

## ENZYME SCIENCE AND ENGINEERING

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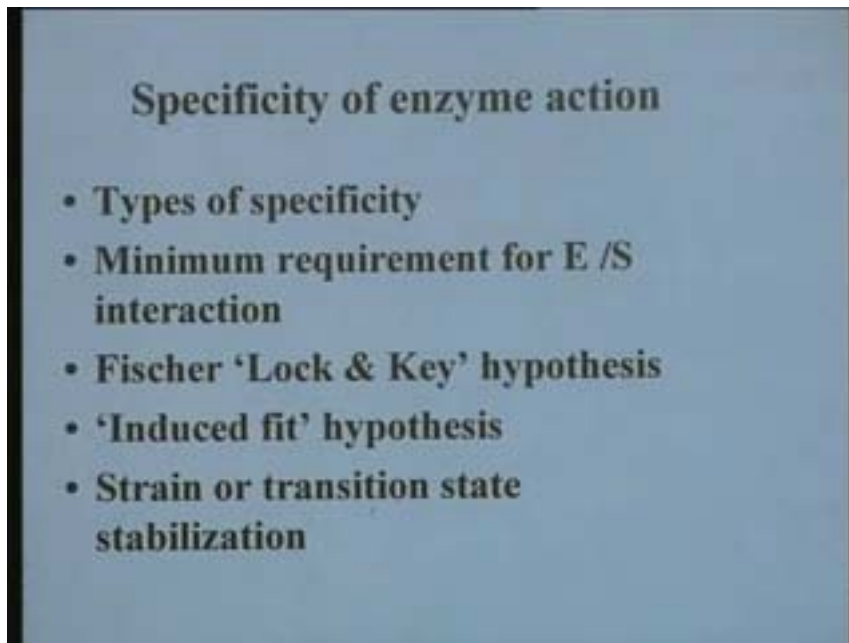
DEPARTMENT OF BIOCHEMICAL ENGINEERING  
AND BIOTECHNOLOGY  
IIT DELHI

LECTURE – 5

### **SPECIFICITY OF ENZYME ACTION**

You may recall that earlier we have outlined various ways of the mechanisms by which enzymes catalyze chemical reactions. One of the unique features of the enzymes' catalytic function is their specificity.

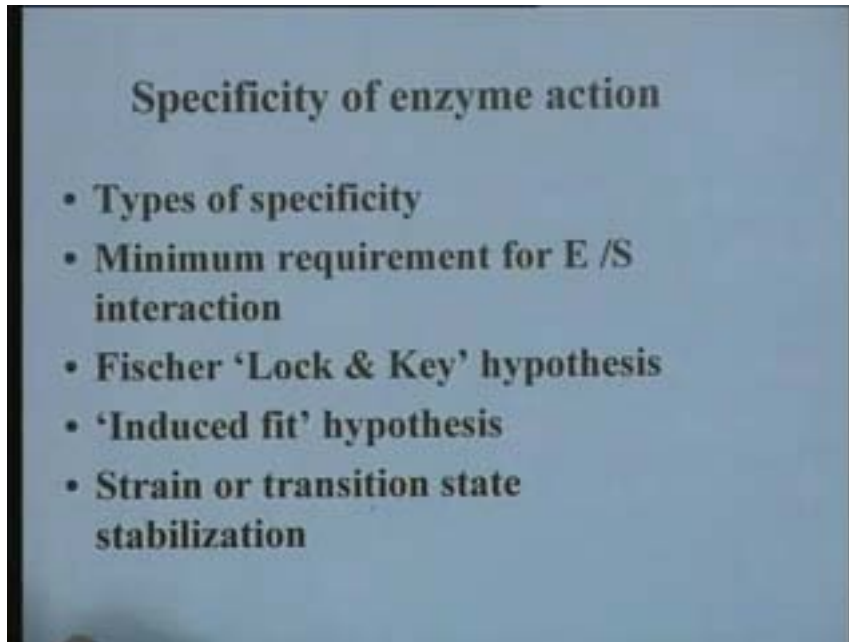
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In fact when we distinguish them or when we compare them with a variety of chemically catalyzed reactions or even uncatalyzed reactions they are distinguished by virtue of their specificity. In the case of chemical catalyst a single catalyst may catalyze a large variety of similar reactions. If you consider nickel as the catalyst all the hydrogenation reactions can be catalyzed by that. Similarly in the case with colloidal platinum or vanadium pentoxide or even acids acts as a catalyst. For large variety of hydrolytic reactions acids or hydrogen ions can act as a catalytic function. On the other hand when we talk about enzymes these functions are highly specific. In most cases the enzymatic catalytic

function is both substrates specific as well as product specific. That means you need different enzymes to act on different substrates or to produce a particular product different enzymes are needed.

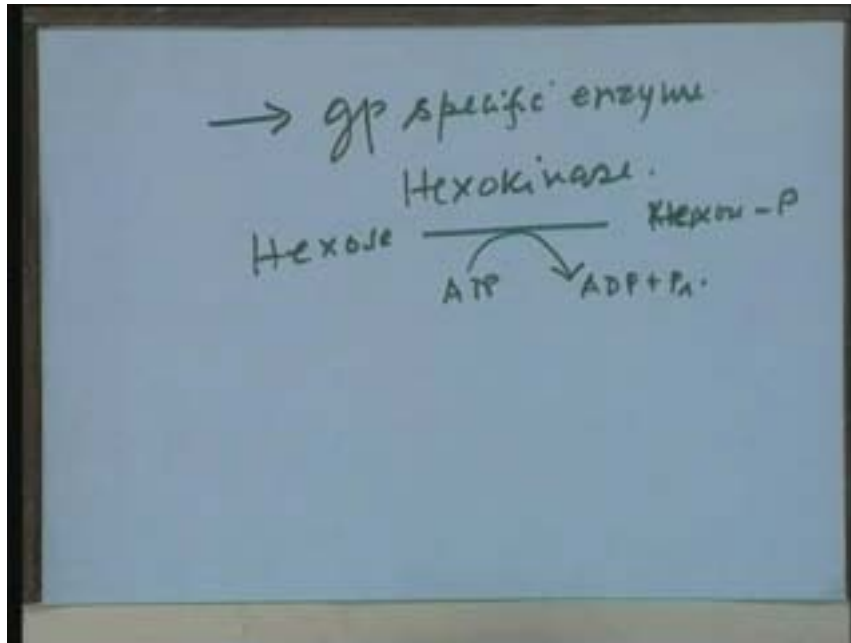
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There is a significant variety as for as specificity is concerned. All the enzymes are not specific to the same extent. There is lot of variation in terms of their characteristic features required as a specific catalyst. In many cases the enzymes may exhibit a very broad specificity not too strictly specific. A large number of examples which you may be familiar with are what we call as group specific enzymes.

A characteristic example of these kinds of enzymes is hexokinase. Hexokinase phosphorylate hexose to a hexose phosphate through involvement of ATP, a very characteristic enzyme.

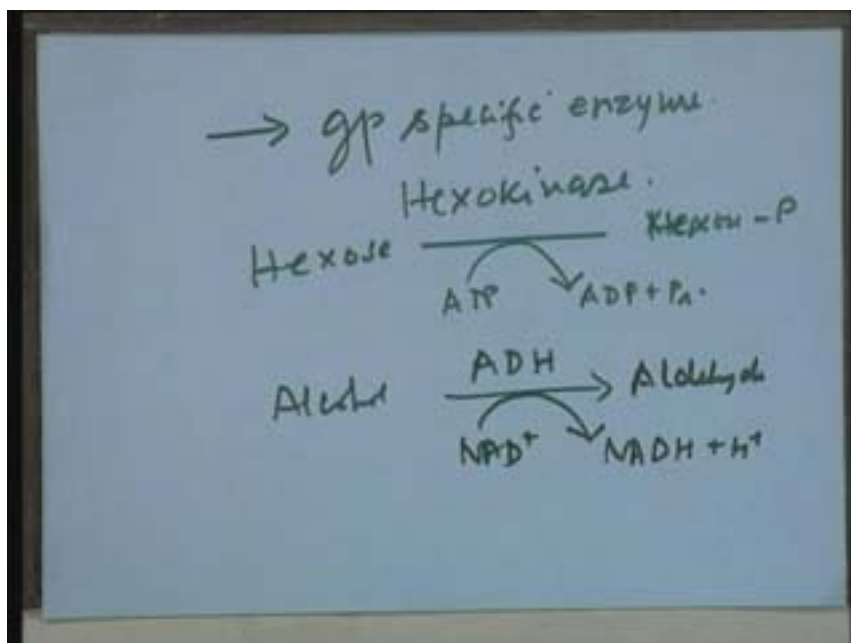
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This enzyme particularly will be able to act upon a large variety of hexose as a substrate and it can phosphorylate. It is not very strictly specific in that sense and we call it as group specific. That means it is involved in the transfer of a phosphoryl group from ATP to a given range of substrate.

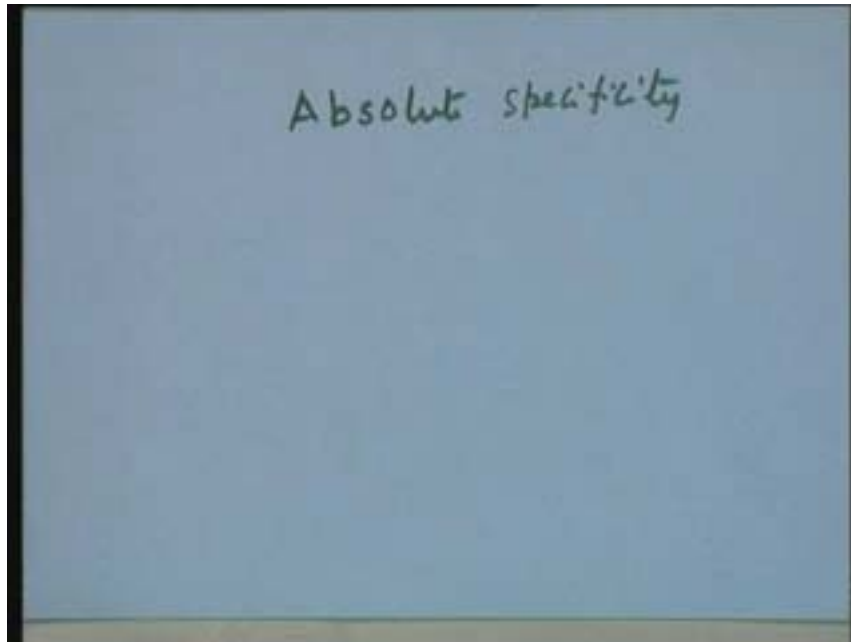
Similarly in the case of alcohol, an alcohol dehydrogenase which also involves NAD gives the corresponding aldehyde.

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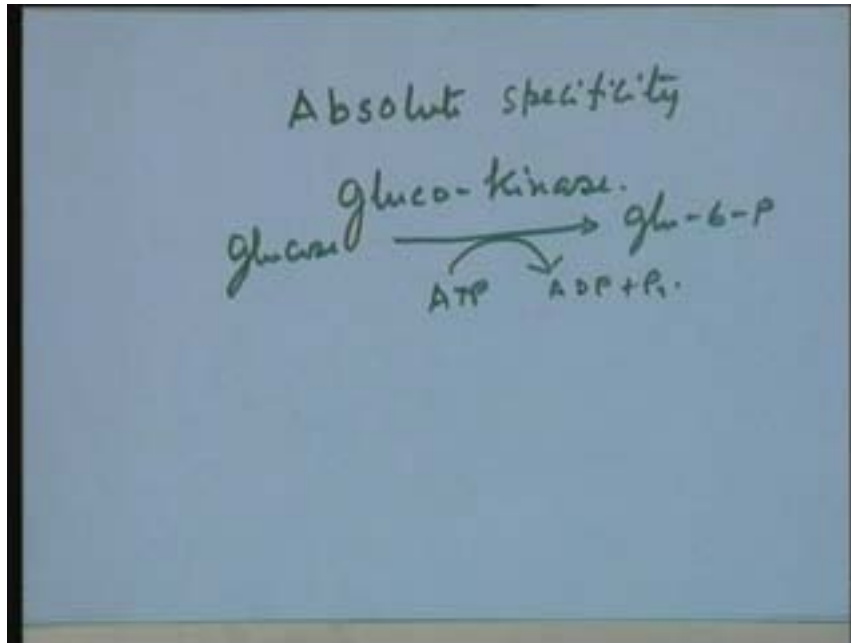
Another example of a group specific enzyme, whereby the variety of alcohols particularly primary alcohols are oxidized to their corresponding aldehyde involve NAD, NADH system as a cofactor. These enzymes are often involved in many of the constitutive functions of a living cell where the cell wants a very broad specificity and that's why the nature has evolved these group specific enzymes. There are certain enzymes which are highly specific and exhibit absolute specificity. When I say absolute specificity I mean that the enzyme can recognize only one substrate.

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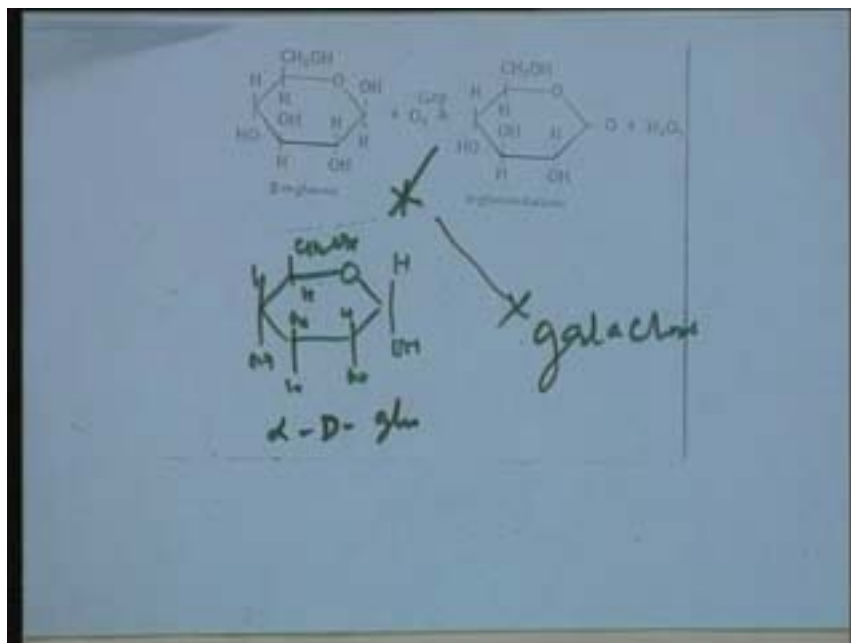
If you consider the same class of hexokinase a very closely related enzyme is what we call as gluco-kinase. This accepts only glucose as a substrate and phosphorylate to gluco-6- phosphate. No other excess sugar is acted upon by such an enzyme. They show absolute specificity.

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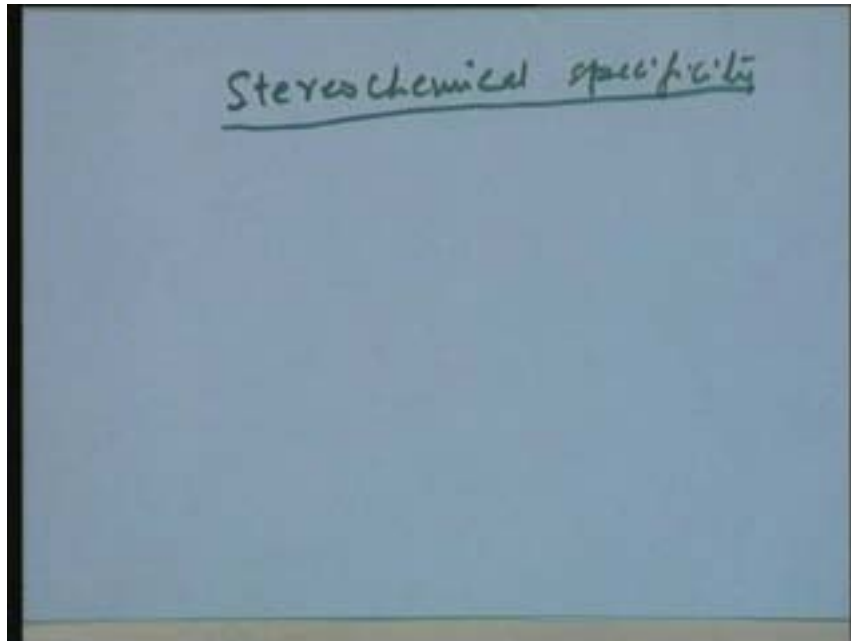
The most absolute specificity in terms of an enzyme, with which some of you may be familiar, is oxidation of  $\beta$ -D-glucose to glucono lactone by glucose oxidase a fungal enzyme which is produced by *Aspergillus niger*. This acts on not only on D-glucose, but it accepts only  $\beta$ -D-glucose and  $\alpha$ -D-glucose is just not accepted. If you consider  $\alpha$ -D-glucose the particular enzyme doesn't act or for that matter even if you go to another isomer of glucose say galactose it doesn't act on even galactose, which is not very different, excepting that these hydroxyl and hydrogen atoms are inter changed.

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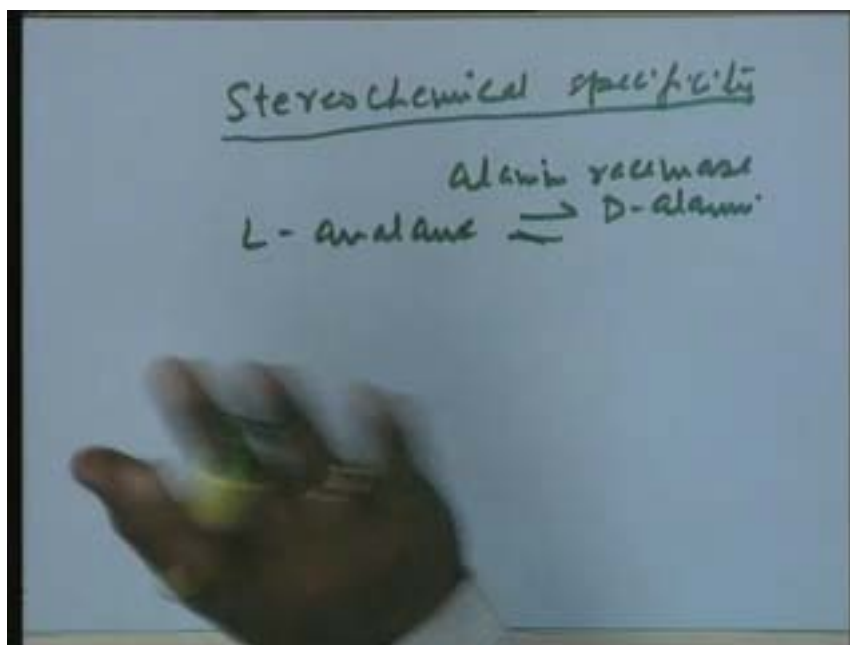
These are the types of the enzymes which fall in the category of absolute specificity. Then we have a number of enzymes which show stereo chemical specificity. That means they recognize the arrangement of atoms at a carbon atom at which stereo isomerism can occur. There are large varieties of enzymes. If you consider the example of let us say lipase.

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Lipase from various sources can exhibit specificity, only one of the isomer either l or d isomer. There are a large number of such examples in the chemical literature or the bio chemical literature. The only enzyme which is known to act on both the stereoisomers or the enzymes whose function is to interconvert L and D isomers are usually called as racemases. For example L-alanine to D-alanine. While alanine racemase can act on either side of the reaction, it is a reversible reaction and therefore it can act on both L-alanine as well D-alanine.

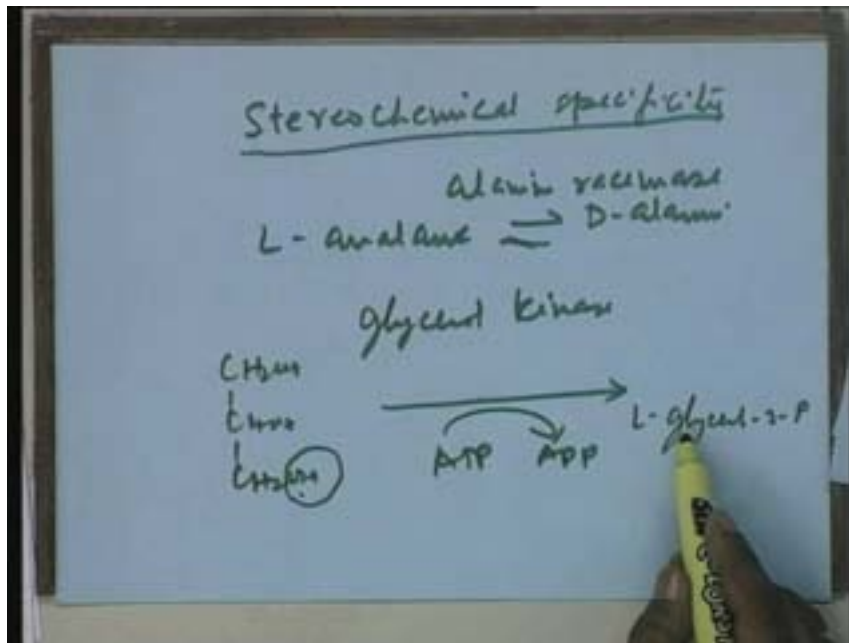
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This is the only one classical example where an enzyme can act on both stereoisomers. Otherwise most of the enzyme will exhibit stereochemical specificity and if there is an asymmetric carbon atom it will only accept one of the isomers and not the other.

The stereochemical specificity can extend to the level that enzyme acting on a non asymmetric molecule can produce one of the isomers as a product. The typical example is glycerol kinase. Glycerol as we know is  $\text{CH}_2\text{OH}-\text{CHOH}-\text{CH}_2\text{OH}$ . It is a non-asymmetric product. The carbon atom in both the  $-\text{CH}_2\text{OH}$  are identical. But the enzyme acts on glycerol molecule and produces L-glycerol-3-phosphate. That means the enzyme transfers again the action of ATP to ADP. The enzyme transfers phosphoryl group to the third carbon atom of the glycerol molecule. Although the one and three are identical still it is able to exhibit the stereochemical specificity in terms of product formation.

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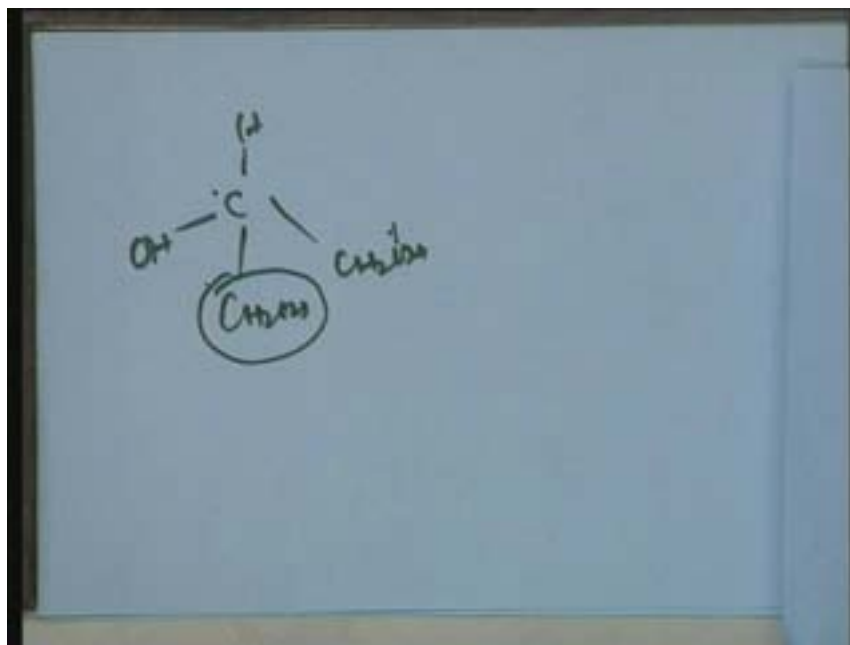


What we must appreciate is that the variety of specificity that can occur in the case of enzymatic reaction can be very loosely specific in terms of group specific enzymes, absolute specificity with respect to a single substrate; it can be stereochemically specific with respect to substrate or with respect to product. Therefore in summary the enzyme will exhibit specificity both with respect to substrate as well as with respect to product.

As I explained earlier the glycerol molecule is a symmetric molecule. There is no asymmetry in this. But the enzyme glycerol kinase acts only on the third carbon. You can analyze the product and see where the phosphoryl group has gone. That's why I'm saying first and third are similar but the enzyme transfers phosphoryl group on to the third carbon atom. Consider the structural conformation of glycerol. This is one, two and three and this acts only on the third one which is on the left hand side.

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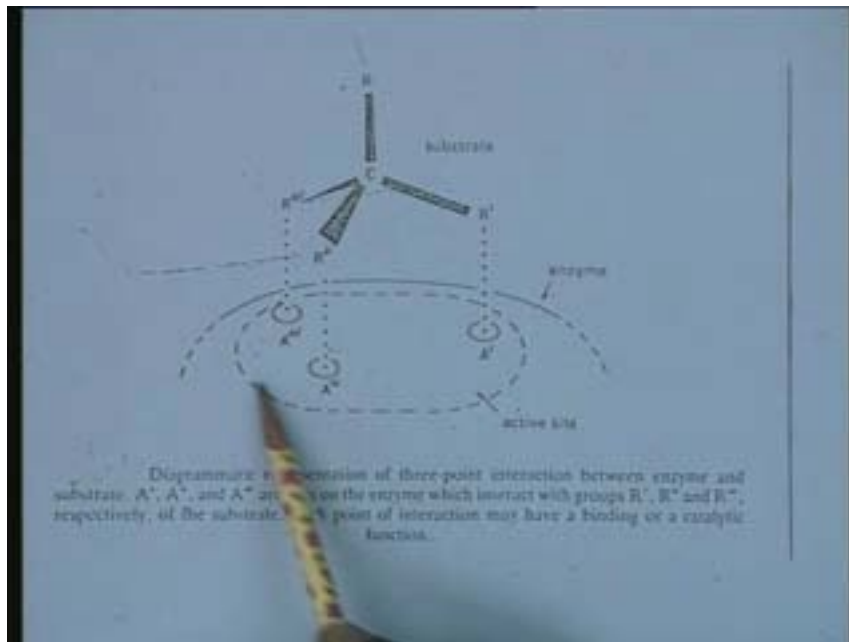
We are expressing here our conformation on a planar diagram. When you look at a three dimensional space, in a three dimensional space the one and three will not be in the same plane. The specificity can have a very large variety and one must also appreciate that the specificity of these enzymes in terms of their catalytic action is attributed to the active sites. As we also saw in the last lecture where the catalytic function was studied, the catalytic functions of the enzymes are responsible for the role played by active site. The specificity also is the result of the conformation of active site. We also saw that the active site will include the binding process. That means certain functional group on the substrate will bind to certain active sites and the active sites will have certain catalytic sites which will interact with the functional residues on the substrate molecule which has to undergo change, the bonds that has to be broken. The active sites must be considered as consisting of two different parts: the binding sites and the functional sites.

Another factor of stereospecificity is that prediction of stereospecificity by any ground rule is difficult. We are able to know the specificity only while we determine the amount of product formed on a particular substrate. In the case of a chemical reaction to take place you have  $\Delta G$  value, the free energy change of the reaction, which will give you an idea whether the reaction will take place in the forward direction or reverse direction or there will be an equilibrium established. But in the case of specificity of enzyme action the only way to understand the specificity is that we monitor the product formed on the substrate and then we can say yes this is specific to the substrates. Once we find the specificity then we can propose hypothesis or mechanisms by which the stereo specificity can be explained. But there are no other thermodynamic or kinetic interpretations which can be applied to the specificity. That is another feature.

One of the most interesting features of enzyme specificity is their stereochemical specificity because when there is a single molecule the functional groups are different, active sites also may have different functional groups to bind them and the steps can be

understood. But to understand the stereochemical specificity that how enzyme is able to identify the two different stereoisomers, the minimum requirements on the function of an active sites was proposed by Oxten as early as 1948. He proposed that at least there must be three sites of different points of interaction on an enzyme active site.

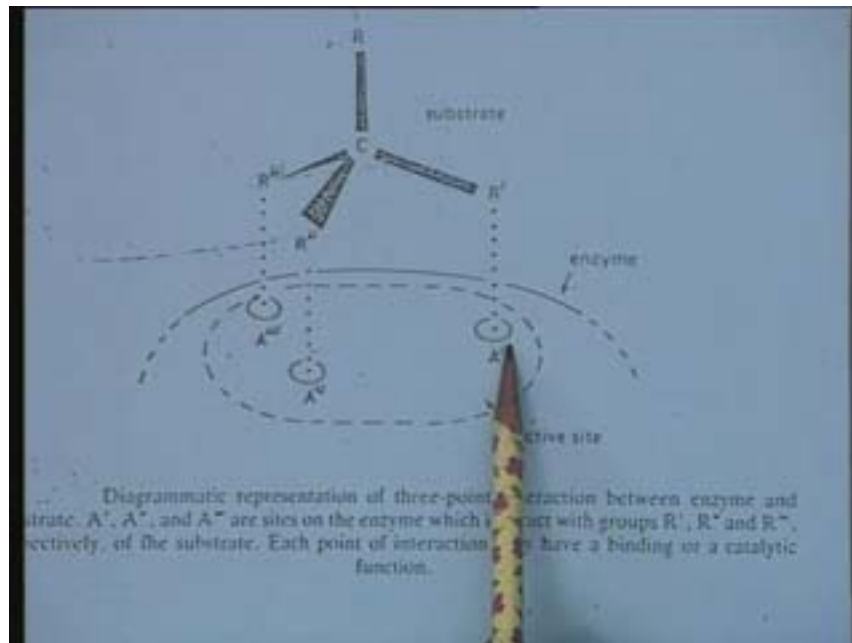
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Here the bottom one is the whole enzyme molecule and the dotted line represents the active sites. I must again remind you that this is not a planar site. It is a three dimensional structure on the surface of the enzyme molecule and not that all the amino acids on these active sites are located in proximity in the amino acid chain. They are brought in proximity by folding of the enzyme protein. Let us say a substrate has to undergo reaction which has three functional group represented by R', R'' or R''''. At least it is understood that as proposed by Oxten that the enzyme active site also must have at least three different functional residues on the active site which can interact with the substrate molecule.

These three interacting sites let us consider that they are the interaction sites. Minimum two of them must be binding sites, out of the three at least two on which the substrate molecule can bind to the active site and put the substrate in a very fixed orientation. We have two points. We are holding the enzyme molecule bound to the substrate. This binding has to be oriented in such a way that the third functional group on the substrate which has to undergo reaction is brought in vicinity of the catalytic site.

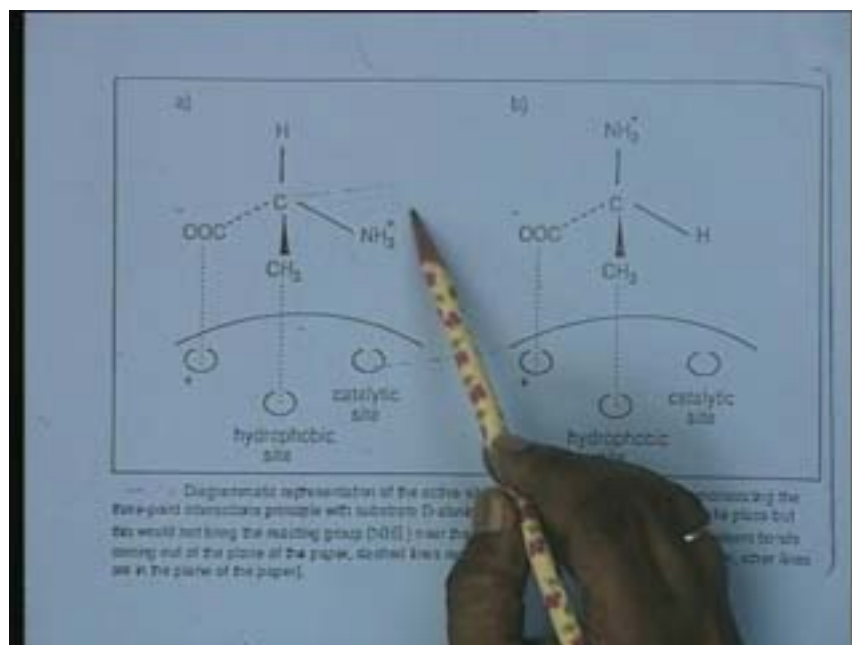
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That is one minimum requirement for explaining the specificity of enzyme action that there must be minimum three sites which interact between the enzyme and substrate. Two of them must be at least binding sites and the substrate binds on these two sites to the enzyme molecule whereby it orients the substrate molecule along with the enzyme such that the reactive functional group and the reactive site on enzymes active site come in proximity or in vicinity. Of course the various mechanisms that we discussed it could be a strain on the bond, it could be the entropic factors and it could be various covalent interactions or electro static interactions that might play a role to carry out the catalysis. But as far as the binding is concerned, it has to be specific in terms of bringing the functional group on which the reaction has to take place.

I will try to explain the same points with a particular example of oxidation of a D-amino acid, using D-amino acid oxidase where you have this amino group which undergoes reaction.

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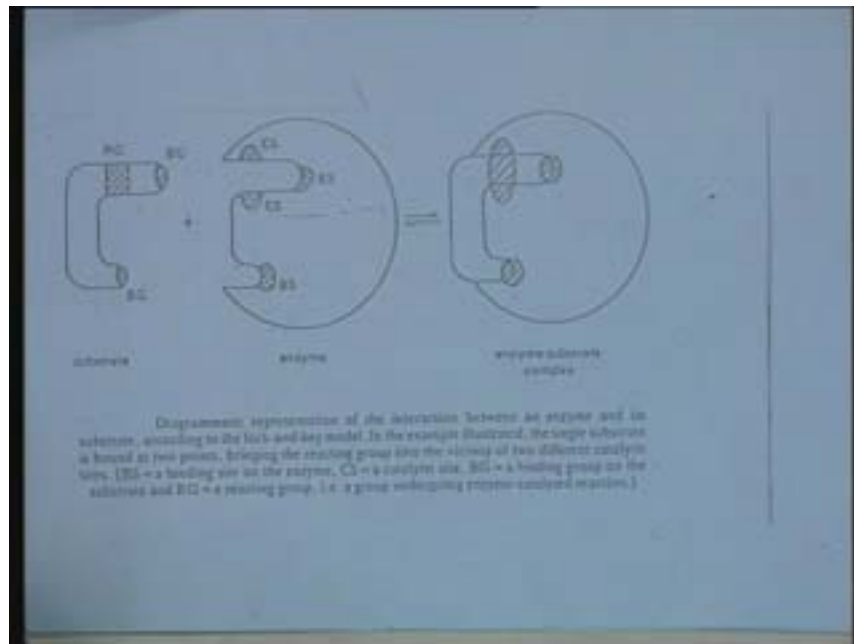


In the molecule the carboxylic group and the methyl group are bound onto the enzyme active site. The one which binds methyl group will be hydrophobic in nature and this will be bound predominantly by electrostatic interactions. These two binding brings the catalytic sites in close vicinity of the amino group which is available for reaction and the catalytic reaction can take place. This is your D-amino acid. You consider corresponding L-amino acid on which the location of H and amino group will be different. Even if the binding takes place this amino group cannot come in the vicinity of the catalytic site, cannot approach the catalytic site and no reaction can occur. That is why the concept of three interacting sites fairly explains the position of enzymes catalytic action as far as their specificity with respect to stereo isomers is concerned. The situation in the case of different substrates which can have different functional groups can be much simpler. Because the binding site will bind two of the functional group, the third functional group which has to undergo reaction will be only recognized by the catalytic site and no other functional group will be recognized and so reaction cannot take place.

One of the very early understanding that as early as 1890, Oxten proposed such a mechanism which puts a limit just like in the case of rate of a reaction we have a thermo dynamic limit. If there is a reversible reaction, these are the kind of limits like equilibrium constant will be achieved at this point and so on. Similarly Oxten proposed a limit that minimum there must be three points of interaction. In case of very hard core specific enzymes the point of interaction can be more than three also. Instead to two binding sites there can be three binding sites also. That depends on the requirement for the enzyme.

As early as in 1890, Fischer proposed Lock and key hypothesis which explained the enzyme specificity. This explanation of the specificity was purely based on certain observations and analysis of the situation mostly mental and not analytical because not many analytical facilities were available.

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It was suggested that the enzyme active site and the substrate have a conformation which is analogous to lock and the key. That means the ..... (23.24) on the key are exactly fitting to the site at which it has to interact with the lock. They are exactly fitting.

Here you have the active site, the sites on which the binding groups available on the substrate binds and this portion shows the substrate which is analogous to the key. This is the active site which is analogues to the lock. The binding sites on the substrate come in proximity or bind to the binding site on the active site of the enzyme molecule. Similarly the reactive group on the substrate comes in contact with two catalytic sites and then once the reacting groups gets entangled with the reaction sites on the active sites, the binding is complete and it can exhibit the catalytic function to take place.

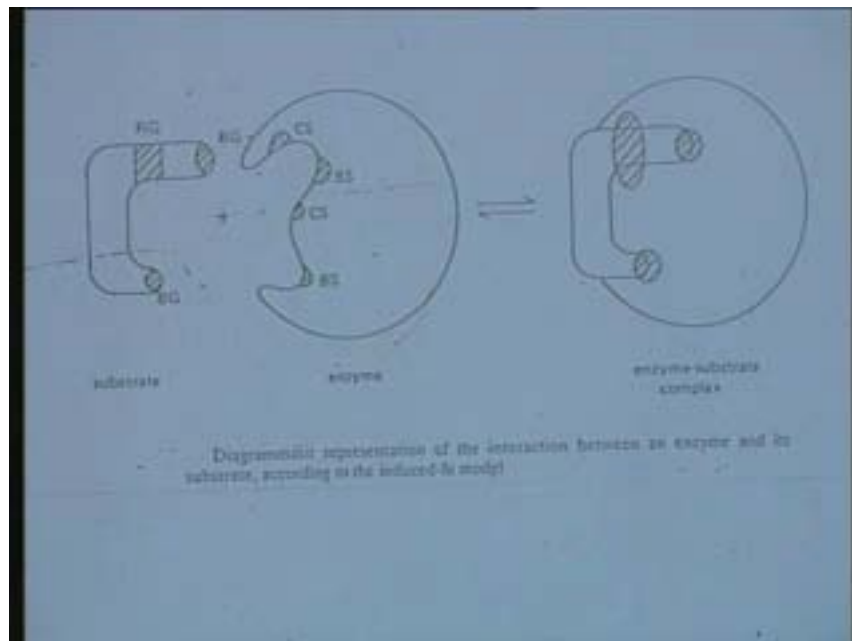
Now that was the understanding and they also very clearly understood at that time that both substrate as well as the active site on the enzyme molecule have rigid structures. There is no question of change if they are rigid in nature. They are just like a lock and key; there is no flexibility. If there is slight modification either in the conformation of the key it will not fit to the lock and thereby the reaction will not occur.

In fact the Fischer's lock and key arrangement was not based on any of the analytical probes. It was only a conceptual explanation given because at that time, one thing we must appreciate that, not a very large number of enzyme reactions were understood. Even the stereospecificity was not clearly understood. It was mostly some very common reactions like hydrolysis of sucrose to glucose and fructose, most of the hydrolytic reactions were understood and this mechanism proposed only such a macro

understanding that the substrate and the enzyme have rigid conformations which are complementary to each other and which fit in strictly. At the time of Fischer's Lock and key mechanism the most understood enzymatic reaction was hydrolysis of sucrose by invertase to glucose and fructose and obviously they would look into variety of other sugars like lactose on which the enzyme could not act. They could distinguish between the conformations of the substrate molecule and have much detailed information.

But still the surprising thing is that the concept is not totally invalid today. In many cases it can still explain the whole theory or whole requirements of specificity. Then came number of other developments and particularly the development with respect to the availability of analytical data on enzyme substrate binding. People were able to make enzyme substrate complexes and their analogs, analyze them by NMR or EPR or variety of analytical physical tools and they found that when the enzyme and substrate do bind there is a significant change in the conformation. The conformation is neither the same what was available in the case of enzyme molecule. So there is something going on and keeping those data in view Koshland proposed a hypothesis as late as 1958 what we understand today as Koshland induced fit model. His proposal was a slight modification of the lock and key arrangement.

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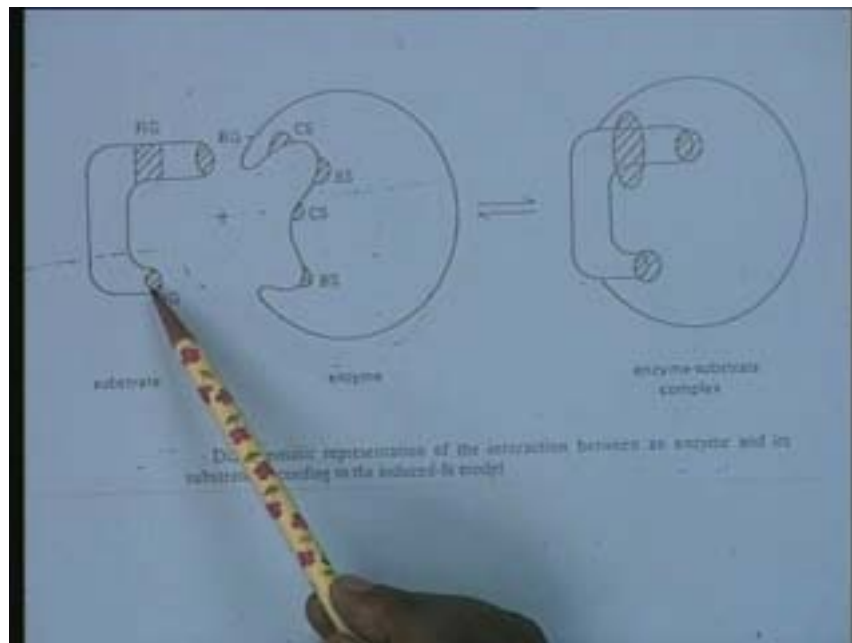


He said that the substrate has a rigid conformation with its binding sites and the functional site or the reacting groups present at a particular conformation. But the enzyme active site is not a rigid conformation. It's a flexible conformation. That means as soon as the substrate comes in the proximity of the enzyme active site, initial binding takes place. The enzyme active site binds onto the substrate molecule.

For example this is a functional group and this let us say is substrate. The enzyme active site as soon as it comes in proximity, it wraps around the substrate molecule. He gave an example that the interaction between the substrate and the enzyme is some thing like a hand and the woolen glove. You wear a woolen glove. Your hand is analogues to substrate. Then when you put in the woolen glove it exactly fits into hand depending upon the conformation of your fingers. Everything goes in line and interacts with different fingers and one can be comfortable and the reaction can proceed.

The basic difference was while the lock and key mechanism or hypothesis proposed both the components to be rigid, in the Koshland induced fit model it was very clearly understood that the substrate may be rigid but the enzyme active sites are not rigid conformations as you can see here. For example you have two binding sites.

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The free active site on the free enzyme molecule without interaction with the substrate has its particular configuration where the catalytic sites and binding sites are distantly located. As soon as the binding groups come in contact with the binding site as they have interacted here the active site undergo a change in conformation and this catalytic sites are brought in proximity so as to wrap around the reactive group on the substrate molecule and then the ultimate conformation of the enzyme substrate complex which can use one of the mechanism by which catalytic reaction can take place, can be applied. Such a mechanism very effectively can explain the high degree of the specificity for the enzyme molecule. In the case of induced fit hypothesis one can also appreciate that in certain reactions for example even if there is reaction contaminant in the reaction mixture which are very similar to nature in the terms of their conformation to the actual substrate, they will not be able to interact. For example in the case of lock and key mechanism if there are molecules which are similar in conformation they might be able to partly

interact with the catalytic site and influence the reaction. Whereas in the case of the induced fit mechanism once the enzyme active site is totally wrapped around the substrate, the reaction sites are not at all available for any other contaminant and therefore there is no chance of any other contaminant present in the substrate molecule to be able to interact with the enzyme molecule.

One of the most typical examples of enzyme catalyzed reaction which was responsible for the elucidation of induced fit model was the hexose kinase. The phosphorylation of D-hexose at the expense of ATP to hexose-6-phosphate. In the absence of hexose, the ATP hydrolyzed extremely slowly. The hydrolysis of ATP is a spontaneous reaction but in the absence of hexose it will hydrolyze only very slowly. The hexose is present in the proximity of the enzyme, the enzyme will bring both hexose and ATP along with the binding sites and then it can flop around the two molecules and the reaction can take place in a very fast speed.

The induced fit mechanism can also explain very clearly the bimolecular reactions which were difficult to understand as far as the lock and key arrangement. Lock and key arrangement was very easily able to explain the unimolecular reactions. But in case where two substrates are involved as in the case of hexose phosphorylation, the role of hexose and ATP both participating at the binding site for the reaction to occur could be explained using induced fit mechanism.

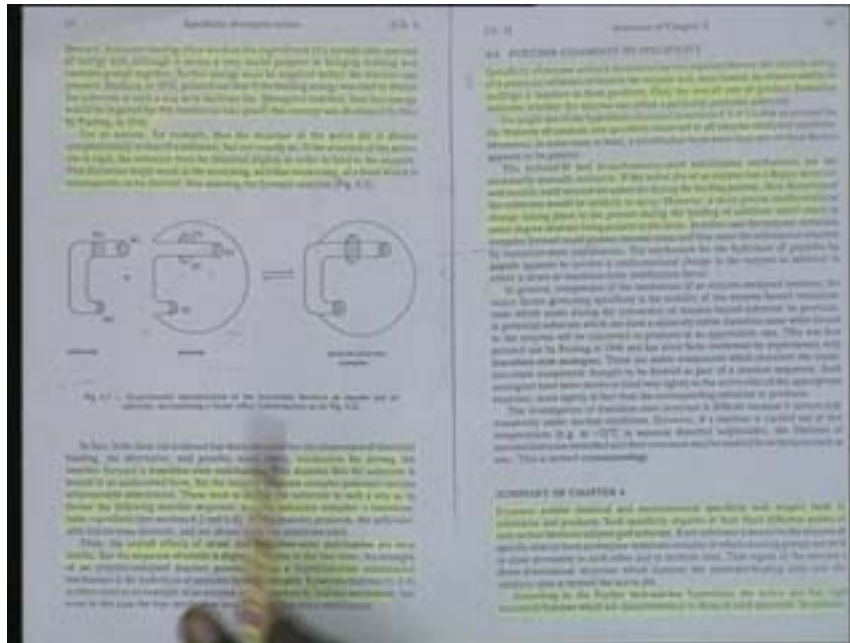
If you consider all the three explanations that we have considered today like Oxten's three site interaction model, lock and key mechanism of Fischer or induced fit hypothesis of Koshland, when we talk about the specificity they don't link specificity by any means to the catalytic function. They consider specificity totally in isolation. They only say that after all these interactions have taken place some of the reaction mechanism responsible for catalytic function will take place and the reaction is catalyzed. None of them is able to provide a suitable explanation for the catalytic function.

When we discussed the various ways in which the enzymes are able to catalyze reactions we understood that binding of the enzyme to the substrate was a very important feature in the acceleration of the reaction rate. Binding of the substrate to the enzyme involves some expenditure of energy that is the binding energy involved in it which ultimately gives you an advantage in the reduction in activation energy and there is an initial energy. It is also presumed that this binding energy is not totally a wasteful energy for the enzyme reactions.

A part of this binding energy also contributes to specificity and therefore if you look at the effect of strains that are developed, a hypothesis which is called as a strain that means at the stage of binding we understood that in the case of lock and key mechanism they are rigid.

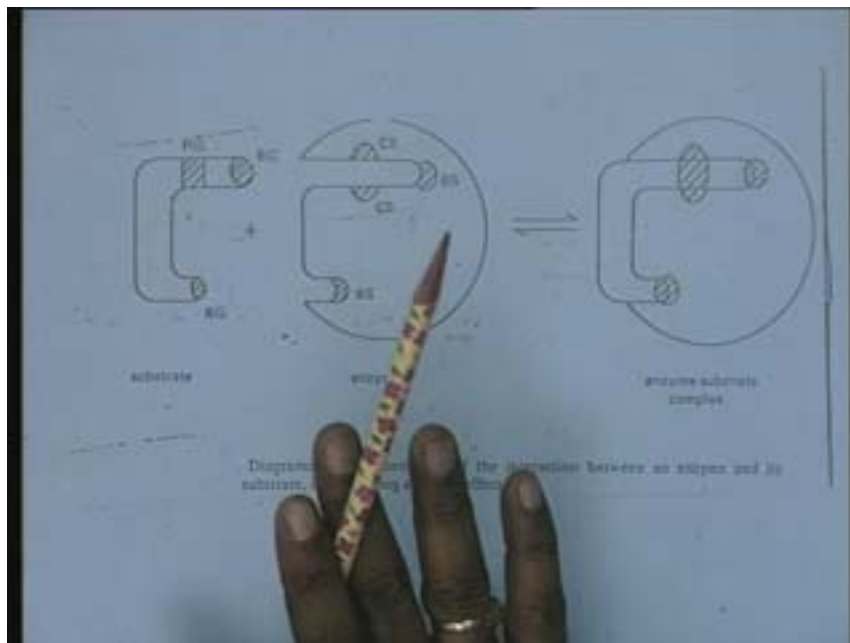
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But we might assume that the active site is almost complementary to the substrate molecule but is not exactly complementary, a situation which is slightly closer to lock and key mechanism not in absolute terms that they are complementary but they are not exactly complementary.

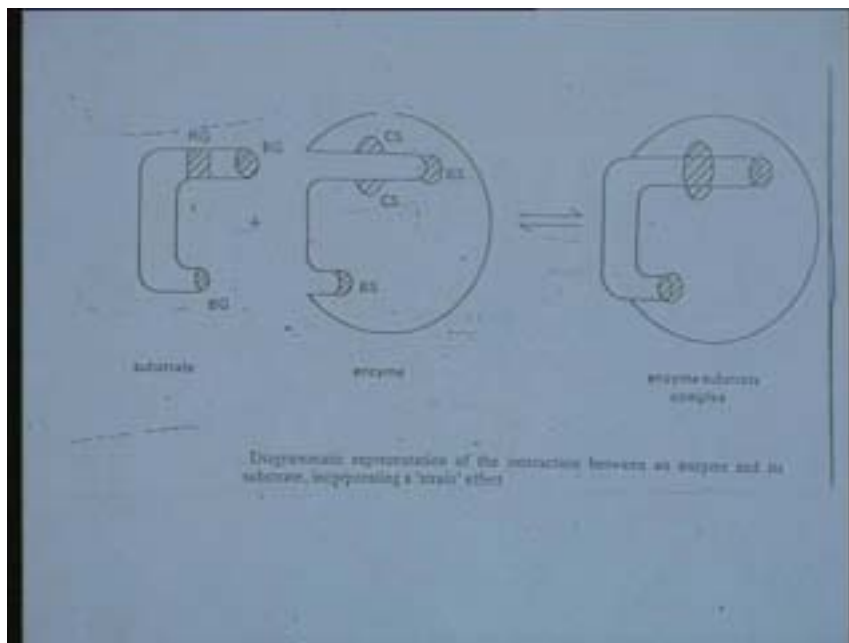
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Therefore when the substrate and the enzyme active sites are brought in proximity and the binding takes place between the substrate and the enzyme active site it brings in a strain. You see that the active site here unlike the lock and key mechanism is not exactly

complementary. This slot is slightly longer and therefore while the binding takes place it makes a sort of a strain onto the reactive group and it is analogous to the mechanism which we proposed for catalytic function that means strain mechanism and which contributes to reduction in the enthalpic component of the free energy of activation. During this interaction of the enzyme active site with the substrate, the strain produced onto the reactive group as a result of binding process is responsible for specificity as well as part catalytic function. These strain developed may contribute towards the reduction in activation energy but it is a result of binding process.

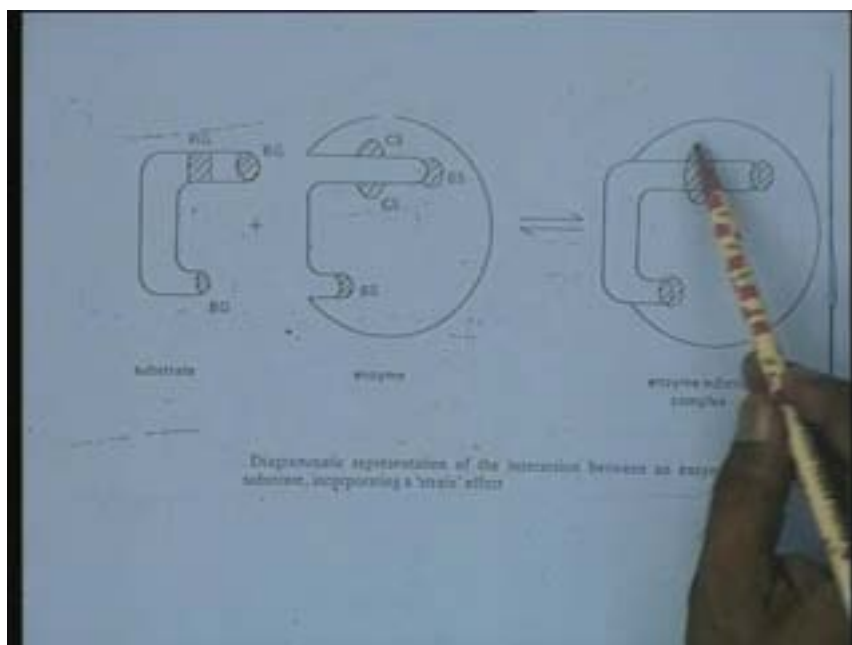
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This binding process brings in the specificity and the nature of the binding site or the active site on the enzyme molecule is slightly complementary or of the similar type but not exactly complementary and the complementarity comes only after binding has taken place which results in the distortion of the reacting group and then the complementarity comes in. So that is called the strain effect which can take into account both catalytic function as well as specificity.

The strain effects have been discussed when we were talking about the ways in which the enzyme reduces energy of activation. The strain on the reacting group was one of the mechanisms. Because the active site is not exactly complementary, after the substrate approaches the binding sites it forces the reacting group to develop a strain so as to bring in complementary to the catalytic sites. Then only these catalytic sites can combine.

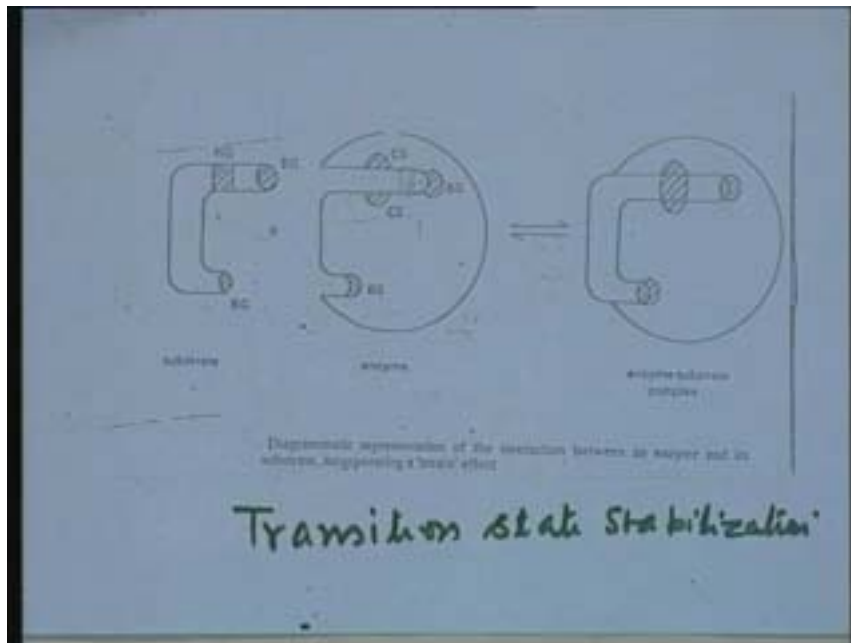
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Otherwise if it was totally complementary without the strain then the reacting group will come down some where here and no reaction can take place. Only when a strain is developed the reaction takes place and the reaction takes place with the suitable degree of specificity introducing to it. Because the binding has to take place and the binding process requires a strain onto the substrate molecule so that it comes in proximity of the catalytic sites. If strain is not developed it will remain in the binding site but not at the active site. The enzyme will not be able to provide the catalytic function. Like in the case of other mechanisms or other hypothesis proposed for specificity while we are able to explain that binding will take place, the functional group comes in proximity to the reacting group but it doesn't explain if the reaction will take place or not. Just coming in proximity could provide you an effect what we discussed like high substrate concentration in the proximity of the active site but does not explain you how the energy of activation is induced. Here in this case this also explains how the energy of activation is reduced in terms of its enthalpic contribution.

Another thing which is very well related to this strain mechanism is the mechanism what we understand as transition state stabilization.

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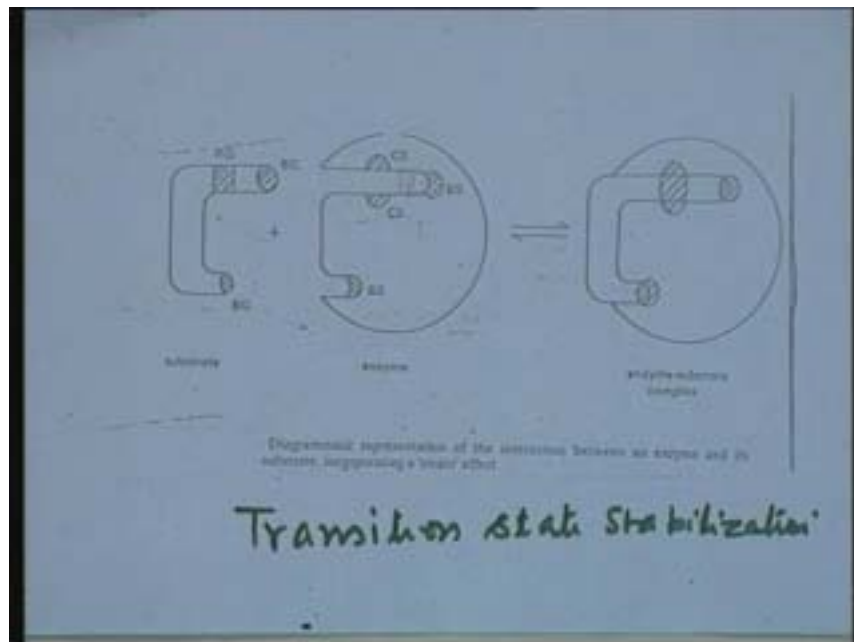


Here in the case the effect is almost very similar to what we call as strain effect but while the substrate enzyme binding takes place at the active site there are still some unfavorable interactions at the active site of the substrate. That means the substrate molecule in the vicinity of the active site is not very comfortable in terms of its interactions. There are many other unfavorable interactions available at the active site and these interactions tend to distort the substrate in such a way so that it favors the formation of a complex which is analogous to structure in transition state. The presence of certain unfavorable interactions at the active site for the substrate when the binding has taken place tends to distort the substrate molecule in the direction so that the enzyme substrate complex tends to become complementary to the transition state.

In the first characteristic feature of catalysis we saw that the transition state is complementary to enzyme substrate complex and not the substrate. The enzyme is complementary to enzyme substrate complex, the enzyme active site and not to the substrate itself. Therefore the role of the binding of substrate molecule to the enzyme at the binding site, the substrate doesn't remain very comfortable. There are many unfavorable interactions that is present there and they distort the substrate molecule so that these enzyme substrate binding approaches towards that of transition complex.

The first step is binding of the binding sites onto the active site. Once that takes place, still the catalytic sites are not approached by the functional groups which are likely to undergo reaction. But in addition to the binding site there are many unfavorable interactions present between the substrate and the active site. Consider three different functional groups. In addition to that on the active site there will be many more amino acid residues which will be in the proximity and they might provide some kind of unfavorable interactions.

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Now these interactions tend to distort the substrate and this distortion of the substrate is favored towards reaching the transition state. In fact the basic role is played by the unfavorable interactions present in the active site. In other words we are developing a strain onto the substrate molecule so that the substrate molecule reacting groups are coming in proximity to the catalytic site.

In the hypothesis of the transition state stabilization it is stated that the unfavorable interactions available at the active sites on the substrate favors the distortion of the substrate in such a way that the final enzyme substrate complex approaches the structure of that of transition state. The energy required to reach the transition state is already reduced. Because partly you have already taken up that path. That is what happened in strain development also. Part of the energy required for energy of activation is already met by strain onto the substrate molecule.

So these are some of the major mechanisms that have been proposed for explaining the specificity of the enzyme action. It is over all rate of reaction in terms of rate of product formation that indicates whether the enzyme is specific to a substrate or not. What is the order of specificity, to what extent it would be specific, what are the impurities which can interfere in the specificity they are all related to the results of reaction that is the rate of formation of product. The specificity also has a very significant role in terms of the industrial applications of enzymes. We must also appreciate that while in the case of the catalytic function we understand that if the rate of reaction is increased its usefulness as a industrial catalyst is also very high because higher the rate of reaction you can very efficiently use it for industrial purposes. But what is the role of specificity. The specificity in fact has both negative as well as positive role. Positive role in the sense, that you can restrict the formation of any of the by-products. Even if your feed history substrate is impure to some extent you will know that only reaction which can be

catalyzed specifically by that enzyme which you are providing will take place and no other reaction will take place. Even the side products formed by action of water usually most of the organic chemists consider water for organic chemical reactions as the culprit because it can lead to variety of side products. So all the side products can be absent, which can make our life comfortable as far as the purification of the product is concerned. That is one of the major advantage but the major disadvantage or drawback from the industrial application point of view is that the specificity requires a single enzyme to be produced for each reaction, as the engineer will appreciate that the benefits of scale of operation for the enzyme production is lost. That means many of the enzymes cannot be produced on a very massive scale because the requirement will be only for single reaction and therefore for a single reaction the requirement will not be very large. So obviously you have to produce them on a very small scale and producing on small scale will lead to high cost of production. That is why the enzymes still remain expensive catalyst compared to chemical catalyst.

For example in the case of chemical catalyst Raney's nickel, it is the catalyst used for all hydrogenation reactions in the organic chemical field and we can produce them in bulk quantities and the cost of production can be reasonably low. On the other hand if you have to produce glucose oxidase, which acts only on  $\beta$ -D-glucose not on  $\alpha$ -D-glucose or any other hexose, its requirement will be only for oxidation of glucose which will restrict its application and therefore the scale of operation. The specificity must be looked in from both the angle and in many cases today people are applying the development of molecular biology particularly in sense of protein engineering to modify the substrate specificity both when we want the absence of side products to make it more specific as well as when we want broad specificity so that the same enzyme can be applied to a large variety of substrates. We are trying to broaden the specificity through protein engineering methods.