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Paper 05: Molecular Enzymology and Protein Engineering

Module No. 05: pH and temperature dependence of enzymes, pH optimum curve and determination of p*K* Values

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Objective:

The objective of the current module is to understand effect of the pH and temperature on the conformation and catalysis of the enzymes. This module is divided into following sections-

1. Introduction
2. Effect of pH on the stability and catalysis of Enzyme
3. pH optimum curve
4. Determination of p*K* Values and its significance
5. Effect of temperature on the stability and catalysis of Enzyme
6. Summary
7. Questions
8. Resources and suggested reading

1. Introduction

Enzymes are large globular proteins that act as biological catalysts. They are central to every biochemical process and can catalyze hundreds of stepwise reactions ranging from degradation of nutrients, conservation and transformation of chemical energy to the formation of biological macromolecules from simple precursors. They increase the rate of reaction without being used up by themselves. In the absence of

an enzyme, the reaction may hardly proceed at all, whereas in its presence the rate can be increased up to 10^{17}- fold. Enzyme catalyzed reactions usually takes place under relatively mild conditions (temperatures well below 100°C, atmospheric pressure and neutral pH) as compared with the corresponding chemical reactions.

Enzymes are stabilized by a number of different interactions categorized as covalent and non-covalent interactions. Covalent interactions comprises of disulfide (-S-S-) bonds which gives additional stability to the protein structure by making long range interactions whereas electrostatic/ionic interactions/salt bridges, Hydrogen bond, hydrophobic and entropic forces comes under non- covalent interactions. All the interactions providing stability to the enzymes are highly cooperative in nature and their continuous break-remake gives strength and flexibility to their structure. There are certain factors that affect the rate of enzyme catalysis adversely such as temperature, pH, salt concentration, detergents, oxidizing and reducing agents, concentration of various preservative (eg glycerol), metal ion, concentration of substrate, co-factors etc. The two major factors (temperature and pH) affecting the enzymatic activity have been discussed in detail below. In the present modules, we will discuss the effect of pH and temperature on the stability and conformation of the enzyme.

2. Effect of pH on the stability and catalysis of Enzyme

The enzymes are affected by the change in pH. The most favorable pH value is known as the optimum pH. This is the point at which the enzyme is most active. Hence, any alteration in the optimum pH results into the decrease in the activity. The correlation of pH with the activity of enzyme is due to the fact that active sites of enzymes have charged amino acids with different ionization states that are necessary for performing critical functions while present in the active sites as well as for the interactions that maintains protein structure. For example, removal of a proton from histidine residue might result in the loss of an ionic interaction necessary for stabilizing the active conformation of enzyme. The pH range over which an enzyme undergoes changes in activity can provide an idea about the type of amino acid residue involved. Variation in pH influences the electric charge of acidic (like carboxyl) or basic (ammonium, guanidium etc.) i.e functional groups on the protein, hence affecting the activity of an enzyme. Therefore, high concentration of hydrogen

and hydroxyl groups resulting from decrease or increase in the pH, disrupts the electrostatic interactions thereby denaturing the enzyme. Many enzymes have optimum pH at around 6.8, but it varies from one enzyme to another due to the different environments in which they work. For example, pepsin, a digestive enzyme works at a acidic pH of the stomach (around pH 2.0) and Chymotrypsin is active at basic pH of intestine (around pH 8.0). Enzyme was stable over a range of pH from 2.5 to 5.5 with an optimum around 3.5. Maximum activity of the α-amylase was observed at pH 3.5 and 85°C in the presence of soluble starch as substrate.

All enzymatic processes involving proton transfer undergoes acid and base catalysis except few which are devoid of any acidic and basic groups at their active sites. Regarding the pH conditions, enzymes have strict limitations that they must operate at physiological pH in the range 5-9. Due to this restriction and the presence of fairly small range of amino acid side chains available for the participation in acid/base chemistry, a remarkably diverse range of acid/base chemistry is achieved. Generally the acid catalysis occurs when the substrate is protonated by a catalytic residue that in turn gives up a proton. The active site residue must therefore be protonated at physiological pH but its pK_a must be just higher (i.e. in the range of 7-10). If the pK_a of a side chain is greater than 10 then it would become thermodynamically unfavourable to transfer a proton. Likewise, the base catalysis takes place either when the substrate is deprotonated, or when water is deprotonated prior to attack on the substrate. Enzyme active sites bases must therefore be deprotonated at physiological pH but have pK_a values just below. The different pK_a values for amino acid side chains can be measured by the analysis of enzymatic reaction rate versus pH. The pK_a values of active site acidic and basic groups can be strongly influenced by their micro-environment. For example, a lysine residue present in the active site of enzyme acetoacetate decarboxylase forms an imine linkage with its substrate and its pK_a value is found to be 5.9 that are much less than the expected value of 9. It was because of the presence of another positively charged lysine residue adjacent to it that makes the protonated form thermodynamically less favorable. Another example is ethylenediamine where the pK_a for the monoprotonated form is 10.7, but the pK_a for the doubly protonated form is 7.5. Halophilic proteins are characterized by increased negative surface charge due to increased acidic amino acid content and peptide insertions, which compensates for the extreme ionic conditions.

3. pH optimum curve

pH will influence the velocity of the enzyme catalyzed reaction. The active sites of the enzyme are frequently composed of ionizable groups that must be in proper ionic form in order to maintain the conformation of the active site, bind to the substrates or catalyze the reaction. Sometimes, substrate may also contain ionizable groups and only one ionic form of substrate will bind to the enzyme and undergoes catalysis.

The graph plotted for pH versus rate of reaction exhibits a bell shaped curve, with the highest peak depicting the optimum pH for the enzyme activity (figure 2). Every enzyme has a functional optimum pH for it activity. It is dependent on the side chain group present on the active site. Therefore, the optimum pH of a enzyme depend on the active site catalytic residues and its ionization state. The acidophilic enzymes have optimum pH in acidic condition while basophilic enzymes have optimum pH in basic condition.

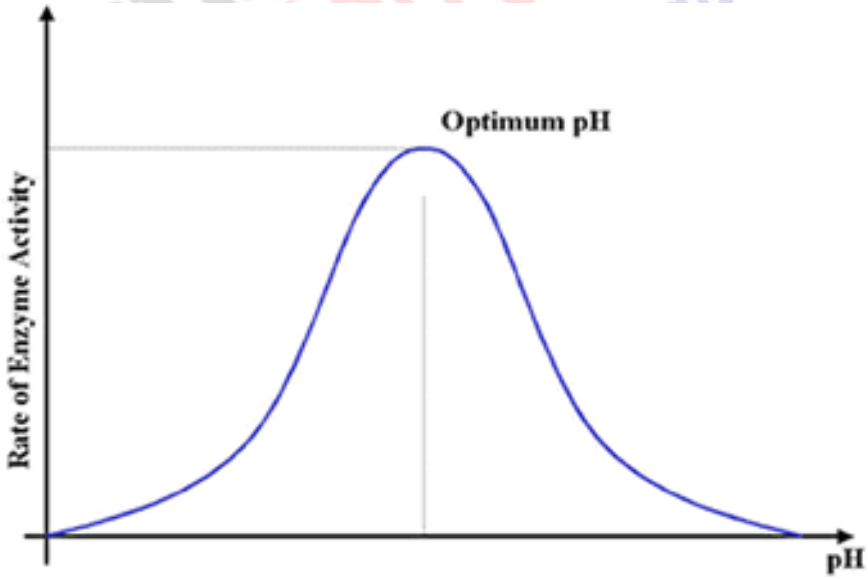

Figure 2: Relative activity of enzyme with reference to temperature

4. Determination of p*Ka* Values and its significance

pH is a measure of the acidity or alkalinity of an aqueous solution. It is defined as the negative of the logarithm to base 10 of the activity of the hydrogen ion.

$$pH = \log[1/H^+] = -\log[H^+]$$

Solutions with a pH less than 7 are acidic and solutions with a pH greater than 7 are alkaline or basic. An acid can be defined as a proton donor and a base as a proton acceptor:

$$Acid \leftrightarrow Base + H^+$$

The species formed by the ionization of an acid is its conjugation base. Conversely protonation of a base yields its conjugate acid. For example, acetic acid and acetate are a conjugate acid-base pair. The equilibrium constant (K) for this ionization is given by :

$$K = [H^+]\,[A^-]/[HA]$$

The pK of an acid is given by:

$$pKa = -\log K = \log\frac{1}{K}$$

pKa is the pH at which half of the acid dissociates, i.e. when $[A^-] = [HA]$. The relation between pH and pK_a can be determined with the help of Handerson-Hasselbach equation.

This equation is given by:

$$pH = pKa + \log\frac{[A^-]}{[HA]}$$

Here, pK_a is equal to –log Ka where, Ka is the acid dissociation constant, [HA] is the molar concentration of the undissociated weak acid, $[A^-]$ is the molar concentration of this acid's conjugate base. The Handerson-Hasselbach equation gives the value of pK_a if the pH and the concentration of the acid and its salt is known and vice-versa. The pK_a of amino acid side chains play an important role in defining the pH-dependent characteristics of a protein or enzyme. The amino acid side chain functional group donates its proton when kept at a pH above than its pK_a whereas, group accept proton when kept below its pK_a. Similarly, if charged group was involved in a salt-bridge with an oppositely charged residue, its pKa would be altered, or if it was in a hydrophobic region of the active site, which would destabilize the charged form of the group. The pKa value of the prototropic group of the active site can often be determined by pH-dependence curve. The side chains of the amino acids are weak acid and base hence the fraction of the side chain present in the ionized form can be calculated by the Henderson-Hasselbalch equation. Hence at pH that is equal to pKa, half of the weak acid will be ionized. The groups donate its

proton at pH above to pKa and accept proton at pH below its pKa. Suppose, active site of the enzyme contain basic group and that must be protonated for catalysis in order to bind negatively charged substrates. The portions of total active enzyme increase with decrease the pH while decrease with increase in the pH. The ionization of the substrate will also change with pH therefore care should be taken to finalize the optimum pH.

The active site of enzyme contains a single ionizable group that must be negatively charged for the catalysis and substrate is positively charged then the velocity equation ca be derived where proton act as competitive inhibitor and equation becomes

$$v_o = \frac{V_{max}.[S^+]}{K_m\left(1+\frac{[H^+]}{K_e}\right)+[S^+]}$$

Where Ke (i.e pKa), rate constant which explain binding of H^+ to the side chain of enzyme. The reciprocal plot will be

$$\frac{1}{v_o} = \frac{K_m}{V_{max}}\left(1+\frac{[H^+]}{K_e}\right).\frac{1}{[S^+]}+\frac{1}{V_{max}}$$

The reciprocal plot of $\frac{1}{v_o}$ and $\frac{1}{[S^+]}$, at different fixed concentration of the H^+ or pH will yield different values of Km,apparent from which Ke (pKe) can be obtained in usual way.

Significance of pKa in ionization of amino acids

Each amino acid comprises of two ionizable groups- one α-amino group and the other α-carboxyl group attached to the C_α atom. Those amino acids with an ionizable side-chain (Asp,Glu,Arg,Lys,His,Cys,Tyr) have an additional acid-base group. Molecules with equal number of oppositely charged ionizable groups are known as dipolar ions e.g. amino acids.

The pK_a values for every amino acid are different due to the presence of differently charged side chains. The pK_a value of α-carboxyl groups of all the 20 standard amino acids lies within a range of 1.8-2.5, whilst their α-amino groups have pKa values in the range of 8.7-10.7.The side chains of acidic amino acids Asp and Glu have pK_a values of 3.9 and 4.1,respectively, whereas those of basic amino acids Arg and Lys have pK_a values of 12.5 and 10.5, respectively. Only the side chain of His, with a pKa value of 6.0, is ionized within the physiological pH range (pH 6-8).

There is certain value of pH for every amino acid at which net charge on an amino acid becomes zero resulting in the formation of zwitter ions. Therefore, amino acid behaves as ampholytes at any pH depending on the pKa of the side chain group. The pH at which amino acid bears no net charge and exists as a zwitter ion is termed as the isoelectric point (pI) for that amino acid. The pI of an amino acid depends on the nature of side chain and its ionization. At this pH, the amino acid does not migrate to any electrode under the influence of electric field. The pK_a is further classified into functional pK_a that refers to the pH at which the group is half ionized between the ionized and unionized form.

Glycine is a simple amino acid with only two dissociating groups, namely, the R-COOH group and R-NH_3^+. The pK_a or pK_1 for carboxyl group is 2.34 and the pK_a or pK_2 is 9.6. Thus, the isoelectric point (pI) of glycine can be determined by taking the summation of both the values of pK_a divided by 2.

$$\frac{pK1 + pK2}{2} = \frac{2.34 + 9.60}{2} = 5.67$$

However the pI for amino acids with additional acidic or basic side chains is different because of extra ionizable group. In this case, pI is taken as the mid-value between pk_a values on either side of isoionic species i.e. acidic amino acids have the pI between the two acid groups while basic amino acids have the pI in between two NH_2 groups. The SH group and the hydroxyl group also behave like acidic group. For example, pI for lysine is the average of the pK_a of two –NH_3^+ groups.

$$pI = pK_2 + pK_3 /2 = (9.18 + 10.79) / 2 = 9.99$$

Therefore ionizable group play a significant role in the determination of the isoelectric point.

5. Effect of temperature on the stability and catalysis of Enzyme

Enzyme is protein complex and its catalytic activity is dependent on the highly ordered tertiary structure where every amino acid side chain group in such a way to form the stereospecific substrate binding site and catalytic center. Tertiary structure of enzyme is mainly maintained by weak non-covalent interactions. Like most chemical reactions, the increase in temperature increases the rate of reaction as high temperature results into greater kinetic energy that is responsible for frequent collisions between enzymes and substrate molecules. Basically, a rise in

temperature increases the thermal energy of the substrate molecules. This raises the proportion of substrate molecules with sufficient energy to overcome the Gibb's free energy of activation (ΔG) and hence increases the rate of reaction. Excessive heat may result into the much higher increase in the thermal energy of the molecules that make up the protein chain that thereby increases the Brownian motion and may lead to the breaking of relatively weak hydrogen bonds, electrostatic interactions and hydrophobic interactions. So the enzymes unfold and get denatured at very high temperatures that disturb its stereospecific tertiary structure. This in turn leads to distortion of the three dimensional structure of the enzyme. Even the small changes in the three dimensional structure of an enzyme can alter the structure of active site, resulting in the decrease or loss of catalytic activity, and ultimately causing decrease in the rate of reaction. A graph of temperature plotted against V_0 is shown below depicting a curve for well-defined temperature optimum (figure 1)

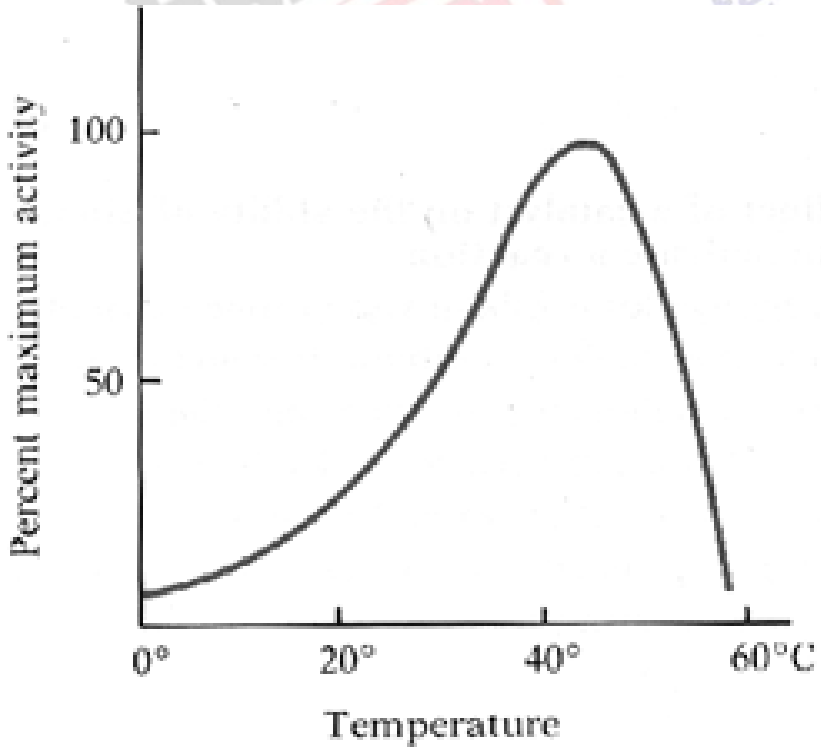

Figure 1: Relative activity of enzyme with reference to temperature

The temperature at which the half of the enzyme gets denatured is generally referred as the melting temperature(T_m) of a enzyme. The T_m decides the stability of an enzyme. For example, in *E.coli*, the DNA polymerase enzyme melts or denature at 41°C. On the other hand, another DNA polymerase enzyme from *Thermus*

aquaticus, commonly known as Taq polymerase used in the polymerase chain reaction is found in a bacterium that lives at high temperatures in hot springs and thus is adapted to work optimally at very high temperatures. The temperature at which Taq polymerase denatures is 99°C. The high number of salt bridges contributes the high melting temperature of Taq polymerase. Thermophilic proteins tend to have a prominent hydrophobic core and increased electrostatic interactions to maintain activity at high temperatures. If the temperature is too low, there can be no noticeable reaction rate due to the inactivation of the enzyme. Thus, an optimum temperature is needed for an enzyme to achieve its maximal rate of reaction. The temperature optimum for each enzyme is different and it varies within organism too. In general, low molecular weight enzyme with single polypeptide chain and having disulfide linkage usually more heat stable as compared to the high molecular weight oligomeric proteins. Psychrophilic proteins have a reduced hydrophobic core and a less charged protein surface to maintain flexibility and activity under cold temperatures. The relationship of rate constant of reaction with temperature will be explained by Arrhenius equation that will be explained in the other module.

6. Summary

In this module, we have discussed about the influence of the temperature and pH of the enzyme. The different non-covalent interactions involved in the stability of the enzyme. The effects of different pH and temperature have effect on the stability of the enzymes. Increase in the temperature will causes increase in the thermal energy of the enzyme s that make up the enzyme chain that thereby increases the Brownian motion and may lead to the breaking of relatively non-covalent bonds which result into denaturation. The thermo-stable enzyme and cold-stable enzyme have different amino acid composition as compare to the mesophilic enzyme. The pKa is the pH at which net charge of the molecule is zero. The pKa of the side chain present in the active side will have effect the activity of the enzyme. Every enzyme have specific active site residue therefore it will be influenced by pH of the medium. The acidophilic and basophilic enzyme have different catalytic properties which make it stable and catalytic active under diverse condition.