Organic Chemistry In Biology And Drug Development
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Lecture - 13
Introduction to Enzymes and its Kinetics

We have just discussed the different techniques that are used to separate the proteins and before that, we have also discussed different techniques that are used to identify the structure of a protein. Now, let us do one problem to clarify any doubt to further reinforce our concept.

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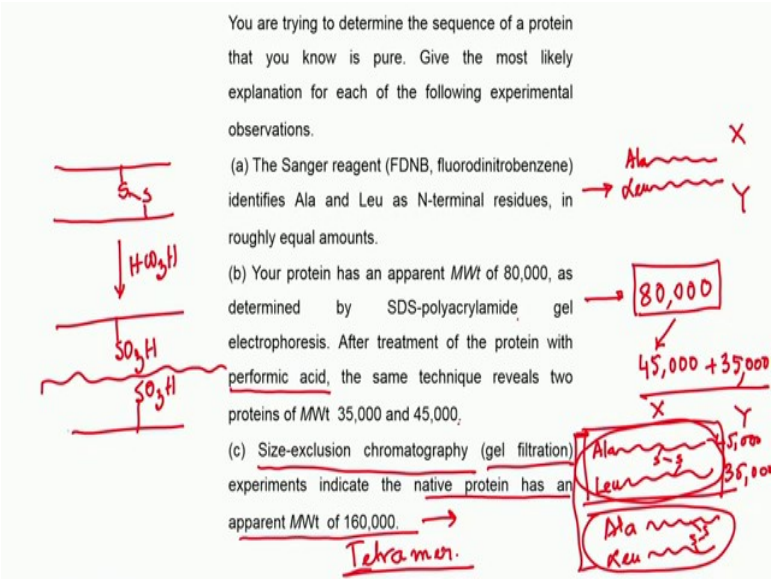

Now, there is a problem that, you are trying to determine the sequence of a protein that you know is pure. I will give the most likely explanation for each of the following experimental observations. Now, what are the observations?

The Sanger's reagent (2,4-dinitro fluorobenzene or FDNB) identifies two N terminal residues, alanine and leucine in roughly equal amounts. So, what does it indicate? It indicates that the protein at least has two chains where one chain has got alanine as the N terminal amino acid; and the other chain there has lecine as the N terminal amino acid. This is your X chain and that is Y chain.

And we will come back to the topic that how these are tied together as per the problem itself. The next information is that your protein has an apparent molecular weight of 80,000 as determined by SDS-polyacrylamide gel electrophoresis. After treatment of the protein with performic acid reveals two proteins of molecular weight 35,000 and 45,000. Formic acid which is further oxidized is known as performic acid (HCO_3H). We must remember that SDS basically denatures the protein.

And then after denaturation, you do the gel electrophoresis and then you also run in a molecular weight ladder. And from the molecular weight ladder, you can say that what is the molecular weight of your protein. So, SDS PAGE says that the molecular weight is 80,000. On the other hand, now you treat the protein with performic acid. The point we have not said anything yet that is if there are two chains of proteins and if they are connected by disulfide linkages; that means, there is a cysteine here.

So, that is usually the way of connecting two chains together through disulfide bonds. Now, the question is, if you treat this one with performic acid(HCO_3H) then this disulfide bond breaks. And, this becomes a sulphonic acid and the other chain also becomes a sulphonic acid; that means, the chains are now separated that is the most important thing.

So, what happens when you did this SDS PAGE? At that time, you get only one band 80,000 that corresponds to the denatured protein. But, when you treated with performic acid, then you see that this is actually made up of two chains having molecular weights 45,000 and 35,000. That means, suppose the X is 45,000 then Y is 35,000 or vice versa.

So, now you have a rough estimate that after performic acid treatment, you get these two bands; that means, these 45,000 and the 35,000 molecular weight chains are connected by at least one disulfide linkage. There may be more than one disulfide; the HCO_3H treatment does not say how many disulfides are there. But, at least the minimum structure that you can write here is that these are the two chains and this is alanine and this is leucine. And suppose this is the 45,000 and this is the 35,000, but I again repeat this could be the other way around.

This could be 35,000 and that could be 45,000, because the information is not given that what is the N terminal sequence of the 35,000 chain or what is the N terminal sequence of the 45,000 chain; that is not shown. So, we have to write this, or the other alternative combination. The last one is size exclusion chromatography, that is called gel filtration. we have learnt that. Size exclusion chromatography indicates that the native protein has an

apparent molecular weight of 160,000. Now, size exclusion chromatography does not disrupt any structure of the protein. Whatever protein structure is there in the native state, that is retained in size exclusion chromatography.

So, that means, if the protein is multimeric in nature, then that multimer will remain as one unit. And when you do size exclusion chromatography depending on the rate of elusion and the cut value; it is revealed that the native structure of the protein has a molecular weight of 160,000.I told you that the gel has a cut point that up to this molecular weight it will allow to be retained; or this will be allowed to be passed through. So, you get 160,000 as the molecular weight of the native protein. This immediately tells you that the protein must be composed of two sets of this alanine and leucine structure. So, the actual protein must be having a chain with alanine and another chain with leucine as their N terminal amino acids; then these chains are connected by this disulfide linkage. And then these two proteins are having some interaction to make a dimeric ensemble.

So, basically this is a tetramer, but dimer of hetero dimers;this is a heterodimer and then this heterodimer is again dimerized. So, actual protein is a dimer of a heterodimer; that means, this is a tetramer. So, the actual protein is a tetramer. This is how the development of different techniques ultimately helped us to determine the structure of the protein. Suppose if you only did the size exclusion chromatography, you would not have been able to know that this protein existed in tetrameric structure. So, you have to do SDS PAGE, you have to also do the performic acid treatment, if there are disulfide bonds that you need to break. And then your Sanger's method actually told you initially that there were two strands. So, that completes our separation techniques and characterization techniques of proteins.

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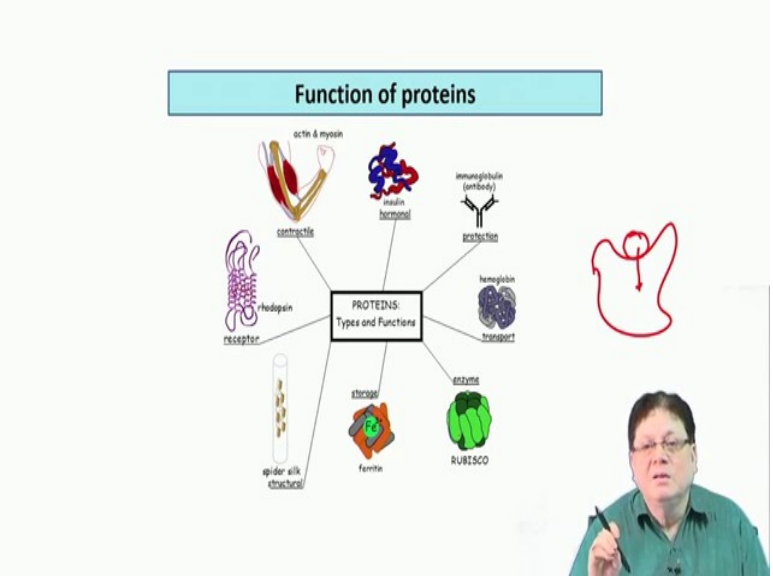

Now, we will go to our next topic that is the functional aspect of proteins. We have completed the structural aspect, now we will go to the functional aspect of proteins. Proteins have different functions in our body: one of the major function is that they act as the catalysts and they catalyze various reactions. Such proteins are called enzymes. Some proteins act only as receptors. Receptor means that you have a protein like this, and some molecule binds here and then that sends a signal after binding. Receptors do not really catalyze any reaction, it is only a site for binding and that binding is associated with generation of a signal.

Like what is shown here is rhodopsin, rhodopsin is the protein that is present in our eyes; when light falls on it, it induces a change in the structure of rhodopsin and that creates the signal. And finally, the signal is processed in the brain and we see the image.

Proteins are present in the muscles; even the silk that makes the beautiful sarees are also made from proteins. Proteins are also present in storage materials ferritin; andtransport proteins which transport (like hemoglobin transports oxygen to the cells). Proteins give us immunity against the invading organisms that affect our body. These are called the antibodies or immunoglobulins. Then they can be also hormones like insulin which metabolize the glucose. There are structural proteins present in muscles; they are made up of proteins like collagen. We will right now discuss the enzymes which are basically proteins that are acting as catalysts. Later on, we will come back during our medicinal chemistry section to the receptor chemistry.

The similarity between the the receptor and the enzyme is that in both, there are sites which are called active sites pockets, where small molecules bind. But, in receptor there is no reaction, there is a signal that is generated and in enzymes there is some reaction that takes place so, that is the difference.

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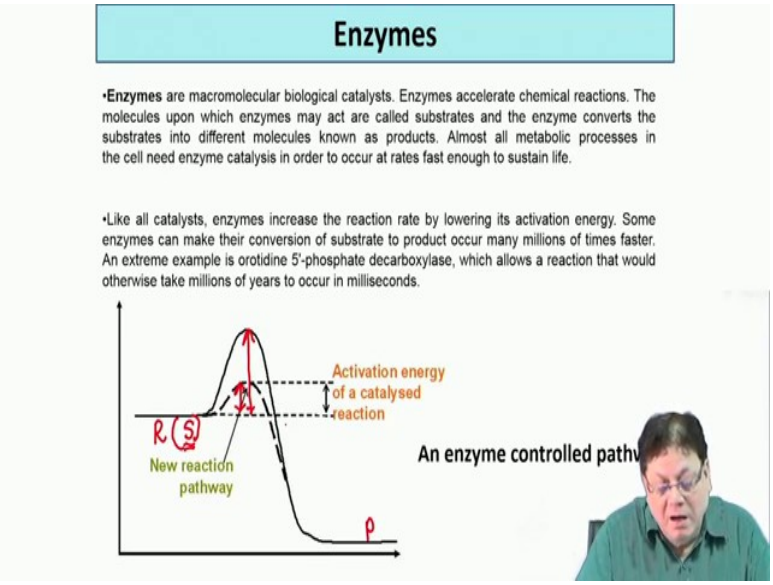

Now, let us go to the next slide. Enzymes are macro molecular biological catalysts. They catalyze the reaction; catalyze means accelerating the reaction. The enzymes are made up of proteins that already I have told, act like ,catalysts; enzymes increase the reaction rate by lowering the activation energy. That means, we know that in any reaction if you want to speed up the reaction, a catalyst only speeds of the reaction both ways: the forward and the backward reaction. Ultimately, allowing the reaction to come to the equilibrium at a much faster rate than the uncatalyzed reaction.

But, a catalyst cannot give higher yield that is a mis-concept that we have; catalysts only lowers activation barrier which is directly related to the rate processes of the forward and the backward reactions. This is the energy diagram. For the reactant, in case of biochemistry, generally we do not write R. Instead of R, generally that is called substrate, the reactant is called the substrate and the product is shown by P. So, if it is uncatalyzed, suppose this is your activation energy; this is the pathway that the reaction takes. That means, now the activation energy for the forward reaction is this.

When you have the catalyzed reaction, then what happens? It takes another path where the activation energy is much lower. So, the enzymes acting as the catalyst, lower the activation barrier for the reaction and so, that the reaction becomes faster.

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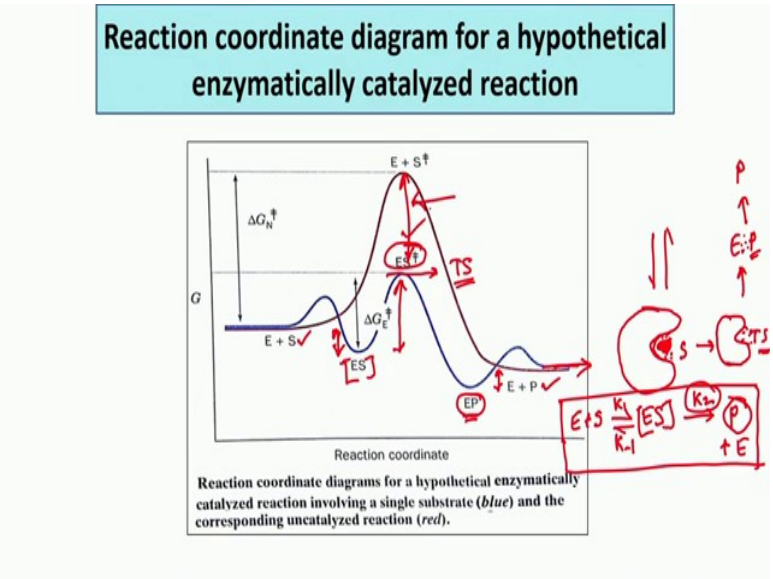

There are still some interesting details here. The mechanism of enzyme catalysis will be discussed now. Suppose enzyme has this type of shape. And this is what is called the active site, where the substrate molecule comes and binds. And this ES complex is formed; then the ES complex breaks into product. And then the enzyme is regenerated, to form the ES complex, you have E plus the substrate. So, the substrate goes and binds to form the ES complex. For simplicity, we are not considering the reversibility in this step, that could be also reversible.

The initial analysis of enzyme catalyzed reaction was done by Michaelis and Menten and for simplicity, they did not consider the reversibility of that step. First it is the enzyme plus substrate to form the enzyme substrate complex; that is a reversible step, k_1 and and k_{-1} are the rate constants for the forward and backward reactions respectively; k_2 is the rate constant for the step involving the decomposition of the ES complex to yield the product. So, this k_2 will ultimately give the product, the rate process for the formation of the breakdown of the ES complex into the product. Now, because its catalysis, we know that the activation barrier is lowered. But, interestingly when substrate binds to the enzyme, the energy of the substrate also is decreased because the substrate is stabilized.

It is like when a baby is alone, the baby is crying with more energy, but as soon as the mother comes and takes the baby into her lap, the baby stops crying; so baby is stabilized now. So, the enzyme is like the mother, substrate is like the baby. Earlier the substrate energy was this and now as soon as its complexes with the enzyme, it forms this ES complex.

And that energy has to be lower than this because it is stabilizing the whole process as there are stabilizing interactions. Why the substrate will go here? Because, there are weak interactions that stabilizes this complex like hydrogen bonding, or a salt bridge formation, or electrostatic attactions, or hydrophobic interactions.

So, all these weak interactions play a part in this binding. But that has to cross an activation barrier because every process needs an activation energy. So, there is an activation barrier like this, but that is a very small activation barrier to form the ES complex. That means, your original energy level now is being lowered. Then that goes into the ES complex which is the transition state; where the bond starts breaking and new bonds are being made. So, this is basically the activated complex or the transition state.

And then that goes to the product; at that point the product is still bound to the active site of the enzyme and ultimately the product is released. So, this also lowers the actual energy of this product that is released. Now what if I ask that between this substrate, product and the transition state, which one is most stabilized by the enzyme? Suppose this is the substrate,so the substrate binds and then it goes into the transition state. This is the transition state; where something is happening on the substrate. So, now this will be TS and the transition state is stabilized by the enzyme and then the transition state will first go into E and P. There will be some bonding, definitely some stability; and then P goes out from the active site of the enzyme and this makes the active site free for the next molecule which comes and binds. Now, out of these three: S, then TS and the product P, which one is most stabilized by the enzyme? The answer is that it is the transition state that is most stabilized by the enzyme, because this goes down a little bit and this also goes down slightly.

They are also stabilized, but to have the lowering of the activation barrier, you need much more lowering of the energy of the transition state as compared to the uncatalyzed reaction. So, that is ultimately what I am trying to say that the enzyme active site must be most complimentary to the transition state followed by the substrate, and the last is the product; because product is released very easily from the enzyme.

So, earlier usually people used to think that the enzyme stabilizes the substrate, substrate goes and binds. Yes it stabilizes, but then when it goes to the transition state, that is the highest extent of stabilization of this. We will go to this aspect later on when we deal with synthetic biology; that is why I spent so, much time discussing this one.

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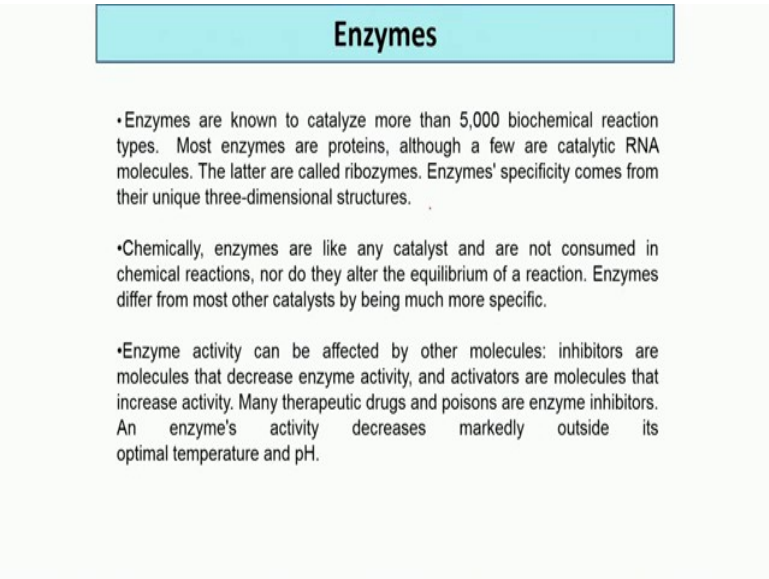

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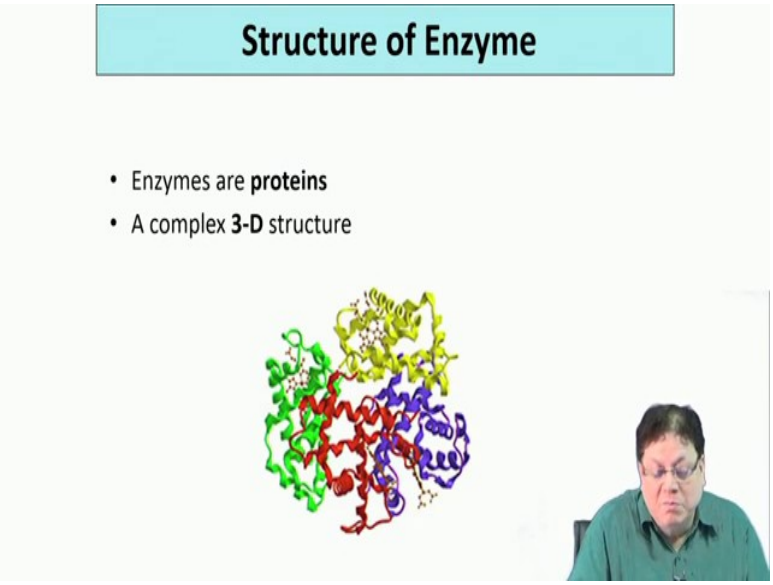

For enzyme, some structure is given here. The enzymes are proteins; this is a complex 3-D structure of an enzyme.

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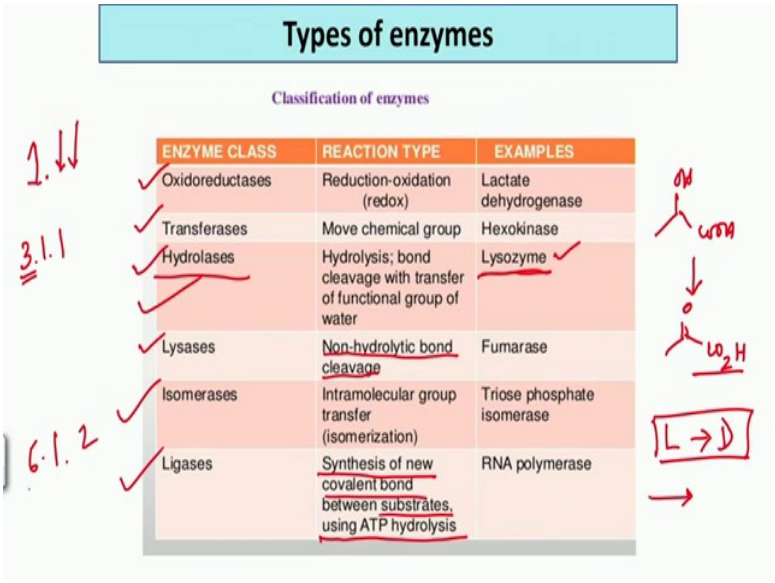

But, let us go to the different classes of enzymes. It is like different organic reactions which are classified according to their pathways and reagents. Like we have nucleophilic addition; we have electrophilic addition; then substitution reaction; elimination reaction. Similarly, based on the type of reaction, the enzymes are classified into six groups. So, whatever enzymes we have in the living world, they will do one of these reactions. The first class is what is known as oxidoreductase, the second one what is transferases. Oxidoreductase carry out redox processes; like lactate dehydrogenasecarries out the conversion of lactic acid to pyruvic acid.

What is lactic acid? Lactic acid is this, and that is getting converted to pyruvic acid by lactate dehydrogenase. Then the second one is transferase; that means, they transfer a chemical group from one place to another like an amine transferase; the amine group of one amino acid is transferred to another substrate which lacks the amine and puts the amine there. So, they are called transferases. Then comes the hydrolase. You already have some idea like the protease which hydrolyze the peptide bond. There could be other types of hydrolase. So, that means, when your bonds are broken by water, then those enzymes are called hydrolases.

So, a specific group is created for that; and there are many enzymes. Lysozyme is one of them; we have chymotrypsin, trypsin, pepsin so, many. Why is lysozyme written here? It is quite interesting that the lysozyme is present in our tears; lysozyme is an enzyme which breaks the peptide bonds of proteins. And this lysozyme gives us a very good antibacterial

activity in the tear which is the water that is constantly washing our eyes. Thus hydrolases are the ones which break a bond by water.

Then you have lysase. Lysases are enzymes responsible for non-hydrolytic bond cleavage. So, if you have a bond cleavage which is done by something other than water, then you have lysase type of enzymes. Then you have isomerase; isomerase is involved in some isomerizations like say L to D conversion; an amino acid going from L to D, that is an isomerization reaction. And then finally, there is a ligase; ligase is basically joining of two things together (like synthesis); that means, your synthesis of new covalent bonds between substrates using ATP hydrolysis; because, for any synthesis you need some energy and that energy is provided by this adenosine triphosphate.

Now, it is important point to note that if I ask you that what are the different types of enzymes? If you change the order of these classifications, then there is a problem. Because, the International Union of Biochemistry says that if you have an enzyme which is oxidoreductase, that should be given a specific number like our IUPAC nomenclature in organic chemistry.

So, there is a nomenclature system for enzymes. So, any enzyme which is oxidoreductase, that will get an initial number 1. And then the species from where it is isolated, and also how many are already isolated from that species. And then the number is put like that; say see if I say 3.1.1, the number 3 means that it belongs to hydrolase.

So that means, you cannot change the order that is written here, that is very important. So, if some something is said at 6.1.2 so that means, it is a ligase.

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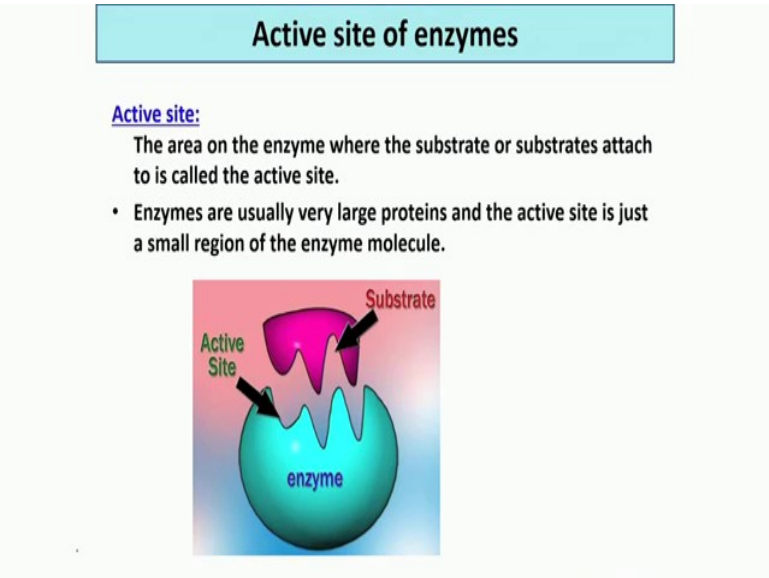

Now, come back to the active site and what is the mechanism of this enzyme catalyzed reaction? What is the mathematical expression of an enzyme catalyzed reaction? So, for enzymes, as I said, this is , one type of active site and this is your substrate. See the substrate has a geometry which is complementary to the active site of the enzyme. But again I warn you that this shows as if the substrate is the best fit, but that is not so; it is a transition state which has the best fit with the active site of the enzyme.

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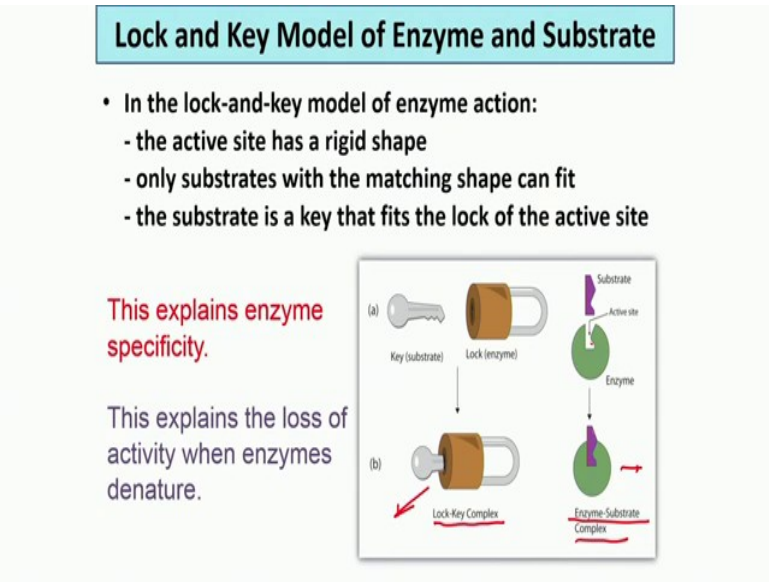

This is called the lock and key hypothesis. The enzyme mechanism was earlier described like this that as if you have this active site which is a kind of a lock and the substrate is like a key. As the key goes and fits into the lock, the substrate goes and binds to the enzyme. As you rotate the key, the lock opens up. So, similar to this lock and key complex, this is the enzyme and substrate complex.

If you want to open the lock, you have to spend some energy on ths lock and key; similarly if you want to get a product you have to do something on this substrate. So, that is the the homology between this lock and key complex and the enzyme-substrate complex .

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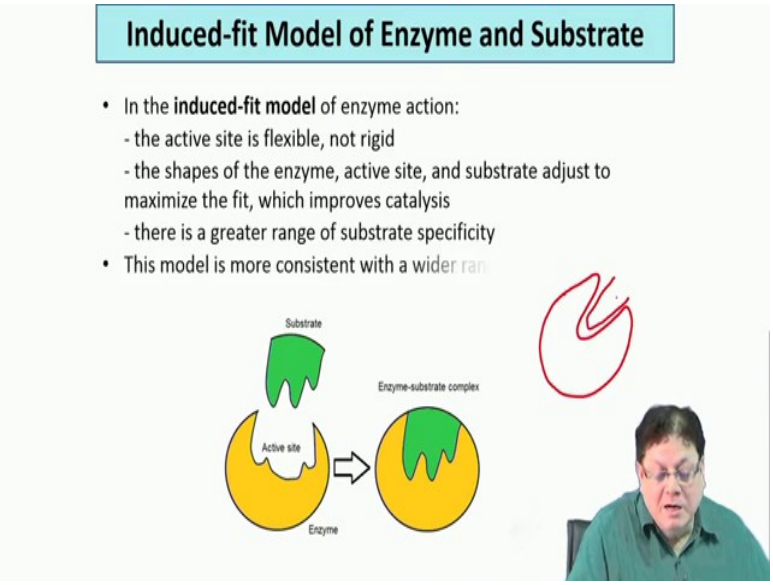

Now, sometimes or actually frequently it is found that initially this active site may not be perfectly complementary to the substrate. So, what happens? Like we have people of different body weights and different dimensions. So, if we have a chair, initially the chair may not fit properly according to your size. But what we do? We ultimately try to feel comfortable by pushing our self inside the chair, so that ultimately there is a best fit between me and the chair. The same thing happens here; the active site may not really have an exact complementarity with the substrate. But, as the substrate goes inside the active site, the enzyme adapts to fit the substrate maintaining the perfect complementarity.

So, this is called induced fit model of enzyme and substrate. So, it is not that the enzyme is already having a perfectly tuned active site. It is like going to a shopping mall and buying a

shirt. The shirt may not fit that well. So, what we do? We go to the tailor and he cuts little bit here and there and then it fits to the body very well. So, the same thing happens here, here the enzyme itself is a kind of a tailor. So, it itself adjusts and then tries to fit the substrate into it.

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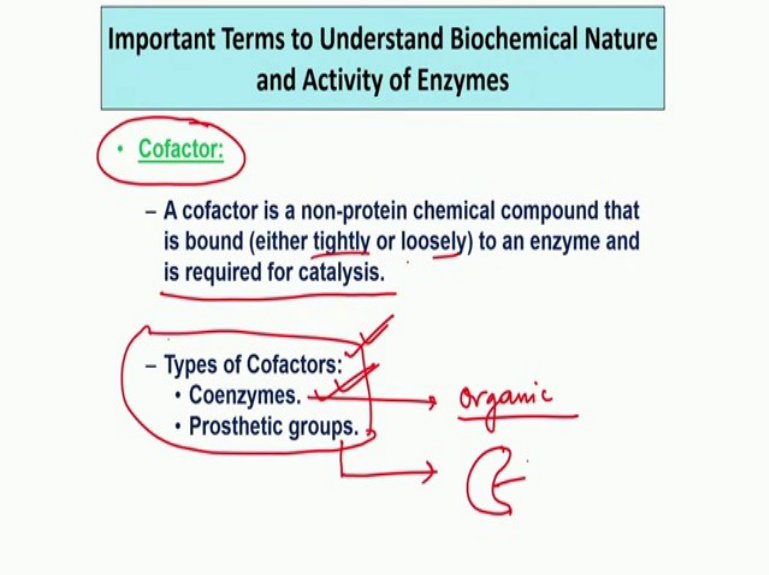

Now, let us go to the Michaelis Menten equation. Before we go into that, it should be known that some of the enzymes (of the six classes that have been mentioned) require a small molecule for their activity to be shown. And those small molecules are called he cofactors. So, cofactor is a non-protein, chemical compound that is bound either tightly or loosely; both are possible for an enzyme; and they are required for catalysis. See that means, it is like you have a big house, everything you have inside; there is refrigerator, TV everything; you have gone outside and then you forgot or you lost your key.

So, if you lose your key, you cannot enter into the house. So, these cofactors are like the entry point. The protein is made by the living system and if the cofactor is not there, the protein is basically useless. Sometimes very simple things like a screw can be the most vital component for some machine to be operational. Cofactors are small molecules which some proteins need to show their catalytic activity. There are two types of cofactors: coenzymes and prosthetic groups. If the cofactor is organic in nature, then that is called coenzyme.

On the other hand, prosthetic group is basically the cofactor which is tightly bound to the protein.

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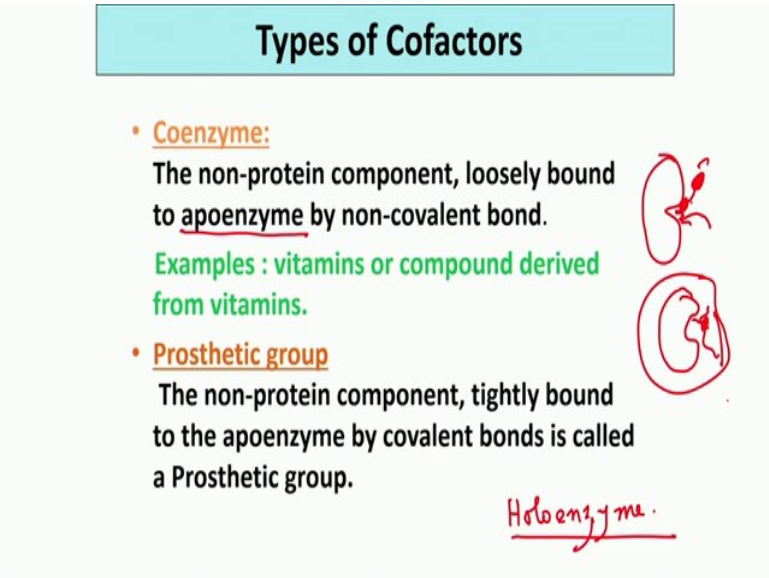

Coenzyme is the non-protein component, loosely bound to the apoenzyme; apoenzyme means the enzyme devoid of the cofactor. See we have said that there is an enzyme like this, there is the active site here, but to do the catalysis, you need this small molecule (cofactor).

Now, there are many cases where cofactor is actually outside the enzyme. But, at the time of reaction that comes and binds to the enzyme and then the substrate comes and the reaction takes place. In some cases, the cofactor may already be bound to the enzyme through a covalent bond. The prosthetic groups are the non-protein components, tightly bound to the apoenzyme by covalent bonds. So, when will the cofactor be called a prosthetic group? When it is tightly bound (attached through a covalent bond) to the enzyme.

Now, if you can strip this cofactor off, then this enzyme without the cofactor is called apoenzyme. And when this whole thing is there; that means, the cofactor is attached to the enzyme, then that is called the holoenzyme. If the cofactor is organic then that is called also called coenzyme. Along with organic molecules, cofactor also includes metal ions; because sometimes metal ions can also be very critical for some enzymes to show their activity.

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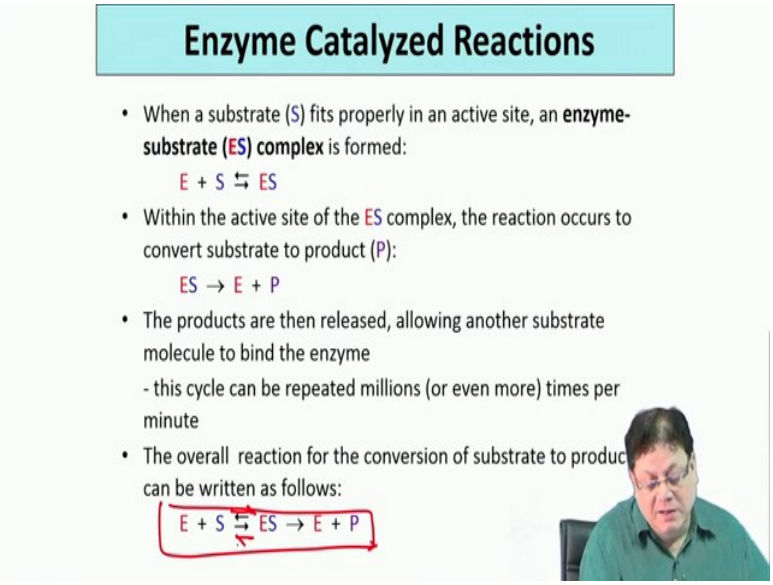

Now, let us go to this famous equation called the Michaelis Menten equation. So, the reaction that is there, that E plus S reversibly gives ES and then this ES going to the E plus product (P). The enzyme becomes free and that can again bind with a new substrate molecule. So, this is the kind of equation that we use.

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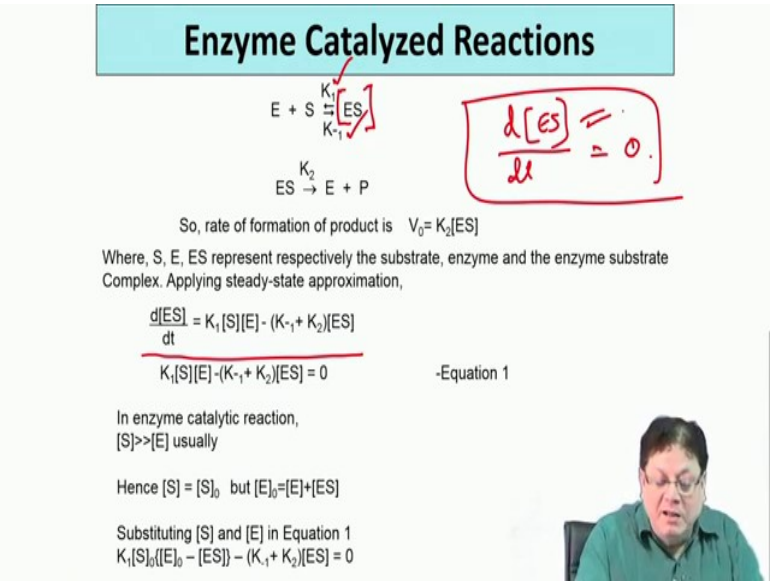

And then, if you solve this, the first step is E plus S forming the ES complex. So, you have two rate constants k_1 and k_{-1}, k_{-1} is the dissociation of the ES complex and k_1 is the association

of E and S. k_2 corresponds to the breakage of this ES complex. Usually we put it in a third bracket because this is a transitory; it does not ultimately remain in the medium. It is produced and then broken down either to product or again or it again reverts back to the substrate and the enzyme.

So, we may solve this equation by assuming the steady state approximation. What is the steady state approximation? It states that initially that substrate will go and bind to the enzyme and then it will also dissociate, this is a reversible process and then after some time if state is reached where the rate of dissociation of ES into E plus S and the rate of association of E and S to form the ES will be same. If that be the case; that means, the change of concentration of ES with time is equal to 0 because, if something is constant; that means, the differentiation is 0. To be more precise, we can say that the rate of dissociation of ES into E plus S and its dissociation to E and P is equal to the rate of association of E and S to form the ES complex.

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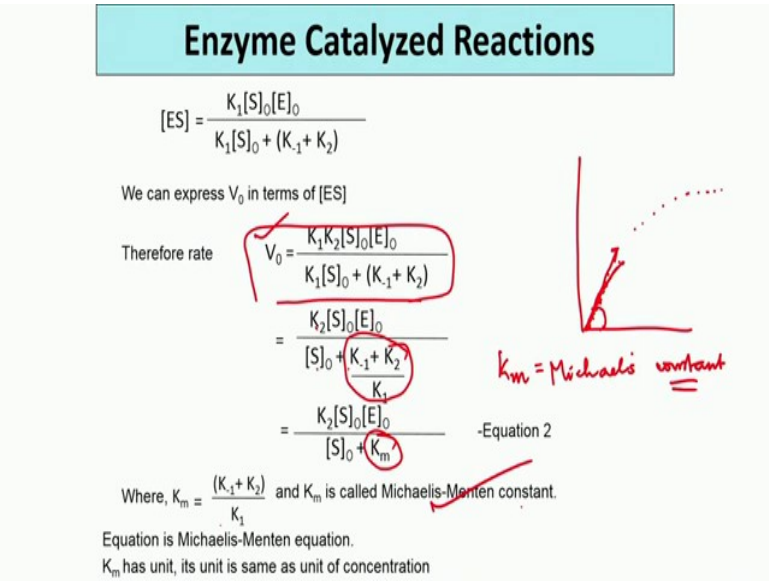

So, you apply this steady state and finally, you come to an equation which is the one shown here. Initially you get this V_0. In enzyme kinetics, as soon as you add the substrate, the reaction starts. So, V_0 is the initial rate; and that is given by gradient of the initial linear line or initial straight line..

So, this V_0 is the initial velocity. ou cannot have a velocity at time 0, it is very difficult to measure that. So, what you can do is that you determine the velocity at some points and say T = 1 minute then 2 minute then 3 minute. And as long as it is linear you can determine the gradient and get the V_0. So, if the V_0 is there, then you can solve this equation. I am not going into the solution; you can do it yourself or refer to any standard test book of this. V_0 ultimately becomes k_2(where k_2 represents the breakage of the ES into E plus product) multiplied with S_0, (where S_0 is the initial substrate concentration) and E_0 (which is the initial enzyme concentration, how much enzyme you have taken) divided by S_0 plus K_m .

Before that ,the equation is like this; initially you get V_0 equal to k_1k_2 S_0 multiplied with E_0 divided by k_1 multiplied to S_0 plus k_{-1} and k_2. And finally, if you divide the whole expression by k_1, you get this $k_2S_0E_0S_0$ plus k_{-1} plus k_2 divided by k_1. And this is what is called the Michaelis Menten constant (K_m). And so, K_m is basically k_{-1} (that means, the rate constant corresponding to breakage of the ES complex to E ans S) plus k_2 (that means, the rate of formation of the product from ES) divided by k_1 (that is the rate constant for the association of E and S to give ES).

This K_m is the Michaelis Menten constant and this equation is what is called Michaelis Menten equation.

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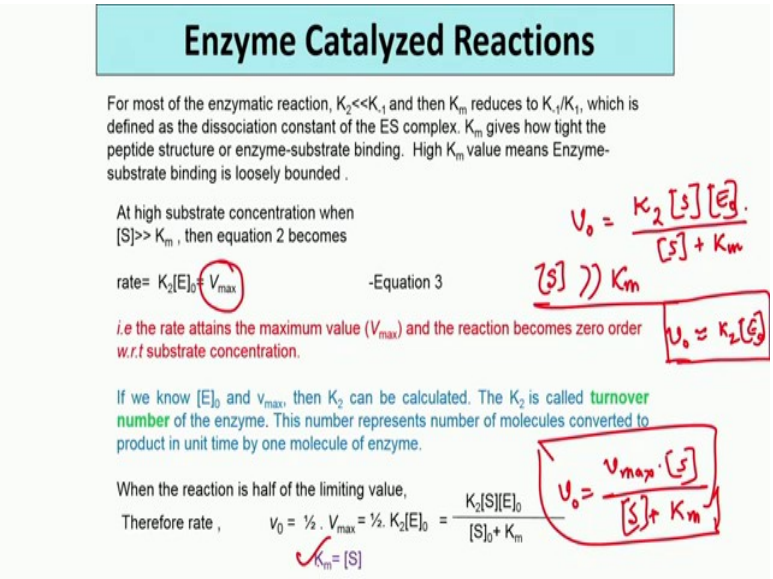

Remember this is also an interesting issue that K_m is basically the ratio of rate constants. But, some of these rate constants are unimolecular rate constants, some are bimolecular rate constants. So, overall it gets a unit which is a unit of concentration because, you have to ultimately add a concentration to this part; K_m. So, K_m has to be in concentration unit otherwise how can you add one concentration which is S and the K_m.

So, you can say in both ways that, because the equation takes this form: S plus K_m in the denominator. So, K_m must be having the unit of concentration. You can also do it by plugging in the rate constants (with their respective units like litre per mole inverse) in the expression for K_m; this will give a concentration unit. You can actually simplify this equation for V_0 if S (which is the concentration of substrate) is much larger than K_m then what happens? So, the equation that earlier you had was V_0 equal to k_2 multiplied to S_0 and E_0 divided by S_0 plus K_m, Now, when S is much larger than K_m, then what happens? You can now neglect the K_m part of it. If you do that, you will see that V_0 is becoming equal to k_2 multiplied with E_0 and that is what is called the V_{max}. The maximum velocity that you can get if you keep the enzyme concentration constant is the V_{max}.

Now, you can simplify the equation; earlier V_0 was given by $k_2S_0E_0$ divided by S_0 plus K_m; now you just write that it will be k_2 multiplied with E_0 is V_{max}. So, you can write V_{max} multiplied with S_0 divided by S_0 plus K_m equals to V_0. So, this is the final form of the equation; some interesting observations are that when your V_0 equals to half of V_{max}; if you put this value as half of V_{max} then you will see that K_m is equal to S.

I think that in the next session, we will do the further analysis of these parameters. So, there are two important parameters that we learnt: Firstly K_m (Michaelis Menten constant) is characteristic of the enzyme that you have taken. And there is another parameter which is V_{max}; because this will ultimately give you that what is the efficiency of the enzyme. If V_{max} is very high; that means, the enzyme appears to be very efficient; maybe in the next session we will start discussing on the K_m.

Thank you.