III.6. Influence of physicochemical factors

Enzyme, substrates, cofactors are the chemical bodies which are necessarily involved in the enzymatic reaction. Among the factors that are implicated there are also physical factors: pH and temperature for example.

III.6.1. Influence of pH

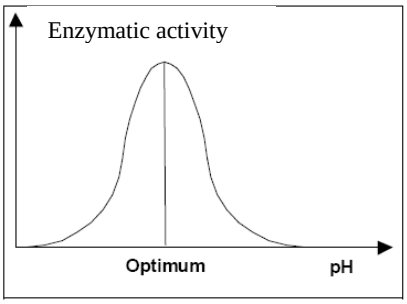
Enzyme activity is profoundly affected by pH, buffer species used, ionic strength, and the dielectric constant of the solution. Enzyme-catalyzed reactions always involve ionizable groups on the enzyme and/or on the substrate. As proton transfers are crucial, maintaining a well-defined pH (H+ concentration) for an enzyme assay is important. Since a range of pH values (between 0 and 14 in an aqueous environment) are in use, more than one kind of buffer ion may be required in an experiment. This is achieved by the judicious use of suitable buffers.

Most enzymes display a bell-shaped pH-activity curve with maximal activity around neutral pH. However, there are enzymes with an extreme pH optimum in the acidic (such as pepsin) and alkaline (such as arginase) range as well. There is therefore a pH of the reaction medium where the electrical charges of radicals of the active site of the enzyme will be most favorable to the enzyme-substrate bond: this pH is called the optimum pH of the enzymatic reaction. The decrease of activity on either side of pH optimum may result from (a) instability of the enzyme and/or (b) changes in the kinetic parameters of the enzyme due to pH. It is important to know whether the effects of pH on enzyme activity are reversible or they result from irreversible changes leading to inactivation.

The pH affects the ionization of molecules:

- Conformation of the enzyme protein;

- Availability of the chemical functions of the enzyme and/or the substrate (i.e. the substrate must be in a certain form, which is not necessarily the form of neutrality).



The pH involves in two different ways: either by modifying the secondary or tertiary structure of the enzyme, or by modifying the electrical charges of the amino acid radicals of the active site. When an enzyme is stored in an environment whose pH is unfavorable for maintaining its structure, it will undergo denaturation.

With the exception of some enzymes (ex: pepsin and alkaline phosphatase), the enzymes that have been most widely studied are active in aqueous solution at pH values between 5 and 9.

Enzyme pH stability can be evaluated by incubating it at different pH values (with and without substrate, effector, etc.) before readjusting to a pH where it is known to be stable. Subsequently, the activity remaining in these samples can be determined in a standard assay. Information about the stability of the enzyme over the pH range studied is necessary in designing correct kinetic studies

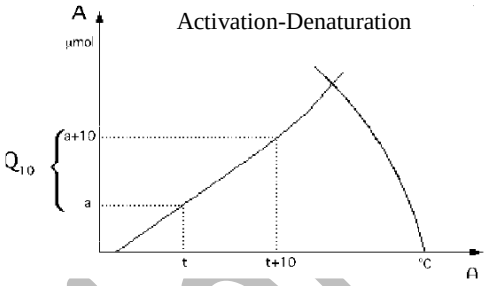
Experimental determination of pH optimum (plots of pH versus activity) serves two purposes. firstly, it is of practical importance in enzyme assay optimization, and secondly, the ascending and descending limbs of such profiles give some idea about the range of pKas and hence possible ionizable groups involved.

III.6.2. Temperature effect

Rate of chemical reaction is directly affected by temperature. Normally the rate doubles for every 10 °C rise in temperature. While this is also true for enzymatic reaction rates, there is one major difference. Like any other protein, an enzyme undergoes thermal denaturation at higher temperatures. Beyond a particular

temperature, enzyme-catalyzed rate starts to decline – due to inactivation of the catalyst. Optimal temperature for the same enzyme may vary depending on the presence of stabilizers, pH, etc.

Most enzyme activities are measured at a standard temperature of 25 °C or 30 °C. However in some cases it may be desirable to use an appropriate physiological temperature. It is usually 37°C for mammalian enzymes and is upward of 72 °C for *Thermus aquaticus* DNA polymerase used in polymerase chain reaction (PCR), for example.



1) Increase in temperature: energy is supplied to the system.

Q10 = activity increase factor when increasing by 10°C

2) Denaturation: the temperature at which it occurs can vary, but only a few exceptions really move away from the 40 - 60 °C range (heat-stable proteins).

III.7. Inhibition of enzymatic reactions

Enzymes are delicate protein catalysts with subtle conformational flexibilities. This makes them vulnerable, and a number of environmental conditions and/or ligands could bring about decline in the net catalytic activity. An enzyme may be irreversibly killed (inactivation by high temperature, extremes of pH, nonaqueous solvent, etc.) or inhibited by ligands that bind to them.

Enzymes can be irreversibly deactivated by heat or by the action of chemical reagents, inducing denaturation. They can also be inhibited (without being denatured) by reversible or irreversible binding of natural or synthetic inhibitors. An enzyme inhibitor is a ligand, not transformed by the enzyme, which modifies the behavior of this enzyme. Many types of inhibition have been described.

Inhibitors are usually small molecular weight ligands that bring about a decrease in the rate of enzyme-catalyzed reaction. For a molecule to act as an inhibitor, it must physically interact with the enzyme. Interactions with the enzyme that do not affect its catalytic activity (that are kinetically silent) are of no inhibitory consequence. For example, a molecule may bind to the enzyme without changing any of its kinetic properties. Although such ligands may serve as potential baits in enzyme purification but are useless in study of kinetic mechanisms.

A study of enzyme inhibition provides powerful insights into their reaction mechanisms. We can classify inhibitors based on their chemical nature and also the unique features of inhibition exhibited by them. The nature of enzyme inhibition may be reversible or irreversible.

The regulation of cellular metabolism is largely based on physiological mechanisms of inhibition of enzyme activity. It is interesting to cause the inhibition of an enzymatic reaction by compounds of known structure in order to obtain information on the mechanism of action of the enzymes. Enzyme inhibition *in vivo* is increasingly the basis of many chemotherapeutic processes.

We will take three examples (reversible inhibition), competitive inhibition, non-competitive inhibition and uncompetitive inhibition. In these types of inhibition, the presence of the inhibitor on the enzyme completely abolishes its activity, but there are also partial inhibitions.

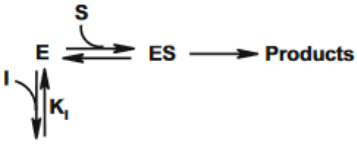
1. Competitive inhibition

If an inhibitor binds to the enzyme and thus prevents the substrate from binding to it, then the substrate and the inhibitor are competing for the active site. The inhibition and the inhibitor are of the competitive type. This is often seen when at least part of the inhibitor has a structure similar to that of the substrate.

Km= [E] [S]/ [ES] => [E]= Km [ES]/ [S]; and Ki= [E] [I]/ [EI]

$[E]_0$= [E]+ [ES]+ [EI]:

[EI]= [E] [I]/ Ki = Km [ES] [I]/ [S] Ki

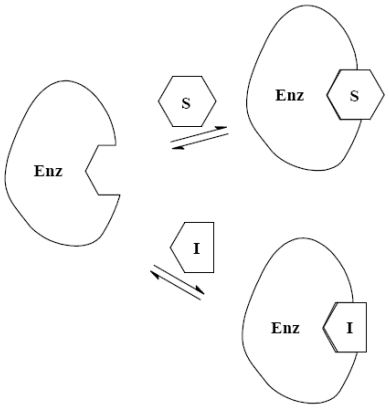


$$[E]_0 = \frac{(k_2+k_{-1})[E\bullet S]}{k_1[S]} + [E\bullet S] + \frac{(k_2+k_{-1})[E\bullet S][I]}{k_1[S]K_i}$$

$$[E]_0 = [E\bullet S]\left(\frac{(k_2+k_{-1})}{k_1[S]} + 1 + \frac{(k_2+k_{-1})[I]}{k_1[S]K_i}\right)$$

$$[E\bullet S] = \frac{[E]_0}{\left(\frac{(k_2+k_{-1})}{k_1[S]} + 1 + \frac{(k_2+k_{-1})[I]}{k_1[S]K_i}\right)}$$

$$v = \frac{k_2[E]_0}{1+\left(\frac{(k_2+k_{-1})}{k_1[S]}\right)\cdot\left(1+\frac{[I]}{K_i}\right)} = \frac{k_2[E]_0[S]}{[S]+\left(\frac{(k_2+k_{-1})}{k_1}\right)\cdot\left(1+\frac{[I]}{K_i}\right)}$$

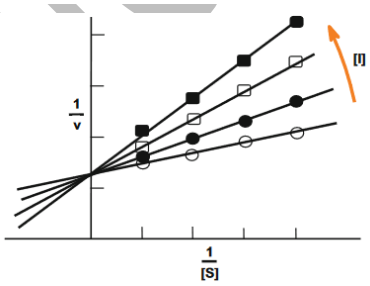


Schematic representation of competitive inhibition

$K_M = (k_{-1} + k_2)/k_1$ **$k_2 \cdot [E]_T = V_{max}$** **$v_o = k_2 \cdot [ES]$**

$$v = \frac{V_{max}[S]}{K_M\left(1+\frac{[I]}{K_I}\right)+[S]}$$

When we vary [I], the lines obtained in double-reciprocal intersect, after extrapolation, at an ordinate point1/Vmax. ([I] = 0).
The inhibition can be lifted by an excess of substrate.
In the presence of an inhibitor, the Michaelis constant for the substrate appears to increase, with the inhibitor competing with it. The affinity seems to be decreased.



Double reciprocal plots for the competitive inhibition of the enzyme with S as the varied substrate

The intersection of the line with the 1/[S] axis has the abscissa:

$$-\frac{1}{K'm} = -\frac{1}{Km}\frac{1}{(1+\frac{[I]}{Ki})} \Rightarrow K'm = Km(1+\frac{[I]}{Ki})$$

$$\frac{1}{v} = \frac{K_M\left(1+\frac{[I]}{K_i}\right)}{V_{max}}\cdot\frac{1}{[S]} + \frac{1}{V_{max}}$$

It is natural to expect that a competitive inhibitor structurally related to either a substrate or a product occupy the active site. Malonate and fumarate are well-characterized succinate dehydrogenase competitive inhibitors of this kind.

IC_{50} for competitive inhibition

The IC_{50} value is the concentration of inhibitor necessary to reduce the reaction rate to 50% of its maximum uninhibited value. An IC_{50} value alone does not allow mechanistic conclusions, but can be used as an index of inhibition efficiency compared to other inhibitors if the IC_{50} values are determined under the same reaction conditions. So, in the case of competitive inhibition.

$$v = \frac{V_{max}[S]}{[S]+K_M\cdot\left(1+\frac{[I]}{K_i}\right)}$$ and $$v = \tfrac{1}{2}\frac{V_{max}[S]}{[S]+K_M}$$ when: $[I] = IC_{50}$

So,

$$\tfrac{1}{2}\frac{V_{max}[S]}{[S]+K_M}=\frac{V_{max}[S]}{[S]+K_M\cdot\left(1+\frac{IC_{50}}{K_i}\right)}$$

$$\frac{1}{[S]+K_M}=\frac{2}{[S]+K_M\cdot\left(1+\frac{IC_{50}}{K_i}\right)}$$

$$[S]+K_M\cdot\left(1+\frac{IC_{50}}{K_i}\right)=2([S]+K_M)$$

$$[S]+K_M+\frac{K_M}{K_i}IC_{50}=2[S]+2K_M$$

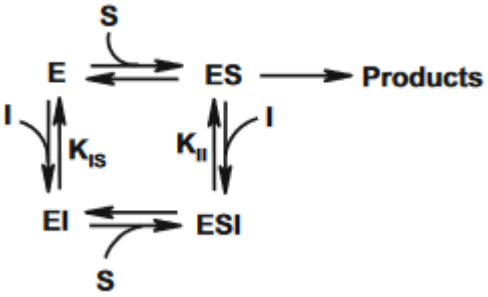
$$\frac{K_M}{K_i}IC_{50}=[S]+K_M$$

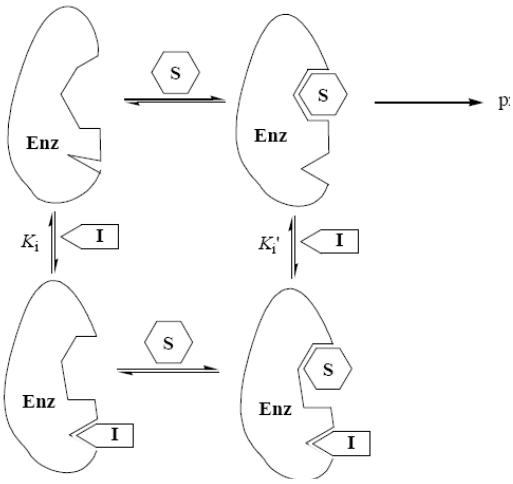
$$\boxed{IC_{50}=\frac{K_i}{K_M}[S]+K_i}$$ and therefore $IC_{50}=K_I\left(1+\frac{[S]}{K_M}\right)$

Note that the IC_{50} approaches the Ki value at very low substrate concentrations

2. Non-competitive inhibition

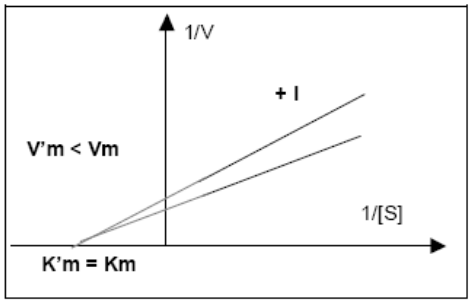
When the binding of an inhibitor to the enzyme occurs on a site completely independent of the active site, there is obviously no competition between the substrate and the inhibitor. By binding, the inhibitor renders the enzyme molecule incapable of catalyzing the reaction: this is called non-competitive inhibition.





Schematic representation of non-competitive inhibition

The inhibitor combines with the enzyme independently of the substrate: both can therefore be fixed simultaneously. There fixation of I on the enzyme does not modify Km. Fixation of S on the enzyme does not does not modify Ki.



Km= [E] [S]/ [ES] => [E]= Km [ES]/ [S];

Ki= [E] [I]/ [EI] => [EI]= [E] [I]/ Ki = Km [ES] [I]/ [S] Ki

Ki= [ES] [I]/ [EIS] => [EIS]= [ES] [I]/ Ki

Km= [EI] [S]/ [EIS] => [EIS]= [EI] [S]/ Km= ([ES] Km [I]/ [S] Ki) [S]/ Km

$[E]_0$= [E]+ [ES]+ [EI] + [EIS]= [ES] (Km/[S] + 1 + Km [I]/ [S] Ki + [I]/ Ki)= [ES] (1+ Km/[S]) (1 + [I]/ Ki)

$$\frac{vi}{Vm}=\frac{1}{(1+\frac{Km}{[S]})(1+\frac{[I]}{Ki})}=\frac{1}{1+\frac{[I]}{ki}+\frac{Km}{[S]}+\frac{Km\,[I]}{[S]\,Ki}}$$

$$\frac{vi}{Vm}=\frac{[S]}{[S]+[S]\frac{[I]}{ki}+Km+Km\frac{[I]}{ki}}=\frac{[S]}{[S](1+\frac{[I]}{ki})+Km(1+\frac{[I]}{ki})}$$

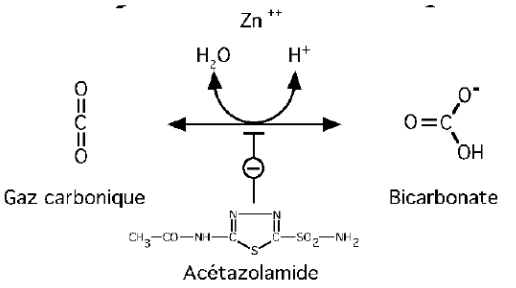
$$\frac{vi}{Vm}=\frac{\frac{[S]}{(1+\frac{[I]}{ki})}}{Km+[S]}\Rightarrow vi=\frac{\frac{Vm}{(1+\frac{[I]}{ki})}[S]}{Km+[S]}\Leftrightarrow V'm=\frac{Vm}{(1+\frac{[I]}{ki})}$$

$$v=\frac{V_{max}}{1+\frac{[I]}{Ki}}\cdot\frac{[S]}{Km+[S]}$$

$$\frac{1}{v}=\frac{Km(1+\frac{[I]}{Ki})}{V_{max}}\cdot\frac{1}{[S]}+\frac{1+\frac{[I]}{K}}{V_{max}}$$

Example of non-competitive inhibition: Carbonic anhydrase

Carbonic anhydrases are zinc-containing metalloenzymes that catalyze CO_2 hydration to bicarbonate and hydrogen ions. Carbonic anhydrase inhibitors are a medication used to manage and treat glaucoma for example

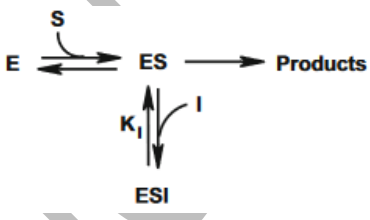


• Carbonic anhydrase is inhibited by the drug acetazolamide.

• This inhibition is non-competitive with respect to carbon dioxide, the substrate of the enzyme.

3. Uncompetitive inhibition

Inhibition by blocking the intermediate complex. If an inhibitor binds to the enzyme-substrate complex, but not to the free enzyme, we have a case contrary to competitive inhibition, which is called uncompetitive inhibition



$Km = [E][S]/[ES]$

$[E]_0 = [E] + [ES] + [ESI]$ and $Ki = [ES][I]/[ESI]$

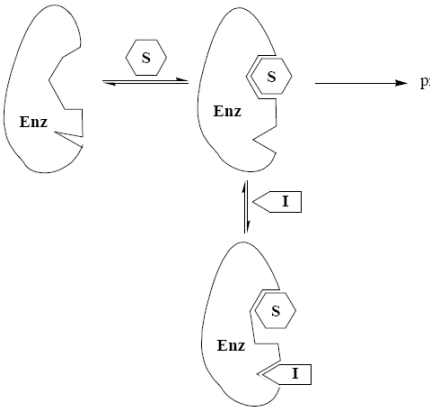
So, $[E]_0 = [ES]\,Km/[S] + [ES] + [ES][I]/Ki$

$[E]_0 = [ES](Km/[S] + 1 + [I]/Ki)$

$[ES] = [E]_0/(1 + Km/[S] + [I]/Ki) = [E]_0[S]/[S](1 + [I]/Ki) + Km$

$\mathbf{v_o = k_2 \cdot [\,ES\,] = k_2 \cdot [E]_0}\,[S]/[S](1 + [I]/Ki) + Km$

$$v_o = \frac{\mathbf{Vmax}\,[S]/(1 + [I]/Ki)}{[S] + Km/(1 + [I]/Ki)}$$

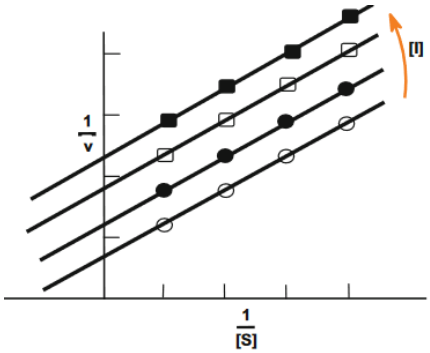


Schematic representation of uncompetitive inhibition

Vmax as well as Km are reduced by the factor: 1+([I]/ Ki)

Km and Vmax vary depending on the concentration of the inhibitor

Everything happens as if the presence of the inhibitor facilitates the formation of the intermediate complex since Km decreases when (I) increases, but at the same time prevents the reaction from occurring.



Double reciprocal plots for uncompetitive inhibition of the enzyme with S as the varied substrate

Notes:

Inhibition by binding of the inhibitor to the substrate

In some enzymatic reactions, in particular in proteolysis reactions where the substrate is a large molecule, there may be fixation of the inhibitor on the substrate and not on the enzyme. This case is not common, but must nevertheless be considered.

Inhibition by high substrate concentrations

Certain enzymatic reactions obey the Henri-Michaelis law for low substrate concentrations, but for high concentrations, the speed, after reaching an optimum, decreases. This occurs when the enzyme is likely to attach several substrate molecules to the active site. Only the complex in which the substrate binds in a

favorable orientation is active. When the concentration of substrate increases, two or more molecules of it attach to each of the subsites of the enzyme, none being this time in a favorable orientation to ensure the reaction, the ternary complex is inactive.

Inhibition by reaction products

Another case frequently observed in enzymology is inhibition by the reaction products, even when the reverse reaction cannot occur. Indeed, the reaction products often have a structure quite similar to that of the substrate, and are therefore likely to form a specific complex with the enzyme.

Activators

If certain effectors have the effect of inhibiting enzymatic reactions, others, on the contrary, are likely to activate them. There are various types of activators, metal ions, anions, molecules of varied nature; the substrate itself can behave as an activator. These phenomena are also involved in the regulation of cellular metabolism; in particular, they make it possible to coordinate the regulation of several metabolic pathways. Coenzymes have sometimes been considered activators.

The mechanisms by which a substance is capable of activating an enzymatic reaction can be as varied as the inhibition mechanisms, either there is **total activation** (the enzyme shows no activity in the absence of the activator), either there is **partial activation** (the enzyme exhibits weak but non-zero activity in the absence of the activator); this only increases the rate of the reaction.