf{Y = \frac{SnH}{KD + SnH}}$$

The Hill transformation allows us to plot **log (v / (Vmax - v)** against **log [S]**. This graphical representation is used to determine two critical values:



- **The Slope (n_H):** This is the Hill coefficient. It indicates the nature and strength of cooperativity:
    - If **$n_H > 1$**: Positive cooperativity. The binding of one substrate molecule increases the affinity of the remaining sites. In this case, the value of n_H is always between 1 and the total number of subunits (N): $1 < n_H < N$.

- If $\mathbf{n_H = 1}$: Non-cooperative. The binding sites are independent of each other. The enzyme follows **Michaelis-Menten kinetics**, and the velocity curve is a rectangular hyperbola.
- If $\mathbf{n_H < 1}$: Negative cooperativity. The binding of the first substrate molecule decreases the affinity of the other sites, making subsequent binding events more difficult.

- **The X-intercept (K_M):** This allows for the calculation of the **dissociation constant (K_M)**, representing the affinity of the enzyme for its substrate.

Example: Hemoglobin

Hemoglobin is a tetramer consisting of four subunits (N = 4). Although there are 4 potential binding sites for oxygen, the experimental **Hill coefficient (n_H) is approximately 2.8**.

This value (2.8) confirms strong **positive cooperativity**, but because it is less than the total number of subunits (4), it indicates that the binding is not "infinitely" cooperative (meaning the four sites do not all fill at the exact same instant.

4. Regulatory mechanisms

The question that can be raised is: *"How can the binding of a modulator to a specific site regulate catalytic activity when that the binding site is far from the catalytic site and may even be located on a different polypeptide chain?"*

Several theories have been developed to answer this, and all have reached the following conclusion:

The binding of a modulator to its binding site induces a **conformational change** in the three-dimensional **(3D)** structure of the enzyme molecule. The resulting structural of the enzyme that is either **more active** (the **R or Relaxed state**) or **less active** (the **T or Tense state**). The characteristic **sigmoidal shape** of the curve is the result of **cooperativity**.

This means that a conformational change in one subunit induces a change in another subunit. Because these enzymes are oligomeric, the message of "binding" is transmitted across the protein's interface from one chain to the next, even over long molecular distances.

4.1. Theoretical developments

Two models explain the structural transition that allosteric enzymes undergo during substrate binding: the **Concerted model** (proposed by Monod, Wyman and Changeux in 1965) and the **Sequential model** (proposed by Koshland in 1966).

- In the **concerted model**, the T → R transition occurs in a single step; it involves all subunits simultaneously.
- In the **sequential model**, the transition occurs step by step, subunit by subunit.

These two models assume that the enzyme exists in at least two distinct conformational states **T (tense)** and **R (relaxed)**:

- **T (Tense) state:** A state with low affinity for the substrate.
- **R (Relaxed) state:** A state with a significantly higher affinity for the substrate.

Both forms exist in equilibrium: **T ⇌ R**

While both models agree on the existence of these states, they differ fundamentally on how the transition between them occurs:

4.2. Hemoglobin Example

Hemoglobin (Hb) is an important model illustrating the behavior of regulatory enzymes. The binding of oxygen to Hb shows a **sigmoidal relationship** between partial pressure of oxygen (pO_2) and Hb saturation with O_2. This curve is remarkably similar to that of a **homotropic regulatory enzyme**.

In 1925, **Gilbert N. Lewis Adair** accounted for this unique behavior of hemoglobin. He postulated that the binding of a single oxygen molecule to one of the four heme groups **facilitates the subsequent binding** of oxygen to the remaining empty sites. This phenomenon is known as **positive cooperativity**. Mechanism of cooperativity in Hb is:

- **Initial state:** In the absence of oxygen, hemoglobin is in the **T (Tense) state**, which has a low affinity for O_2.
- **Transition:** As the first O_2 molecule binds, it triggers a structural shift in the entire protein complex.
- **Final state:** This shift transitions the remaining subunits into the **R (Relaxed) state**, which has a much higher affinity for oxygen.

This "all-or-nothing" or "stepwise" transition (depending on the model applied) ensures that hemoglobin can efficiently load oxygen in the lungs (high p O_2) and effectively release it in the tissues (low pO_2).

4.3. Catalytic sites and regulatory Sites

The existence of distinct catalytic and regulatory sites is perfectly illustrated by the example of **Aspartate Transcarbamylase (ATCase)** from *E. coli*. ATCase has two substrates (aspartate or S1, and carbamoyl phosphate S2) and two effectors: ATP (F1, an activator) and CTP (F2, an inhibitor).

The enzyme binds the two substrates **Aspartate (S1)** and **Carbamoyl phosphate (S2)** on a **catalytic subunit**, present in six copies (α units). It binds the allosteric effectors on another **regulatory subunit**, also present in six copies (β units). The enzyme is composed of a total of 12 subunits; its formula is $\alpha_6 \beta_6$, with molecular masses of 34,000 for the α unit and 17,000 for the β unit.

The action of effectors modifies the equilibrium between the **T (Tense)** and **R (Relaxed)** states:

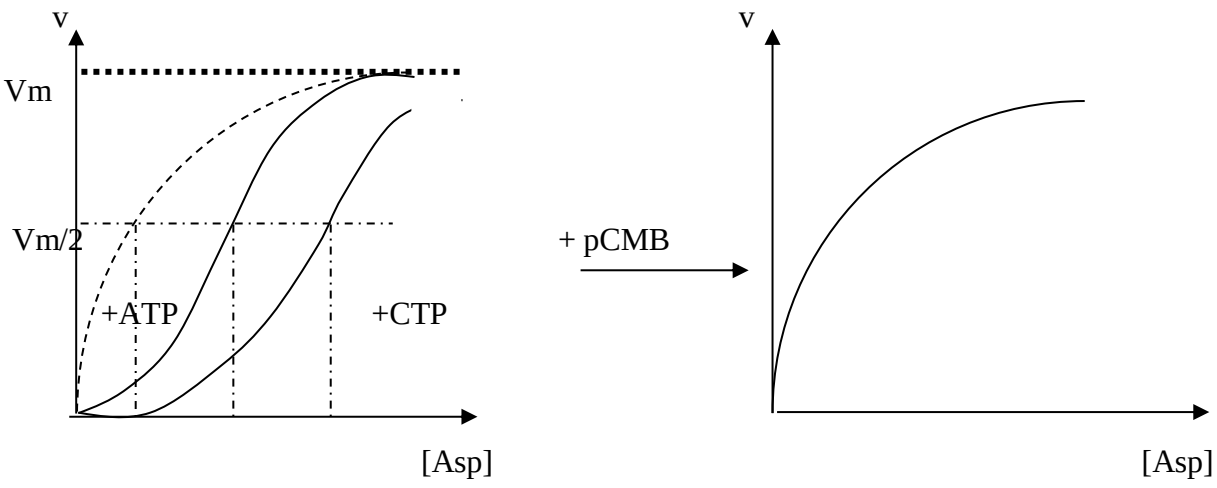
- **ATP (Activator F1)** has a higher affinity for the **R state**; it binding shifts the equilibrium from **T toward R**, increasing the enzyme's activity.
- **CTP (Inhibitor F2)** has a higher affinity for the **T state**; it binding shifts the equilibrium from **R toward T**, decreasing the enzyme's activity.

The reaction catalyzed by ATCase belongs to the pyrimidine biosynthesis pathway:

Asp + carbamyl-P ⇄ asp-carbamyl-P → →→ CTP

CTP is the final product of this pathway and regulates its own production via **feedback inhibition** (retro-inhibition).

The activity of ATCase in the presence and absence of effectors, and after treatment with **p-chloromercuribenzoate (pCMB)**, gives the following curves:



1. **Control (No effectors):** Shows a standard **sigmoidal** curve, reflecting cooperative behavior.
2. **+ ATP:** The curve indicates increased affinity for the substrate.
3. **+ CTP:** The curve indicates decreased affinity for the substrate.
4. **Action of pCMB (para-chloromercuribenzoate):** This chemical agent reacts with sulfhydryl groups and causes the **dissociation** (separation) of the catalytic subunits from the regulatory subunits.

The enzyme becomes **desensitized**. The catalytic units (alpha continue to function independently, but the curve becomes **hyperbolic** (Michaelian) and is no longer affected by ATP or CTP.

The pCMB molecule reacts with the **cysteine residues** of the enzyme. Since these cysteines are essential for the structural link between the catalytic and regulatory subunits. their modification (**denaturation)** leads to:

- **Dissociation** of the subunits enzyme.
- **Loss of allostery:** Once the regulatory subunits are detached, the enzyme is desensitized. The inhibitors (CTP) and activators (ATP) can no longer influence the reaction because their binding sites are no longer physically connected to the catalytic machinery.
- **Michaelian behavior:** The isolated catalytic subunits function independently. Without the inter-subunit constraints that cause the sigmoidal shape, the enzyme follows simple **Michaelis-Menten kinetics** (a hyperbolic curve).

Additional experiments using **SDS electrophoresis** allowed the separation of the different enzyme fractions before and after pCMB treatment. The following results were obtained.

	Before pCMB Treatment (Native Enzyme) (kDa)	After pCMB Treatment (kDa)
peakN°:	1	2
MM	300	
- 1st peak	-	100
- 2nd pic peak	-	34

As a conclusion to this experiment, it can be deduced that the enzyme underwent a separation of its subunits into two fractions, resulting in two peaks:

- **The first peak** corresponds to a trimer of catalytic subunits (α_3). With each alpha unit weighing 34,000 Da, the total mass is approximately **100,000 Da**.
- **The second peak** corresponds to a dimer of regulatory subunits (β_2). With each beta unit weighing 17,000 Da, the total mass is approximately **34,000 Da**.

The reaction induced by pCMB is therefore:

$$\alpha_6\beta_6 \rightarrow 2\ \alpha_3 + 3\ \beta_2$$

The resulting enzyme is **desensitized**. The loss of effector activity can be explained as follows:

- the β subunit contains the regulatory sites that bind ATP and CTP,
- the α subunit contains the catalytic sites.

Thus, the two types of sites are located on entirely different polypeptide chains, they are **structurally distant** from one another. In the intact enzyme ($\alpha_6\beta_6$), they communicate through conformational changes across the subunit interfaces; once dissociated, this communication is broken.

5. Monod-Wyman-Changeux (MWC) theory

5.1. R and T forms

The MWC model (also known as the **Concerted Model**) proposes that an allosteric enzyme exists in two distinct states, **R_0 (Relaxed)** and **T_0 (Tense)**, which are in a state of dynamic equilibrium.

When the enzyme (E) is in strictly in one of these two forms, all ligand-binding sites are equivalent and independent: the binding kinetics are therefore **Michaelis-Menten-like** (hyperbolic).

If a ligand (such as a substrate) has a higher affinity for one of the two forms (typically the **R form**), it preferentially binds to R and the concentration of R_0 decreases; this shifts the equilibrium from T_0 toward R_0 in order to maintain the system's initial balance. As a result, the proportion of R forms increases, leading to **positive cooperativity** during ligand binding.

When the equilibrium has been shifted toward R state, the sites become equivalent and independent, and the enzyme behaves again in a **Michaelian manner** at high substrate concentrations.

5.2. K and V models

Allosteric systems can be further categorized based on which kinetic parameter the effector modifies:

- **K-Model**

In the **K-model**, the focus is on **affinity**. This model assumes that the substrate has a higher affinity for one of the two conformations (by definition R); therefore, the $\mathbf{K_M}$ values of the two forms **(R et T)** are different:

- The substrate has a much higher affinity for one form (R) than the other (T).
- Consequently, the $\mathbf{K_M}$ values for the two forms are different.
- Allosteric activators and inhibitors work by shifting the T⇌ R equilibrium, there by changing the apparent $\mathbf{K_M}$ of the enzyme.

- **V-Model**

In the **V-model**, the focus is on the **catalytic rate**. This model assumes that the substrate has the same affinity for both R and T forms. In this case, the substrate no longer behaves as an allosteric effector, and the kinetics become Michaelian. The allosteric properties of the enzyme are revealed only upon addition of allosteric effectors:

- The substrate has the **same affinity** for both the R and T forms.
- Because the substrate binds equally to both, it does not act as an allosteric effector and cannot shift the equilibrium; therefore, the kinetics remain **Michaelian** in the absence of other molecules.
- The allosteric properties only become apparent upon the addition of **external effectors**. These effectors change the maximum velocity ($\mathbf{V_{max}}$) of the enzyme by favoring a form that is more or less catalytically efficient.