Organic Chemistry In Biology And Drug Development

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Lecture - 15
Enzyme Kinetics (Contd.)

Welcome back to this course on Organic Chemistry In Biology and Drug Design.

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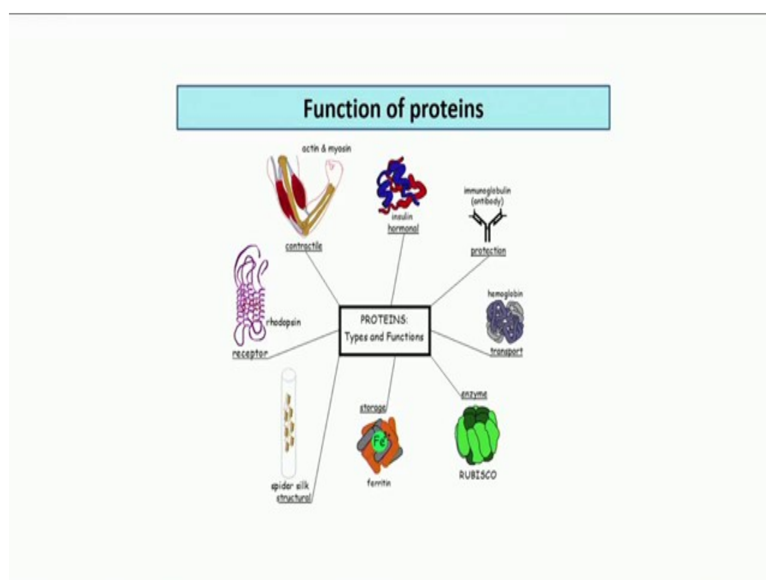

Yesterday we were discussing one of the most important functions of proteins as catalyst of different reactions that take place in the living organism. Now we start with this slide just in order to remind you that proteins have several functions in our body.

Proteins can play a structural component like making the muscle where the triple helical collagen plays the important role. Proteins can work as enzymes that we are going to talk further and then you have these transport proteins or you have antibodies which give us immunity and you have receptors which help in carrying out the signals from one place to another.

Here the particular receptor is rhodopsin, this is the important protein in our eye and it has got a cofactor. Yesterday I told you that what is the cofactor? Apart from the protein, there maybe another non protein part which can play a role in the catalysis and also in signal

transduction. In rhodopsin, the cofactor (retinal) is a carotene like molecule, which is highly conjugated; which in presence of light is isomerized from cis to the trans and that creates a signal. So, these are the signal transduction molecules. Now let us go to that slide where we ended last time. I also want to remind one fact which I told you yesterday that out of the three structures (substrate, transition state and product) that we can consider while a reaction goes from the substrate to the product, the enzyme complementarity is most suitable with the transition state followed by the substrate and finally, the product which is weakly bound.

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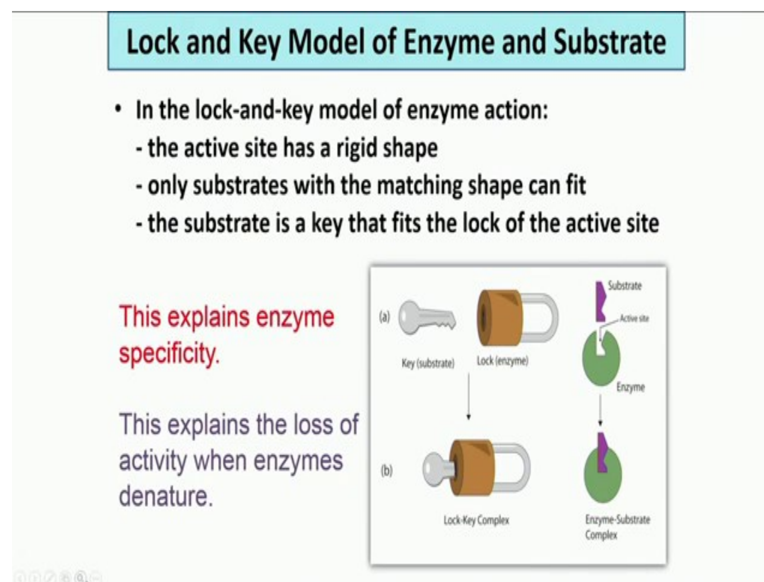

So, the product is released in order to allow the next molecules to be turned over into the product.

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Enzyme Catalyzed Reactions

- When a substrate (S) fits properly in an active site, an **enzyme-substrate (ES) complex** is formed:

  $E + S \leftrightarrows ES$

- Within the active site of the ES complex, the reaction occurs to convert substrate to product (P):

  $ES \rightarrow E + P$

- The products are then released, allowing another substrate molecule to bind the enzyme

  - this cycle can be repeated millions (or even more) times per minute

- The overall reaction for the conversion of substrate to product can be written as follows:

  $E + S \leftrightarrows ES \rightarrow E + P$

So, we were discussing the different classes of enzymes and there are six groups of enzymes which are oxidoreductase, then comes the transferase, then hydrolase, then the lyase, then isomerase, then followed by ligase.

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Enzyme Catalyzed Reactions

$$E + S \underset{K_{-1}}{\overset{K_1}{\leftrightarrows}} ES$$

$$ES \xrightarrow{K_2} E + P$$

So, rate of formation of product is $V_0 = K_2[ES]$

Where, S, E, ES represent respectively the substrate, enzyme and the enzyme substrate Complex. Applying steady-state approximation,

$$\frac{d[ES]}{dt} = K_1[S][E] - (K_{-1} + K_2)[ES]$$

$$K_1[S][E] - (K_{-1} + K_2)[ES] = 0 \qquad \text{-Equation 1}$$

In enzyme catalytic reaction,
$[S] >> [E]$ usually

Hence $[S] = [S]_0$ but $[E]_0 = [E] + [ES]$

Substituting [S] and [E] in Equation 1
$K_1[S]_0\{[E]_0 - [ES]\} - (K_{-1} + K_2)[ES] = 0$

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Enzyme Catalyzed Reactions

$$[ES] = \frac{K_1[S]_0[E]_0}{K_1[S]_0 + (K_{-1} + K_2)}$$

We can express V_0 in terms of [ES]

Therefore rate $$V_0 = \frac{K_1K_2[S]_0[E]_0}{K_1[S]_0 + (K_{-1} + K_2)}$$

$$= \frac{K_2[S]_0[E]_0}{[S]_0 + \frac{K_{-1} + K_2}{K_1}}$$

$$= \frac{K_2[S]_0[E]_0}{[S]_0 + K_m} \quad \text{-Equation 2}$$

Where, $K_m = \frac{(K_{-1} + K_2)}{K_1}$ and K_m is called Michaelis-Menten constant.

Equation is Michaelis-Menten equation.

K_m has unit, its unit is same as unit of concentration

I said that this chronology has to be maintained because IUB has assigned a number to each class of enzyme. When a new enzyme is discovered by somebody then, then according to their reactivity, they fall into any of the classes.

So, they will put a number in front, that front number says that out of the six classes, which one does the enzyme belong to. Then we went on to this Michaelis-Menten equation and analyzed how enzymes catalyzed the reactions within living systems from a mathematical point of view; we got to know that which equation really fits to the enzyme catalysis and it is the very well-known Michaelis-Menten equation. And there are two important parameters that we ultimately saw in the enzyme catalysis. And they are K_m and V_{max}.

So, these are the two important parameters. k_{-1}, represents the dissociation of the E-S complex. k_1 represents the association rate of the enzyme and substrate and k_2 is the rate of turnover of the ES complex into the product. So, all these are important.We say that the K_m is an important parameter because usually k_{-1} or k_1 are much greater than k_2, so you can in all practicality disregard this k_2 and that makes K_m equal to k_{-1}/k_1 which is actually the dissociation constant of the enzyme substrate complex.

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Enzyme Catalyzed Reactions

For most of the enzymatic reaction, $K_2 << K_{-1}$ and then K_m reduces to K_{-1}/K_1, which is defined as the dissociation constant of the ES complex. K_m gives how tight the peptide structure or enzyme-substrate binding. High K_m value means Enzyme-substrate binding is loosely bounded .

At high substrate concentration when $[S] >> K_m$, then equation 2 becomes

$$rate = K_2[E]_0 = V_{max} \qquad \text{-Equation 3}$$

i.e the rate attains the maximum value (V_{max}) and the reaction becomes zero order *w.r.t* substrate concentration.

If we know $[E]_0$ and v_{max}, then K_2 can be calculated. The K_2 is called **turnover number** of the enzyme. This number represents number of molecules converted to product in unit time by one molecule of enzyme.

When the reaction is half of the limiting value,

Therefore rate , $V_0 = ½ . V_{max} = ½ . K_2[E]_0 = \frac{K_2[S][E]_0}{[S]_0 + K_m}$

$K_m = [S]$

And what was V_{max}? You see V_{max} is related to is k_2; that means the rate constant for the formation of the product multiplied with the initial enzyme concentration. In theory, enzyme remains like that, because after the reaction, since it is a catalyst, so the catalyst will be regenerated; so it can be reused later on. Now this k_2 is also important as this is related to V_{max}. Earlier I said that there are these parameters K_m and V_{max} or before that you can say that I have all this important parameters k_1, k_2 k_{-1}; V_{max} is related to k_2 and K_m is related to k_{-1}/k_1.

So, we generally describe the efficiency of an enzyme by these two parameters K_m and k_2, these two parameters are generally used. Regarding V_{max}, as you increase the enzyme concentration, you will reach higher V_{max} values; since this would lead to an increase in the number of active sites. Under certain restrictions, k_2 and Km indicate the true efficiency of the enzymes.

Now same k_2 is also sometimes called k_{cat} because that is the reaction where the product is formed. And this is also called the turnover number; that means it also gives an estimate that how many molecules of the product are formed by turning over the ES complex into the product. So, higher the k_2 means more number of molecules per unit time is converted into the product. So, it is also called turn over number. So, k_2 is equal to k_{cat} and that is also called the turn over number.

Now, the question is that is what really decides the efficiency of an enzyme? Now if you look at the steps that are involved in the Michaelis-Menten equation, E plus S goes to ES complex and then this complex goes to E plus product. We are just removing that EP as the intermediate because that is a very transient species. So, we do not consider that.

An enzyme will be highly efficient if the turnover number is very high; that means k_2 is very high. On what parameter k_2 depends? k_2 will depend on the steady state concentration of ES at a particular time.

So, basically k_2 is dependent on the concentration of ES. Now concentration of ES is dependent on these two parameters k_1 and k_{-1}. So, that means, if I have more ES and higher k_2, then the efficiency of the enzyme will be very good. That means, to have high efficiency, what we need is high concentration of E-S and a high value of k_2.

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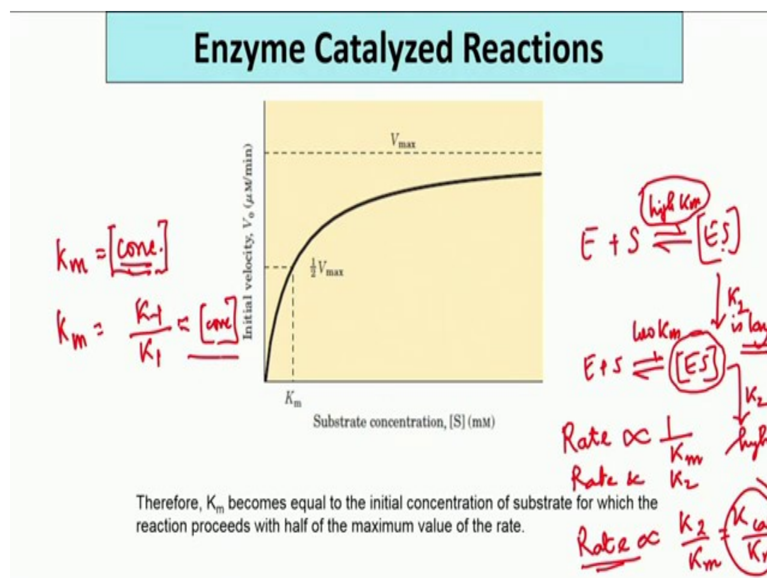

Now, this is interesting that if some enzyme is there which has got a very high K_m value, but it also has very high. k_2; that means, it has got a large turn over number, but it has got a high K_m also. Is this enzyme very efficient or whether an enzyme where there is a low K_m and also a very high turnover number (high k_2) more efficient? Now you know that low K_m means less dissociation of ES complex because K_m is defined roughly as the dissociation constant of the ES complex. So, low K_m is very good because it imples that at a particular time, the concentration of ES will be high because the dissociation will be less.

High K_m on the other hand means that the concentration of ES at a particular time is low because the dissociation is favored. Now that means, the rate of the reaction is actually inversely proportional to K_m, lower the K_m, you get higher concentration of ES. The rate is directly proportional to k_2; larger the k_2, higher is the turnover number of the enzyme.

So, if you combine these, you ultimately see that rate will be dependent on k_2 by K_m. In the books, you will see that it is equal to k_{cat}/K_m. This ratio actually decides the efficiency of an enzyme. I have done in a different method, but you can ultimately prove taking the Michaelis-Menten equation and then derive this aspect that the efficiency of an enzyme is dependent upon k_{cat}/K_m. But without going into deep mathematics, it can be very easily said because rate is inversely proportional to K_m, rate is directly proportional to k_2. If for an ezyme, k_2/K_m is very high, then that is a very efficient enzyme.

Now there is another way to define K_m; it is the concentration needed to reach the half of V_{max}.K_m has a unit of concentration. It is also the ratio of two rate constants. But remember k_{-1} is a unimolecular reaction; when ES goes to E plus S, it is a first order type reaction. So, here the rate constant has a unit of concentration per time, but k_{-1} has two concentration terms per unit time. So, ultimately the one concentration term will be will be crossed out and the time also will be crossed out. So, ultimately this gives a concentration unit. So, in both ways, you get the same thing; that should happen also because both are pointing to the same parameter.

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Enzyme Catalyzed Reactions

A linear plot of $1/v_0$ *versus* $1/[S]_0$ can be obtained by utilizing the equation 2 and equation 3 as follows,

$$v_0 = \frac{V_{max}[S]}{[S] + K_m} \quad \text{-Equation 4}$$

$$\frac{1}{v_0} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} \quad \text{-Equation 5}$$

The equation 5 is called Lineweaver-Burk equation. The plot of $1/v_0$ *versus* yields a straight line with slope K_m/V_{max} and intercept equals to $1/V_{max}$ on the $1/v_0$ axis, and an intercept of $-1/K_m$ on the $1/[S]$ axis.

We also introduced this double reciprocal plot; How can we get the values of K_m and values of V_{max}? That is done by doing a double reciprocal plot also called a Lineweaver-Burk plot. You know that this is done by taking the reciprocal of the equation that you get directly from Michaelis-Menten equation.

So, you have to do a reciprocal of that equation and you get a straight line. So, $1/V_0$ versus $1/S$ will give a straight line like this. Remember that this V_0 is the velocity at the initial point. Because the enzyme velocities will be maximum in the beginning when the enzyme is available in total quantity, but as soon as some active sites are being filled up, the reaction rate will slowly decrease.

So, the initial rate obtained from this Michaelis-Menten equation is V_0; that means, in the initial stage, the line remains a straight line. So, if you take the data points very quickly in the very beginning, say 1 minute, 2 minutes, 3 minutes or so; you get that straight line curve and then you extrapolate and you get the value of the V_0 (the initial velocity). This X-intercept is $-1/K_m$ and the Y-intercept is $1/V_{max}$.

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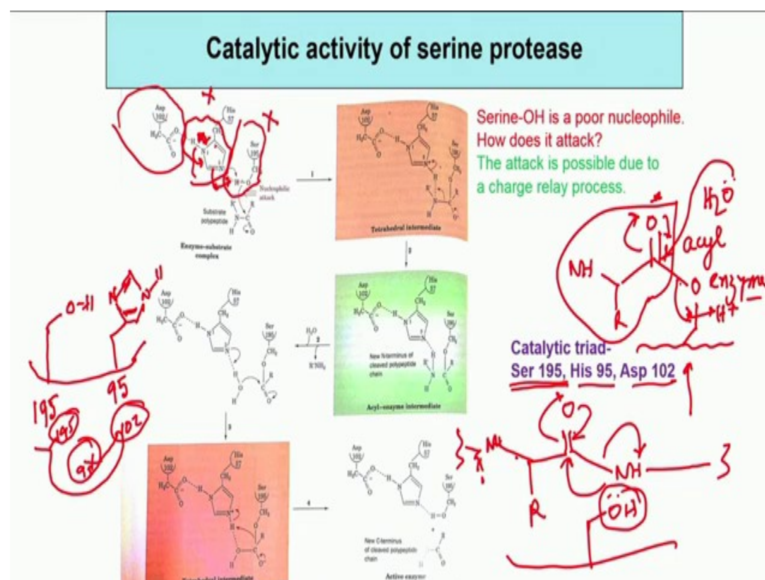

So discussion on all the factors are done. I think this is the slide where we ended last time; that is the mechanism where we want to know the real mechanism; that means, from molecular aspect what really happens during enzyme catalysis. And for our purpose, we have chosen the protease class of enzymes. I told you there are four different classes of proteases;

one is the class of serine protease, where serine is the nucleophilic that assists the hydrolysis of the peptide bond.

Then there is cysteine proteases, where cysteine is the nucleophile; cysteine proteases are also called thiol proteases. And then you have metalloprotease where a metal ion is required and; that means the metal ion is a cofactor. And then another one is called aspartyl protease where aspartic acid remaining at the active site assist in hydrolyzing the amide bond.Now let us talk about all these. So, we have this example of serine protease. One enzyme known to be a serine protease, that is already introduced to you is chymotrypsin. Chymotrypsin recognizes aromatic amino acids and cleaves the peptide bond from the carboxy end..

Now I told you that serine is the nucleophile here. So, mechanistically what is happening? That is CONH and you have this protein chain on this side and on this side you have the R (that is the aliphatic side chain) and you have now NH again and then CO, which is the other part of the protein. Now what we are saying is that there is a serine amino acid that comes and attacks this carbonyl carbon of the amide, this goes here, that comes back and the amide bond is lost. So, this is the mechanism, but this is not the complete mechanism; yet what you have done is that you have broken carbonyl carbon nitrogen bond.

So, one of the peptide that is going out from the right side; that means, from the C terminal side that has got the NH_2 free amine, but this one where the OH is attacking the carbonyl, that ends up as an acyl-enzyme complex; that means, it becomes actually double bond O when the lone pair comes back again and you get this enzyme which is now tied up with serine.

So, this is the acyl enzyme complex. So, I think it is clear what I am saying; that this is our nucleophile that attacks the carbonyl carbon, breaks the carbon nitrogen bond. So, this protein is now free with an N terminus and C terminus, but this protein on the left side that has got the C terminus attached to the enzyme serine and this is what is called acyl enzyme complex because this is an acyl group which is covalently tethering the substrate to the enzyme.

Now water comes again in the next step, it attacks the carbonyl carbon of the acyl enzyme complex and through the same type of mechanism, the serine is released. The serine OH gets its proton back that it has already donated earlier. So, that was the mechanism. However, there is a serious flaw in this in the sense that this serine hydroxy is just an alcoholic hydroxy group; this alcoholic hydroxy group (OH) cannot hydrolyze a peptide bond because OH is not sufficiently nucleophilic to attack the carbonyl carbon.

So, what happens here? People then realized when they checked many of these serine protease class of enzymes; they found that for this enzyme, there are always three particular amino acids which are present, at particular sites. There is a serine which is present for chymotrypsin, this serine is at 195th position with respect to the N-terminal amino acid; that means, 195th amino acid is a serine. Then you have a histidine; histidine basically contains an imidazole moiety in the side chain.

So, N then another N and this is the structure; this is a double bond here and the double bond there. . So, this is the histidine. It has got a NH. So, histidine is always there at position 95. And then there is an aspartate which is at 102. Now these numbers means the position of the amino acid the 195th is serine, 95 is histidine and there is an aspartate at 102.

So, it cannot be a continuous chain because you see 95, 195 and 102 are discontinuous numbers. 95th amino acid is in between these two. So, the possibly it will be like this, that first your 95 then 102 that comes. So, it may be like this that you have a 95 at some position, then you have a 102 somewhere here and then that turns back and then you have 195 here. Because that is the only way you can you can have that. So, this will be your 95, then 95 goes to 102 and you actually go from this N terminals to this C terminals.

So, 195th is a serine, 95 is histidine and 102 is your aspartic acid or aspartate under physiological pH. We generally call aspartate or glutamate in biology because at the biological pH, they will be ionized. So, that is why remember glutamate is different from glutamic acid. Glutamate is the ionized form of glutamic acid. So, here it is this aspartate.

Now what happens here? People have found once they notice that these three amino acids are present in the serine class of proteases; that means, these three are important in doing the catalysis and finally, this is the mechanism that is suggested. Let us see where it has started. This is serine; in this case it is from the left side. So, this is your aspartic acid which is present as aspartate, CO_2 minus then you have the histidine, and finally, you have the serine.

So, what is activating serine? We know that an alkoxide is a very good and powerful nucleophile and not the alcohol. Now the making of this alkoxide kind of thing is assisted from these other amino acids (aspartic acid and the histidine). So, what happens here? Aspartate negative charge abstracts the H from the imidazole moiety of histidine, then the nitrogen becomes highly basic, and this now flows here in this direction and;it takes up the

hydrogen from the OH of serine. So, basically you are making an alkoxide now (that is much more powerful nucleophile than the alcohol itself) which attacks the carbonyl carbon.

So, if you suppose remove the histidine by some process in the enzyme; suppose in chymotrypsin you do some engineering to remove the histidine, you will see that there is no catalysis or even if there is catalysis, that will be very less. So, almost the catalytic activity is gone, if you remove this. Catalytic activity will be gone 100 percent if you remove serine because serine’s presence is absolutely necessary. Catalytic activity is also gone, if you remove the other two (histidine or aspartate). . And that was the beginning that when they removed aspartate, they found that the activity is decreasing; then they removed the histidine, and they found that the activity is almost gone; and when they removed the serine, they found that the activity is lost completely. So, serine is absolutely necessary, but along with that, histidine and aspartate they are also required.

So, now you see a nice relay process, the carboxylate (aspartate) takes the hydrogen from histidine which in turn activates the histidine as a base and it now takes the hydrogen out from the serine. This makes an alkoxide and that is now able to attack the carbonyl. So, it attacks the carbonyl because it is a powerful nucleophile. And then what intermediate will you get? You will get an intermediate like this. I should write it then it will be clearer.

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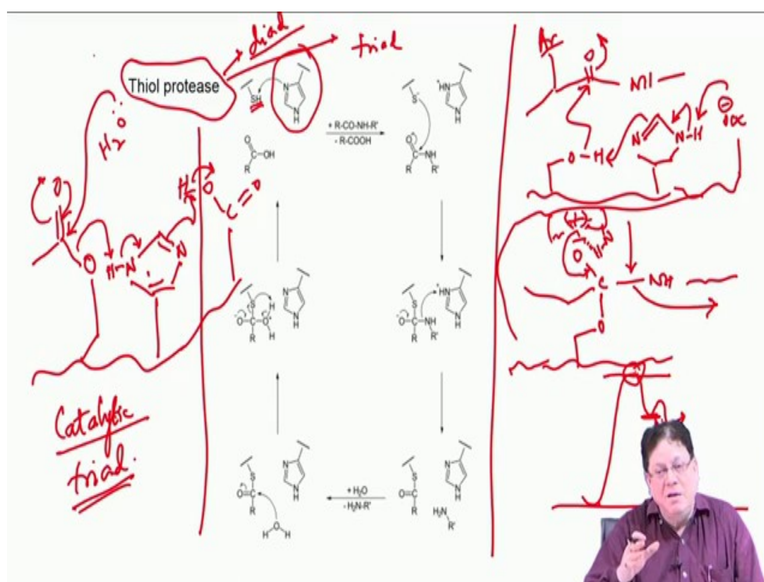

So, we are saying that there is a serine, there is a histidine and then there is an aspartate. So, we are writing COO minus and here is your peptide bond. Remember, if it is chymotrypsin, then you have this R as some aromatic group.

So, the mechanism suggested is a charge which is called a charge relay process. This takes the hydrogen that goes here, that comes there, makes the alkoxide and that goes and initially that will form a tetrahedral intermediate. Initially that will not be broken; the negative charge goes to the carbonyl oxygen. So, O then CO then O minus and this is attached to the left side of the peptide and this is the NH and this is the other side. So we are developing a negative charge in that, this is the intermediate; this is now the transition state because this is the intermediate where the bonds have been formed. In transition state bonds are formed partially, here in the intermediate, the bonds are formed fully. But this is not a very stable system because you have a negative charge on oxygen.

Now enzyme is also extremely clever. Because you know that if you can stabilize the intermediate; that means, the reaction will be faster. I told you what is an intermediate. If there is a reaction which is a two step process; you get an intermediate via a transition state where that oxygen will have δ- charge. There is a postulate which is called Hammond's postulate. We do not know the exact structure of transition state because these are extremely transient species. But what will be its structure? We can approximate the structure by referring to the structure of the species whose energy is closer to the transition state; thus the structure of that species will resemble the transition state. That means, if this is the intermediate then transition state will be structurally close to the intermediate and not the substrate or not the product; because this is energetically much closer to this. So, this is the intermediate.

Now the enzyme stabilizes the intermediate by having an oxy anion hole. Oxy anion hole represents a groove which is positively charged or which has got the ability to donate hydrogen and there can be stabilization by hydrogen bond. So, this oxy anion (intermediate) is an oxide and there is an oxy anion hole, so, that actually stabilizes the O minus.

So, what is the next step? In the next step, this electron density comes back and the NH leaves, but this is not the complete mechanism. Only one of the protein part is gone. The other protein part is now attached to the protease via the acyl-enzyme complex. In the next

step, water comes and then breaks the acyl-enzyme complex by hydrolyzing the acyl enzyme complex;that is shown here; thus you saw all these charge relay processes.

The other important point that I should tell you is what occurs when the acyl enzyme complex is hydrolyzed by water. So, this is your serine, you have the histidine. Now the structure of the histidine what we drew earlier was NH on this side and N on that side, but now this has got the H and this nitrogen is devoid of the hydrogen; now there is a proton transfer, this hydrogen earlier belonged to the serine; the hydrogen belonging to this nitrogen is now taken by the aspartic acid. So, now momentarily aspartate is converted into aspartic acid.

So, now, water comes and the reverse of the charge relay process (with respect to earlier what happened) occurs. Reverse step means, water comes and attacks the acyl carbonyl carbon. So, first it goes there it comes back and then this O of the serine regains its hydrogen what was momentarily with the imidazole moiety of histidine. This comes back, breaks the C-N bond and this nitrogen gets its own hydrogen from the aspartic acid making the aspartate. This forms a catalytic triad, because there are three amino acids that are involved in the catalytic cycle.

So, first electron flowed to the serine. Here it is a proton transfer. So, the proton is transferred to the serine and the imidazole gets back it proton, which was momentarily with the aspartate. So, the enzyme is regenerated and it can do the catalysis again. Similar mechanism happens in cysteine proteases. Here the SH is activated by again an imidazole. Sometimes what was found that in some thiol proteases or cysteine proteases, there could be a catalytic triad.

In some of the thiol proteases, it needs this aspartate and as well as the histidine, but because sulphur is possibly more nucleophilic. So, you may not need the aspartate, the imidazole itself can be able to pull out the hydrogen.

So, in this case of thiol proteases, you have the dyad mechanism as well as the triad mechanism. Triad means now you have the same aspartate, histidine and cysteine or you can have only histidine and cysteine in case of dyad. One of the very well known thiol protease is what is present in papaya and you know papaya juice is specially given while you cook meat because that can soften the meat by hydrolyzing some of the peptide bonds that are present in meat.

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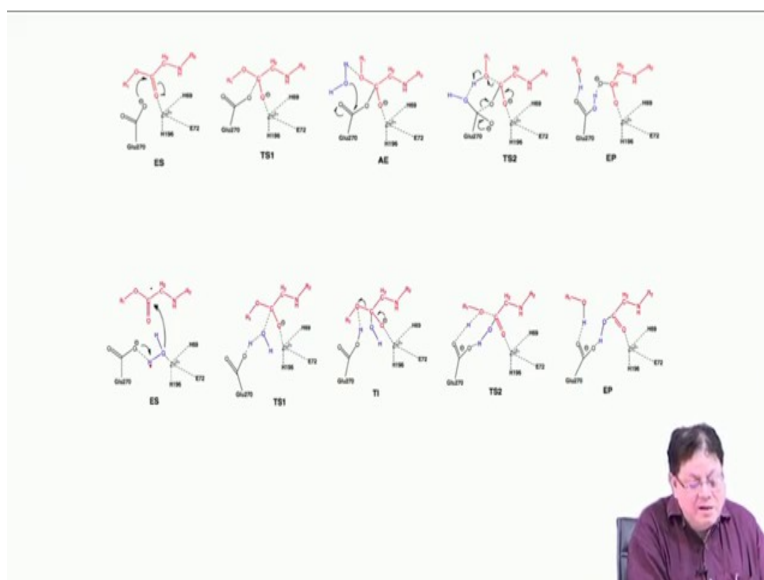

Now, this is what is the called metalloproteases. I told you in that case, a metal ion activates the carbonyl. So, it has a metal ion mediated mechanism. There are two types of actually metalloproteases. In one case, the metal activates the carbonyl and a there is a glutamate that attacks the carbon and this is stabilized by zinc.

So, it is as if the zinc is providing the oxy anion hole that was earlier present in chymotrypsin. Here it is the carbonyl becoming the oxy anion that anion is stabilized by zinc and then it comes back and the NH goes out. So, that goes out and the substrate is momentarily attached to the glutamate. And then water comes and hydrolyzes this intermediate. I am not going into the details as the structures are all here. So, you can see one by one from here.

Other mechanism is that zinc and a carboxylate (a glutamate) can activate the water molecule, like serine was activated by histidine and aspartate. The glutamate can take the hydrogen from water and the oxygen was chelated to zinc. Similarly, if you chelate the oxygen to zinc, then the acidity of this hydrogen increases.

So, the glutamate is able to abstract the hydrogen from water and this virtually becomes hydroxide and the hydroxide being a stronger nucleophile than water, can come and attack the carbonyl and hydrolyze the peptide bond. So, that is the mechanism of metalloproteases. There are two mechanisms that I have shown. In one case, the glutamate directly attacks and the carbonyl which is stabilized by zinc. In the other case, glutamate does not attack the

carbonyl directly; it is the water that directly attacks the carbonyl, but here zinc and glutamate mutually cooperate to activate the water to make hydroxide.

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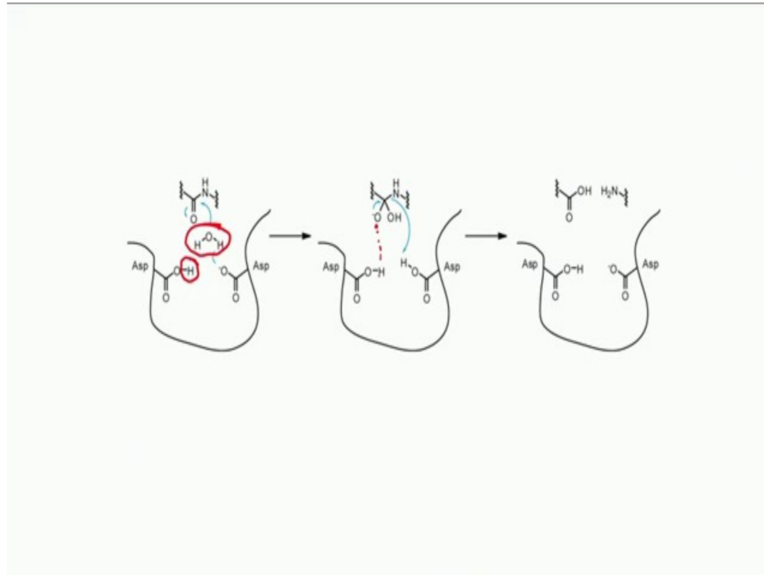

And the last one is what is called aspartyl protease, where there are two aspartate residues; one residue is present in the acid form and the other is present in the carboxylate form and you have water. So, what happens here? Again there is donation of charges in a relay mode.

So, the oxide of the carboxylate takes up the hydrogen from water, this becomes hydroxide and this becomes O minus which is actually stabilized by this other carboxylic acid proton (which is in the acid form) via hydrogen bonding.

So, the next step is the hydroxide (generated from water) attacking at the amide carbonyl carbon; thus the peptide bond breaks. Only the purpose of having two aspartates is that one aspartate is helping the release of hydrogen ion from water, make hydroxide ion; the other aspartate in turn is giving stability to the oxyanion that is being generated, like what I have shown here. That completes these four classes of proteases; only the protease not any other enzyme.

In the next class, we will talk about how to control the activity of an enzyme.

Thank you.