Organic Chemistry In Biology And Drug Development
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Lecture - 14
Enzyme Catalyzed Reactions and Introduction to Catalytic Activity of Proteases

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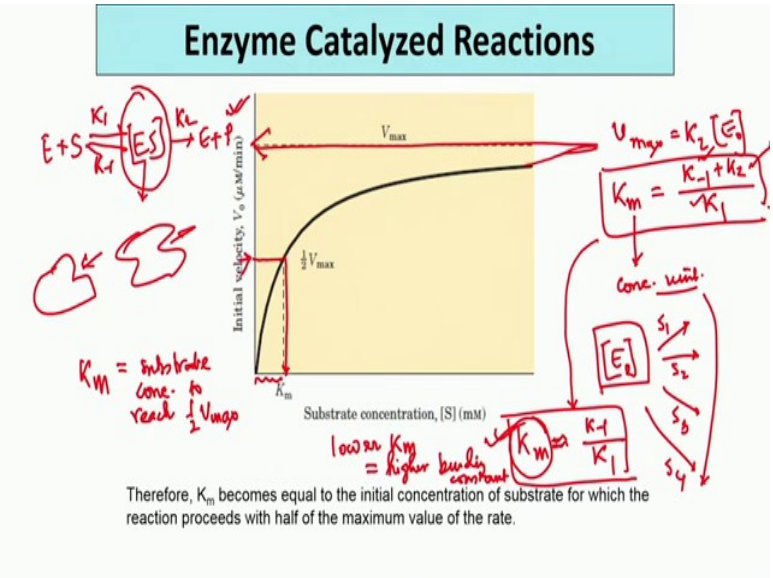

Last time, we ended with the two parameters V_{max} that is equal to k_2 multiplied with E_0. And, we have seen that K_m is equal to k_{-1} plus k_2 divided by k_1; and K_m has the unit of concentration. Now, you may plot the initial velocities for each of these reactions involving various substrate concentrations (S_1, S_2, S_3) and keeping the enzyme concentration constant. These initial velocities will be different for the different values of S.

Now, if you have many points like S_3, then S_4; that means, you slowly increase the substrate concentration and you determine the initial velocity and then you plot the initial velocity with substrate concentration. So, what will happen? You will see that initially as you increase the substrate concentration, the velocity increases. This is justified as there are many enzyme molecules which have these active sites. So, initially the number of active sites are more than the number of substrate molecules. So, initially, all active sites will not be filled up by the substrate molecules. The more active sites you fill, greater the rate.

So, as you increase the substrate concentration, greater number of active sites start getting filled up. So, the rate also increases, but this happens up to a certain point. At some point, since your enzyme concentration is constant, all the active sites will be filled by the substrate molecules. So, beyond that if you increase the substrate concentration, you will not get any rate enhancement. So, the nature of the graph will be like this; it increases and finally, it gradually comes to a constant value. And so, suppose I extrapolate it like this where it attains a constant value so; this is the maximum velocity, and you can get the particular enzyme concentration; maximum velocity V_{max}. So, this is your V_{max}.

So, now you know, this is called the saturation point. The enzyme is saturated with the substrate; like your saturated solution, if your water content is fixed, then after a certain point, the sodium chloride will not dissolve any further. So, that is why, this is called saturation of the enzyme, this is your V_{max}; that V_{max} depends on what?

It depends on your initial enzyme concentration; if you vary the initial enzyme concentration, your value of V_{max} will also change. Now we have earlier told that K_m is equal to the substrate concentration required to reach half of V_{max}. So, what you do? Once you know the V_{max}, now you will see what is the half of V_{max}.

So, this is half of V_{max}. Now, you will draw a line here and then see what is the value in the X-axis. So, that gives you the value of S which is equal to K_m. That is how you determine the K_m. Now what is the significance of K_m? K_m can be defined in two ways; one is that K_m is basically the ratio of these rate constants on the other hand you can define it as the substrate concentration required to reach half of V_{max}.

Both are correct and both are important. But let us analyze this part first. What happens in enzyme catalysis is usually that as you add the substrate to the enzyme, after few minutes, they quickly adopt an equilibrium state; that means that the ES complex is formed very fast and the steady state complex is reached very quickly.

And that is because k_1 and k_{-1} are much higher than the value of k_2; k_2 is the rate constant for the decomposition of ES into the product which is actually much less as compared to the these values of k_{-1} (rate constant for the breakdown of the ES complex) and k_1 (rate constants for the formation of the ES complex). So, these are much higher than k_2. You can now simplify it; K_m is approximately equal to k_{-1} divided by k_1. The reaction scheme is E plus S going to ES and that is going to E plus P.

So, usually k_1 by k_{-1} is the equilibrium constant if we consider the reaction from left to right. Now, here it is k_{-1} divided by k_1; that means, it is the dissociation constant. Earlier k_1 divided by k_{-1} was basically association. Now k_{-1} by k_1 is the dissociation constant of the ES complex; that means, K_m is also roughly equal to the dissociation constant of the ES complex. The question that arises now is that whether higher K_m is better for enzyme efficiency or lower K_m better for enzyme efficiency?

The rate of formation of the product is basically k_2 multiplied to ES. So, then if you can maintain a higher concentration of ES in the system then, you will have higher turnover of ES into the product. Now if the dissociation of ES to E plus S is very high; that means, the association is low; that means, your ES concentration is low; that means, your rate of the reaction will ultimately be low. So, the enzyme will be less efficient.

And on the other hand if K_m is low; that means, your dissociation is low and your association is high; if that be the case; your turnover to the product will be ultimately higher. So, for an enzyme reaction, K_m is very important; lower K_m means tighter binding with higher binding constant. So, you see the same parameter can be defined in several ways that K_m is the dissociation constant of the ES complex or K_m is the substrate concentration to reach half of the V_{max}.

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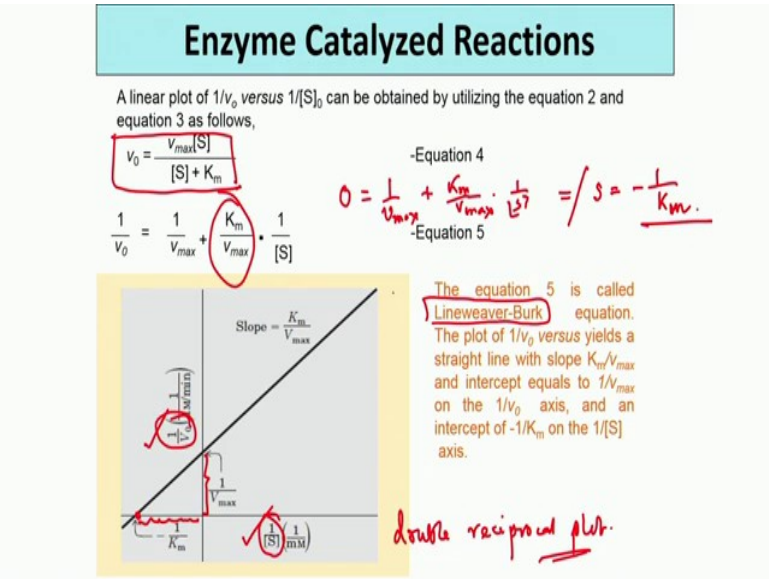

Now, how to really prove that the Michaelis Menten equation is correct? As I said the earlier graph was not a linear graph; it took a shape like this. So, it is very difficult to really plot a graph which is not linear; actually we try to always have a linear graph.

So, we convert the equation in such a fashion that ultimately when we the plot the X and Y, then it gives a straight line and that is much better because you can confidently draw straight lines if your points are more or less colinear. If they stay away a little bit like in the earlier case, it is really difficult to draw this. So, always there is an attempt to represent an equation in such a form that ultimately when we plot X and Y, it gives a linear graph and that is what is done from the same equation if you take $1/V_0$.

So, that becomes $1/ V_{max;}$ that means, it is S by $V_{max;}$ S and S crosses out. So, this $1/V_{max}$ and this becomes K_m/V_{max} multiplied with 1/S. This is called a double reciprocal plot; now if you plot $1/V_0$ versus 1/S, that will be linear because this is now in the form of Y = mX + c.

So now, you get a straight line and in this straight line, what is the value of the X-intercept? Here the value of the X-intercept will be equal to $-1/K_m$ because here your $1/V_0$ is becoming 0; that means, you put 0 equal to $1/V_{max}$ plus K_m/V_{max} multiplied by 1/S. So, if you solve this, you will get that S becomes equal to $-1/K_m$. That means, this X-intercept is $-1/K_m$; the negative value comes because it is on the other side of the X axis.

So, experimentally how to determine K_m? You take the enzyme concentration constant and add the substrates in different concentrations, in different test tubes and measure the initial rate and then you plot $1/V_0$ (that means, 1 by initial rate) against each substrate concentration, take a double reciprocal. This is what is called a double reciprocal plot or it is also known by the name of the scientists who did this; they showed the way to determine or to convert Michaelis Menten equation into a linear form. So, that is also called Lineweaver-Burk plot and this intercept is basically $1/V_{max}$.

Here you put 1/ S equal to 0. So, that will give that Y axis is equal to $1/V_{max}$ and the slope is $K_{m/}$Vmax. So, by doing these type of experiments which involves determining the initial velocity at different substrate concentration and at a particular enzyme concentration, you can determine this V_{max} and the K_m. These are the two extremely important parameters for the equation.

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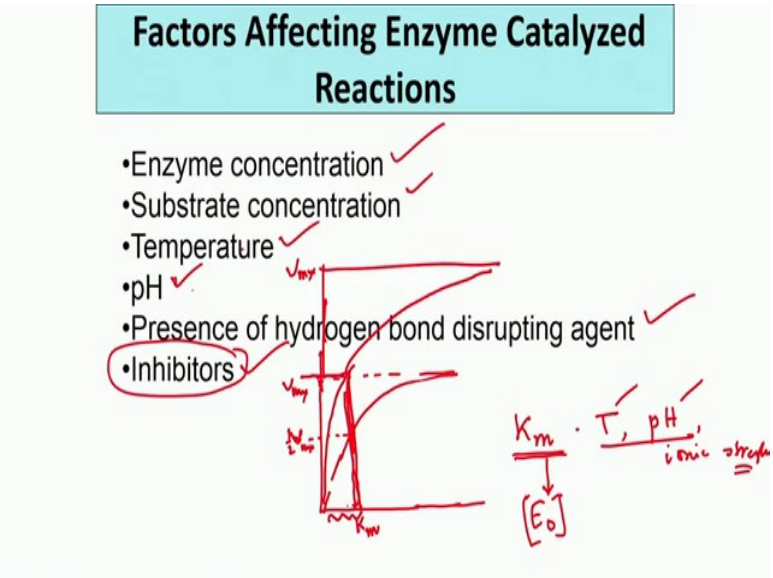

Now, let us discuss the factors affecting enzyme catalyzed reaction. First factor is theenzyme concentration; the more enzyme you have, the greater will be the rate of the reaction. Second factor is the substrate concentration; it depends on the substrate concentration; but you have to be careful that beyond a particular substrate concentration, you cannot increase the rate of the reaction because you will reach the saturation point, at that point, the velocity is what is called V_{max}. Temperature also plays a part because the enzyme works when it is in the native state; if you apply some energy like heat energy to the enzyme, then some unfolding will take place or if you apply some salts into the solution, some unfolding will take place or if you change the pH that also disturbs the conformation of the enzyme.

So the enzyme efficiency will be dependent on these parameters. Further we can say that the presence of hydrogen bonding disrupts the protein conformation. Urea is a hydrogen bond disrupting agent; it is a very simple molecule with the formula NH_2CONH_2. But that has got hydrogen bond donor and acceptor and that is a very good molecule to disrupt the hydrogen bonds that are present in a protein. So, if you add urea, the protein slowly denatures; the hydrogen bonds get broken. And also sometimes or on many occasions, there are other small molecules which look like the substrate, but they are not actually the substrate for the enzyme, but they go and bind to the enzyme and make the active site less available for the actual substrate. Thus some of the active sites will be lost by the presence of those small molecules which are called inhibitors. So, inhibitors are small molecules, that basically slows

down the enzyme kinetics. So, these are the different parameters. Now one more point. When we discuss the factors affecting enzyme catalyzed reactions, we mainly mean the K_m and the V_{max}. These are the parameters which ultimately tell us about the efficiency of an enzyme. So, you have to measure the K_m and the V_{max}, then you will know that what is happening there which is slowing down of the rate.

We know that K_m is dependent on temperature; it is dependent on pH. Provided you do not add any other organic molecules, so no inhibitor is there. So, it is dependent on pH, it depends on the ionic strength of the solution.

But the question is does K_m depend on the initial enzyme concentration or not because K_m can be defined as the concentration to reach half of the V_{max}. Suppose I have a particular enzyme with concentration E_1 where this is the graph. So, this is the V_{max} and half V_{max} is this; that means, the K_m is this. Now if we increase the enzyme concentration so, what will happen? Definitely the V_{max} will increase because, you have more number of active sites; so, you can have more substrates now. So, the value of V_{max} will also increase.

Interestingly what will happen? Suppose the V_{max} now increases and goes to that point; suppose this is now the new V_{max}. But if you calculate half of V max, you will see that it will cut at the same point on the X-axis; it will give the same value of K_m. So, again I repeat V_{max} will change as you change the enzyme concentration, but the concentration to reach half of the V max still remains the same. So, K_m is independent of the enzyme concentration.

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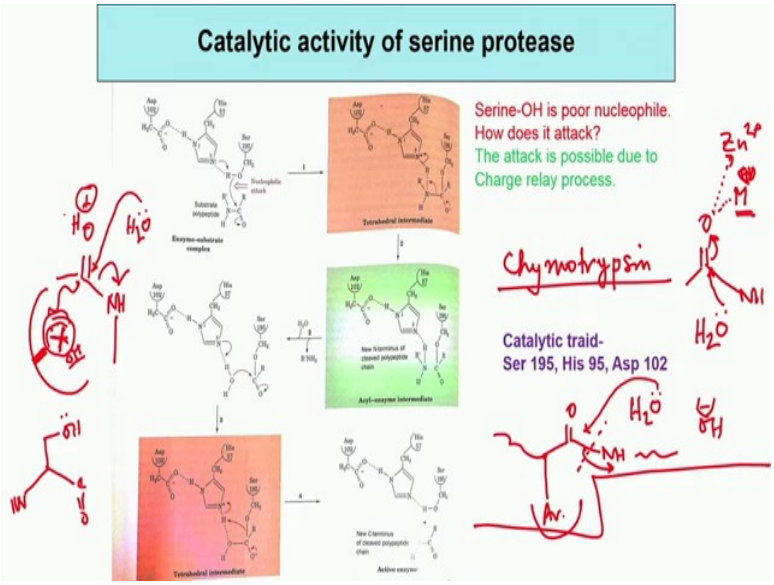

Now, we will go into the molecular details regarding how enzymes catalyze a reaction because what we have seen earlier was really not at the molecular level. We had a very broad perspective. A cartoon type diagram and then an equation and then solve the equation to find the parameters. But the more intricate details involve studies on how these enzymes catalyze the reaction, and what really happens inside the active site? Because we have seen that substrate goes and binds, but something happens to the substrate; how the enzyme is able to do the reaction on that.

Now, we will just show here the mechanism of one protease which we have already encountered and that is called chymotrypsin. Remember what is chymotrypsin? Chymotrypsin is an enzyme which recognizes amino acids or which cleaves peptide bonds from the carboxy end when there is an aromatic amino acids present in the polypeptide. So that means, if you have a peptide bond like this CONH and this is the peptide backbone. So, there must be some aromatic group and that is what is needed. So, the peptide bond that is broken is this one. This is what chymotrypsin does.

Now the question is in order to hydrolyze a peptide bond, what is needed? You need to have water and that ultimately has to come here and break the amide linkage. Now, it is not so simple that if you take a protein and dissolve it in water, the protein never gets hydrolyzed and that is very good thing because if proteins are hydrolyzed that easily, then our body will be fragile; all the proteins will be hydrolyzed. So, fortunately, that does not happen. To make

this hydrolysis by water happen, what you need is some other nucleophile which can attack this carbonyl and then ultimately break this bond and then the nucleophile is finally released by water.

So, that is the mechanism of the protease class of molecules? Actually there are different classes of proteases. Proteases are the enzymes which hydrolyzes the peptide bond. There are different types of proteases depending on their nucleophilic part. I said the enzyme must be having some nucleophile which is a much better nucleophile than water. Water is not a good nucleophile; good nucleophile is OH minus, but in the biological system, at biological pH 7 or 7.2, you do not have this alkalinity which is needed to hydrolyze the peptide bond; instead enzyme provides the nucleophile.

So, internally it attacks leading to breakage of the amide bond and then finally, water comes and releases the X again. So, this goes out and then another molecule comes in. Now depending on the nature of X, you have different types of proteases. One is called serine protease; serine protease is where X basically represents an amino acid containing OH (X = OH); because we are talking about serine; what is serine? Serine is an amino acid with CH_2OH as the side chain. So, in serine, OH is the nucleophile; and then if serine OH is the nucleophile that hydrolyzes the peptide bond, then that class of enzymes is called the serine proteases.

Similarly we may have SH instead of OH, those are called cysteine proteases because SH comes from only cysteine. A third one where you do not have internal nucleophile, involves the enzymes having a metal ion. Because you know that many of the addition to the carbonyl compounds are assisted by acids like Lewis acids. There are many reactions which are assisted by Lewis acids, which activates the carbonyl. Similarly metal Lewis acids are generally metal halides like aluminum chloride ($AlCl_3$) or boron trifluoride (BF_3).

Due to their incomplete octet structure (it has not fulfilled the octet structure), these have got very good Lewis acid character. In case of enzyme hydrolysis, this also can be achieved by simple metal ions, suppose M^{n+} which is a zinc ion in this case. So, oxygen will co-ordinate to the zinc. As a result, the electrophilicity of this amide carbonyl carbon increases.

So, now in presence of metal ions, you do not need any other nucleophile, other than the actual nucleophile water. So, in presence of metal, water can come, attack the carbonyl carbon; this is assisted by the metal ion. Now who is holding this metal ion? The enzyme is

holding the metal ion. So, metal ion becomes the cofactor in that case. So, metal ion activates the carbonyl carbon, water directly comes and breaks the amide bonds. These are called metalloproteases. So, we have serine protease, metalloprotease and then cysteine protease, but there is another category that is called the aspartyl protease.

What is the aspartyl protease? Even in case of this enzyme, amide hydrolysis occurs via direct attack of water upon the carbonyl carbon. What favours the reaction if you want to break a bond involving a carbonyl? I have two options; one is either use a powerful nucleophile to attack the electrophilic carbonyl carbon; break the carbon nitrogen bond or I can activate the oxygen through some processes like metal ion and then water comes and attacks.

For aspartyl protease, instead of metal ions; there is a proton; because you know that many of the nucleophilic addition reactions of carbonyl like addition of DNP, hydrazine etc, are catalyzed by acids. Because the acid protonates the carbonyl oxygen and consequently activates the carbonyl carbon. Then nucleophiles like hydrazine, phenylhydrazine or semi carbazide etc can attack very easily. Now, who will provide this acid? Again the enzyme because the enzyme can be aspartic acid where there is H plus. So, the aspartic acid proton can protonate a carbonyl of an amide bond and then water can come and hydrolyze the amide.

So, these are the four classes. We may have direct attack by water which is assisted by chelation of the carbonyl oxygen either through metals in case of metalloproteases, or by proton from the aspartic acid in case of aspartyl protease; then the water attacks the carbonyl and so, the carbon nitogen bond is broken. This is one side and on the other side, you have a good nucleophile present in the enzyme and that comes and attacks the carbonyl carbon and this amide bond cleaves, but in that case, there is a covalent bond formation between the enzyme and the carbonyl carbon.

So, the enzyme is momentarily stuck with the remaining part of the protein and that is called the acyl enzyme complex. So, the acyl enzyme complex will be finally hydrolyzed when water will come and break the acyl enzyme complex giving your nucleophile back and the result is the overall hydrolysis of the peptide bond. In the next session, we will start from here. We will address the question that what is a good nucleophile in enzymes? Because enzymes do not have good nucleophiles like cyanide, iodide or hydroxide (a moderately

strong nucleophile). So, what is a good nucleophile in case of enzymes? . Serine is nothing, but an amino acid containing an alcoholic OH. The question is that, is the alcoholic OH, sufficiently nucleophilic to hydrolyze a peptide bond? If not, then how can it cleave a peptide bond?In order to answer that question, you need to carry out some control experiments; I take a peptide and add some methanol because that also has alcoholic OH, but nothing happens; if you add ethanol, nothing happens. So, no alcohol can hydrolyze the peptide bond. So, the biggest challenge that was posed at that time when it was discovered that serine protease utilizes serine as the nucleophile to hydrolyze the peptide bond; then the big question came that how is it possible that serine can attack a carbonyl and break a carbon nitrogen bond?

So, we will stop here and we will start from this that how serine hydrolyzes the peptide bond? Thus there is again a question of activation of the nucleophile, the power of the nucleophile has to increase. So, that it can attack the carbonyl. So, we will start from there that how the serine is activated and ultimately that leads to the attack to the carbonyl carbon.

Thank you.