ENZYME SCIENCE AND ENGINEERING

PROF. SUBASH CHAND

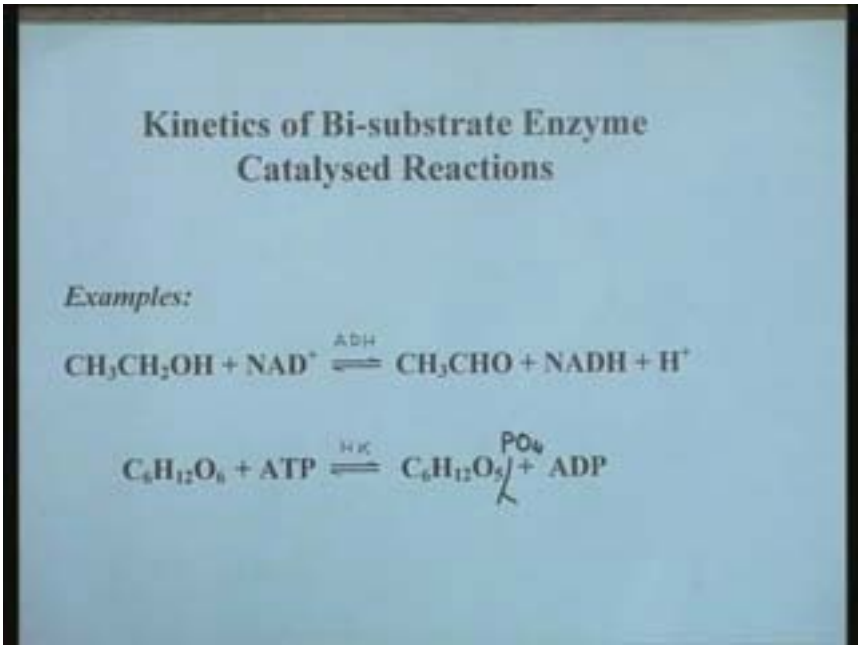
DEPT OF BIOCHEMICAL ENGINEERING
AND BIOTECHNOLOGY
IIT DELHI

LECTURE – 12

KINETICS OF BI-SUBSTRATE ENZYME CATALYZEDREACTIONS

So far we have been talking about the kinetics of single substrate enzyme catalyzedreactions and as we all know that in the living cells a large variety of enzymes interact with the multiple substrates. In fact most of the enzyme catalyzedreaction that do take place in a living cell involves two substrates in most cases.

[Refer Slide Time: 1:31]

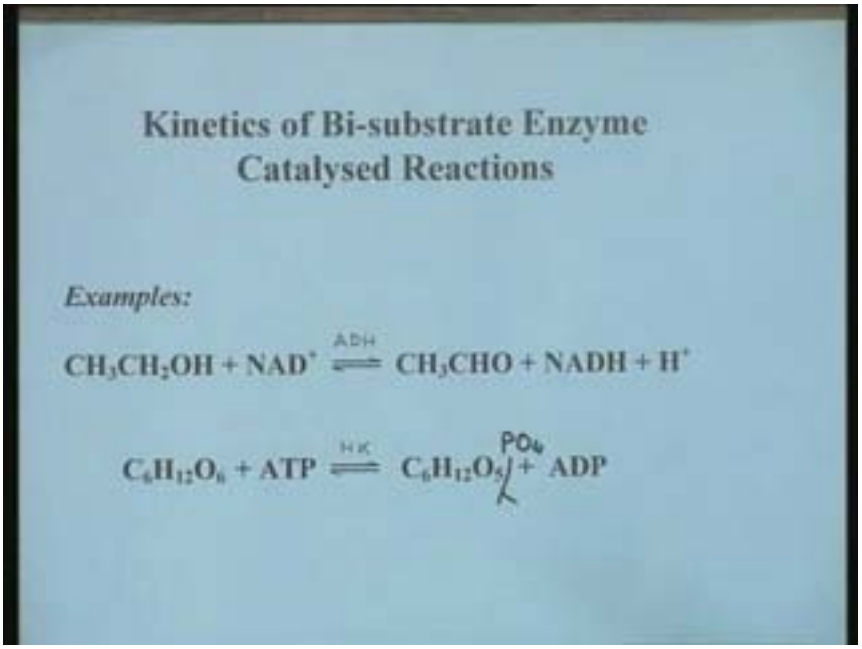


We have also seen that under certain set of conditions the classical Michaelis Menten kinetics which has been proposed for single substrate enzymatic reactions can also be applied to bi-substrate reactions. Certain conditions apply but those conditions are by and large extreme conditions. That is you have to have one of the substrate in a very large excess concentration. Those situations may not really exist in a living cell. In a living cell the concentration of the two substrates that are to interact with the enzyme may be almost in an equal or near by concentration levels. They may not be extremely different and

therefore it is necessary to look at the actual effect of the bi-substrate reactions on the reaction rate.

Two of the examples which I have chosen here are one on alcohol dehydrogenase which oxidizes ethanol to acetaldehyde involving a co-enzyme NAD and the other is hexokinase catalyzed phosphorylation of glucose or any other hexose to the corresponding phosphorylated sugar.

[Refer Slide Time: 2:50]

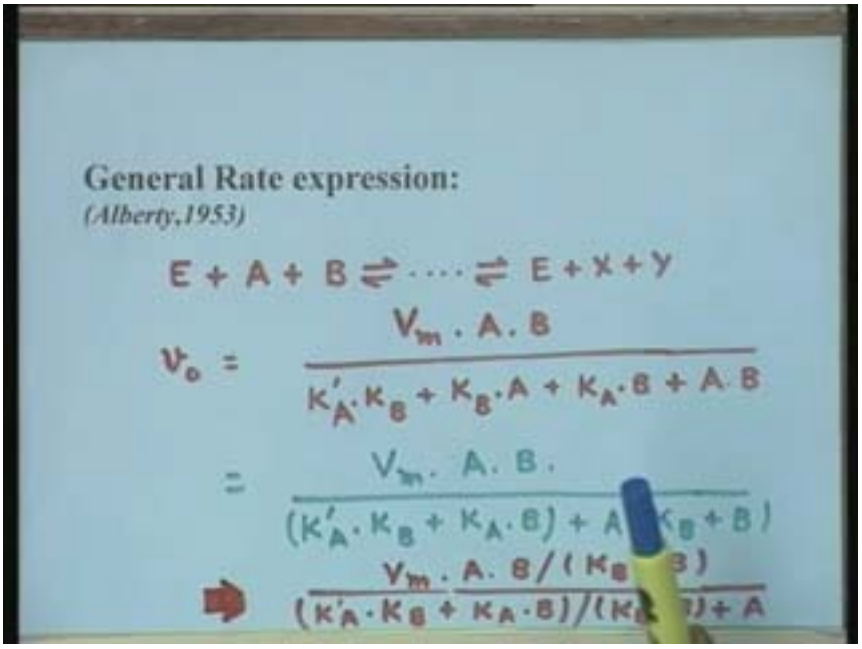


Bulk of the bi-substrate reactions do fall in the category of either oxidoreductases or transferases or lipases. Most of the hydrolytic enzymes isomerizes or often can be analyzed using classical Michaelis Menten kinetics. The oxidoreductases represented by the first reaction or a transferase represented by hexokinase or in many cases the lipases are the real true bi-substrate reactions which in general should not be treated or analyzed with the Michaelis Menten kinetics.

To account for the rate of reaction for such reactions taking example of a typical bi-substrate reaction as I have shown here in general I have left certain dots in between, in between the substrate as well as the product stream. Basically that signifies that the dynamics of the interaction between the enzyme and the substrate molecule may be quite varied unlike in the case of Michaelis Menten kinetics where you have a single substrate and it has to bind on the active site of the enzyme molecule and form an enzyme substrate complex.

Here because the substrates are more than one there are many possibilities for interaction between the substrate and the enzyme molecules and so therefore these dotted figures indicate a variety of interactions that are involved.

[Refer Slide Time: 4:30]



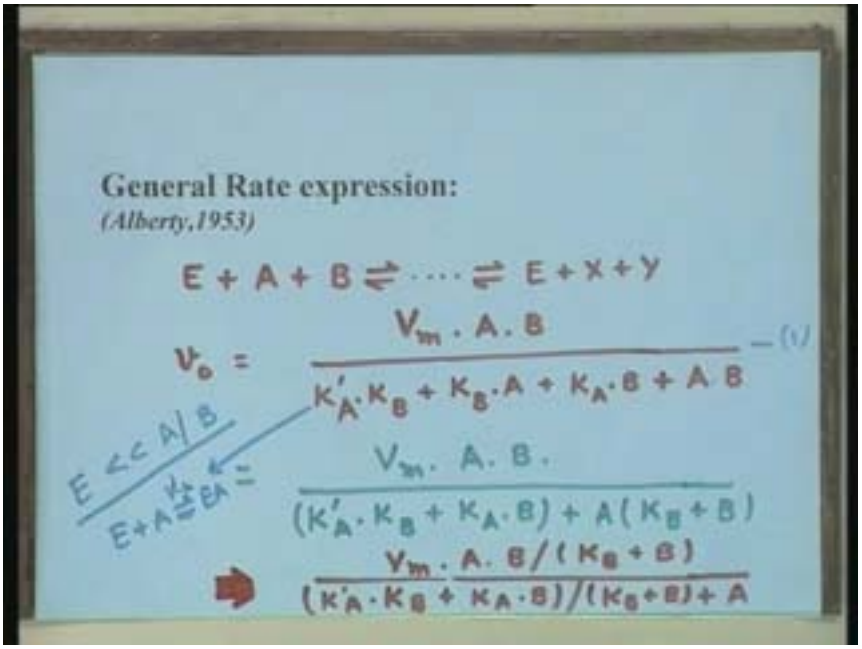
But in general the action of enzymes on a reaction involving two substrates A and B giving rise to products X and Y is expressed here. A generalized rate expression for this was proposed by Alberty based on steady state hypothesis. Again similar considerations as we talked about in the case of Michaelis Menten kinetics. He proposed the initial reaction rate for such a reaction can be given by an expression given here. Let us say this is expression one. That is

$$V_0 = V_m.A.B$$

V_m is the maximum reaction velocity where both the substrates A and B are in saturating concentrations. That means both the substrates are in large quantity and they are able to saturate the enzyme which in concentration terms is much, much smaller than the A or B. The concentration of enzyme is much, much smaller than either A or B. Almost the same assumptions that we made in case of Michaelis Menten kinetics and the A and B can be to a saturating level providing a maximum reaction velocity.

The denominator term gives you another three kinetic parameters; K'_A is the dissociation constant of binding of E to A giving EA. This is dissociation constant and K_B and K_A indicated here are the concentration of the substrates A and B respectively at which the reaction velocity is half the maximum reaction velocity at saturating level of the other substrate.

[Refer Slide Time: 6:37]



That means when we talk of K_B, K_B is that concentration of B at which the reaction velocity V is half of that of V_m at a saturating concentration of A. Similarly for K that concentration of A at which the reaction velocity is half that of V_m at a saturating concentration of B. Or in other words we are analogous to our classical Michaelis Menten constant for the substrate. That means you can consider that K_B is the Michaelis Menten constant with respect to B at saturating concentration of A.

There might be some confusion about K_A and K'_A. K'_A is the dissociation constant for binding of A to the enzyme. KA is the corresponding Michaelis Menten constant with respect to A. This distinction between the two will be more clarified or clear when we proceed ahead but such a situation exists in the case of bi-substrate reactions. In most cases this dissociation constant is different than the apparent Michaelis Menten constant unlike in the case of single substrate reaction where the two are almost identical. You will notice that the apparent Michaelis Menten constant will depend upon the concentration of the other substrate also and this dissociation does not depend upon the other substrate. Out of the several mechanisms, in one of the mechanisms this K'_A may be equal to K and the rate expression will accordingly get modified.

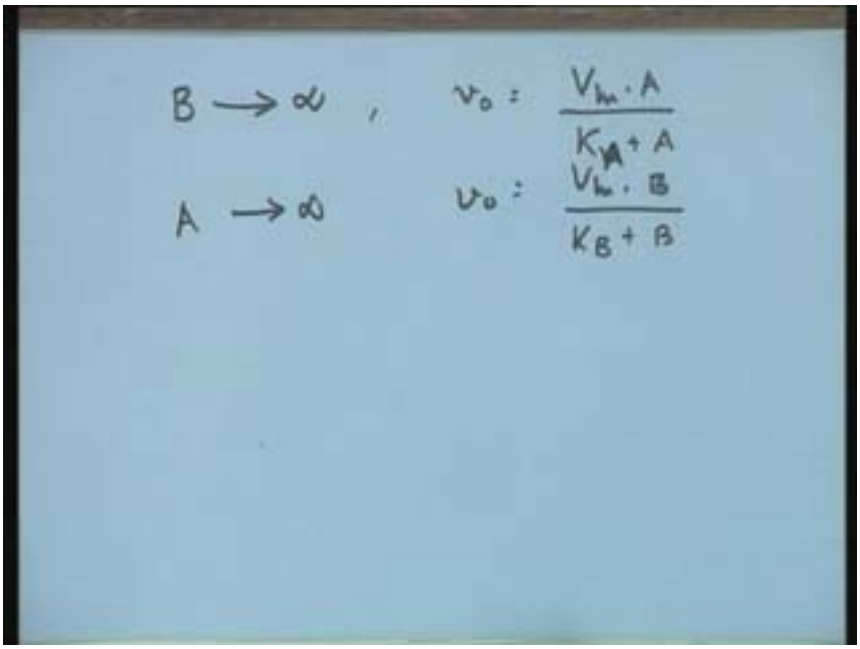
Under those conditions if you take the first expression you will notice that this can also be considered almost like as you mentioned earlier while looking at the validity of Michaelis Menten kinetics for bi-substrate reactions that if we put one of the substrate concentration in excess the expression can represent the bi-substrate reaction kinetically with respect to other substrate. So therefore if let us say B is very large, B tends to infinity then

$$V_0 = V_m.A/K_A+A$$

Both from numerator and denominator divide by V some of the terms will be cancelled and you will end up with a simple expression like Michaelis Menten. Similarly when A tends to infinity analogous expression will emerge

$$V_0 = V_m.B/K_B+B$$

[Refer Slide Time: 9:40]



So you get a similar expression in the case of excess concentration of or large concentration of one of the reactants and that was also the understanding we had when we discussed Michaelis Menten kinetics. You can rearrange this general expression by taking certain factors and divide both and you can end up at the constant concentration of B. If you consider B as a constant at some value of B the expression will turn out to be analogous to Michaelis Menten constant. If this becomes a constant parameter, at constant B you can write

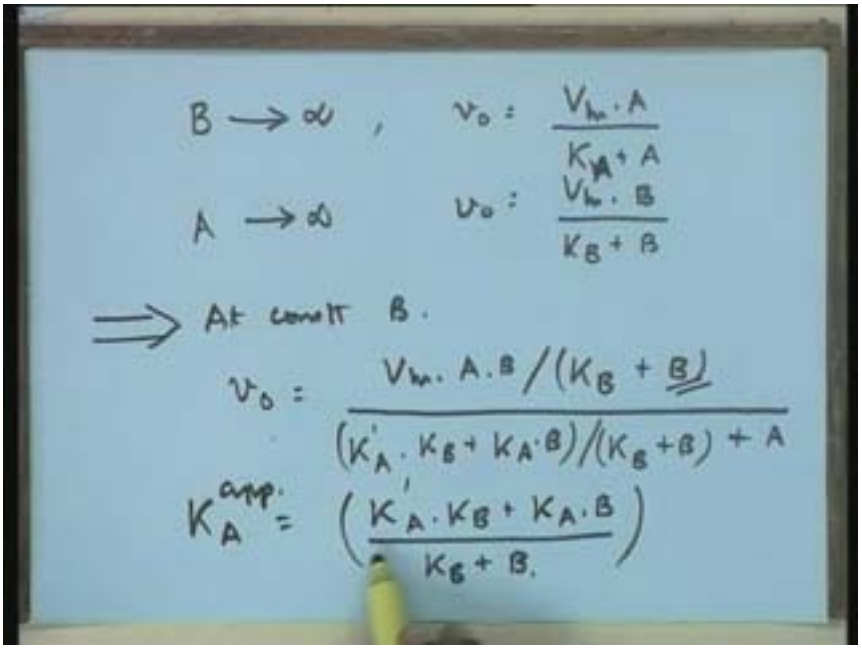
$$V_0 = \frac{V_m.A.B/(K_B+B)}{(K'_A.K_B + K_A.B)/(K_B+B) + A}$$

We have rearranged the general equation in the form of Michaelis Menten expression. So therefore at any constant value of B you can consider that the apparent value of K_m for A K_A is

$$K_A^{app} = \frac{K'_A.K_B + K_A.B}{K_B+B}$$

This is the apparent K_m with respect to A by rearranging this general equation. You notice the relationship between the apparent K_m for A and K'_A.

[Refer Slide Time: 12:02]



Although they are related it also involves the concentration term of the other substrate which is B and a similar relationship like this when you consider constant concentration of A and the apparent K_m for B.

Or in other words in a special case when

$$K'_A = K_A \; ; \; K_m^{app} = K_A$$

The relationship between these two terms implies that when they are same as the Michaelis Menten constant with respect to A it does not interfere and $K'_A = K_A$. It has no effect on the binding of B and so it is independent of concentration of B in the reaction mixture. Similar situation will apply if you consider

$$K_m^{app} \text{ for B} = \frac{K'_A.K_B + K_B.A}{K_A + A}$$

Again here also similarly you will notice

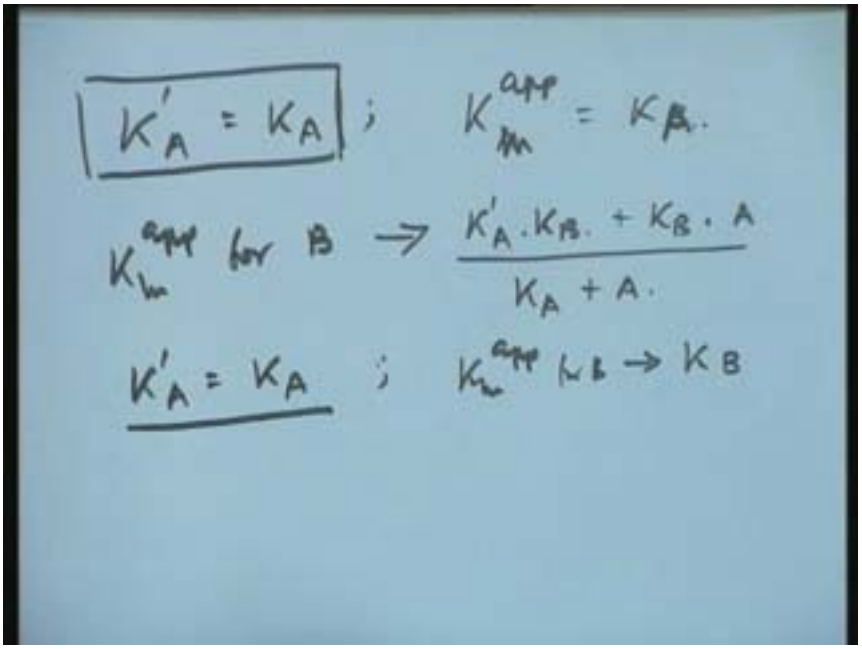
$$K'_A = K_A \; ; \; K_m^{app} \text{ for B} \longrightarrow K_B$$

This here also $K'_A = K_A$. It is with respect to first substrate binding. Under this condition I am trying to give you an analogy or an explanation as to how K_A and K'_A are related. If you consider the single substrate reaction the dissociation constant of substrate with the enzyme is analogous to K_m value, whereas in the case of bi-substrate reaction that situation is not true. That is only a special case of a bi-substrate reaction under which the K_m apparent for B will become equal to K_B when these two become equal. Otherwise this value will be this magnitude although they will have a relationship but they will be a function of other substrate concentration.

The other substrate concentration will influence the magnitude of K^{app}_m in the case of a bi-substrate reaction whereas in the case of a single substrate reaction the K_m value was almost analogous under the assumption that the term turn over number that is K_2 term is not very, very small. That was the only assumption. Otherwise if it is very small they have a difference but if it is not very small the two are analogous.

In the first case this should be K_A.

[Refer Slide Time: 15:50]



You can also look at these expressions in a graphical mode and to have a better visualization. In the first case when we are talking of constant B your profile will be something like this. This is your maximum reaction velocity and your half V_m will be this one where K_A apparent with respect to substrate A will be

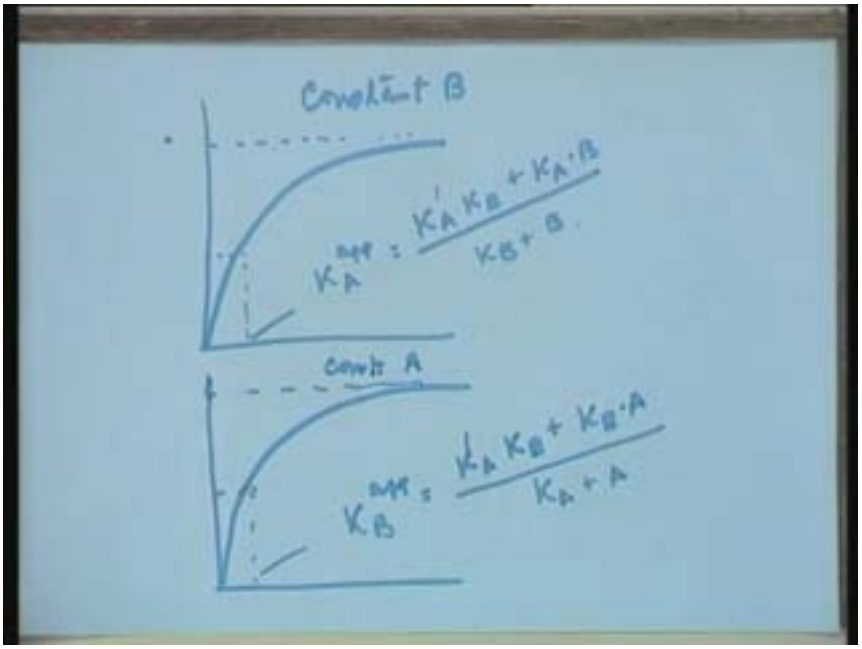
$$K_A^{app} = \frac{K'_A.K_B + K_A.B}{K_B+B}$$

Similarly here this situation will become V_0 when the value of B is saturating. It will be V_m when the value of B is saturating. At any other V value of B the rate profile will be a hyperbolic profile analogous to that of Michaelis Menten constant. Similarly in the case of A constant you will have another Km value

$$K_m^{app} = \frac{K'_A.K_B + K_B.A}{K_A+A}$$

Here also this is your constant A. This will be a maximum reaction velocity when A becomes saturating.

[Refer Slide Time: 17:37]



Such a situation can be conceived as a simplification of Michaelis Menten system. Another way to look at this kinetic expression is, just like we have been looking under all the cases of single substrate reaction, double reciprocal plots which will also give us the magnitude of all the kinetic parameters that are involved. What will be the kind of the double reciprocal plot? In the case of a generalized rate expression one can just write

$$1/V_0 = (1+ K_A/A+ K_B/B + K'_A.K_B/AB)/V_m$$

That is the double reciprocal plot if we divide both the numerator and denominator by A into B you will get rate expression. If you plot this at a constant value of V just like in the first case when we take constant value of V, you will get a profile with B increasing at different constant value of V. If B increases you will get a slope of

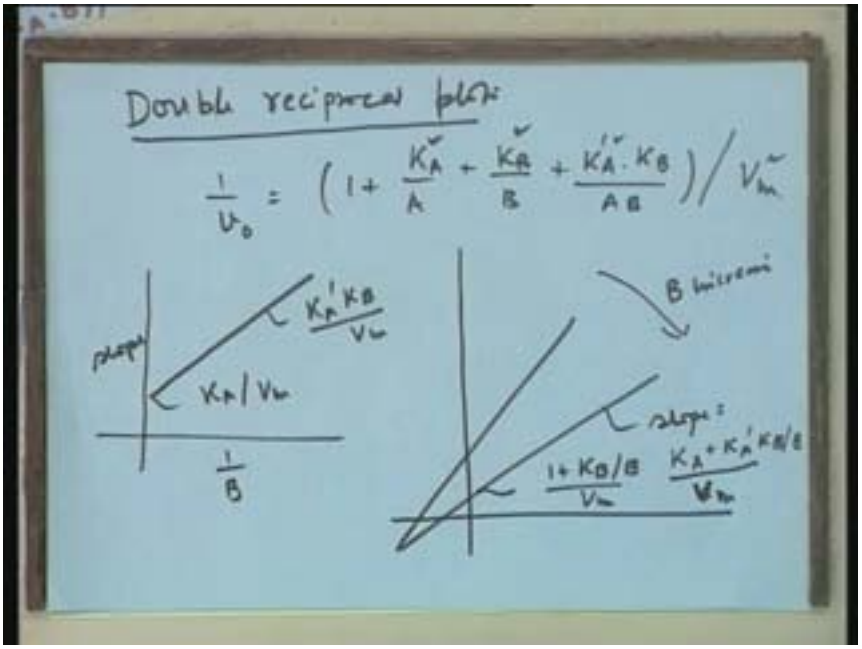
$$\frac{K_A + K'_A.K_B/B}{V_m}$$

The intercept on this factor will be

$$\frac{1+K_B/B}{V_m}$$

So you will notice that both slope as well as intercept of the profiles will decrease as you increase the value of B and ultimately when this B become saturating it will be a typical K value for the substrate A. Experimentally the magnitude of K_A, K_B, K'_A and V_m can be determined by double reciprocal plot analysis. If you recall what we did in the case of the inhibition patterns, we took the double reciprocal plots and then took slopes and intercept and made secondary plot. Similarly here if you plot 1/B versus the magnitude of slope you will get again a straight line with the slope equal to $K'_A.K_B/ V_m$ and intercept of K_A/V_m. In the case of intercept the slope will be K_B/V_m and intercept of $1/V_m$.

[Refer Slide Time: 21:17]



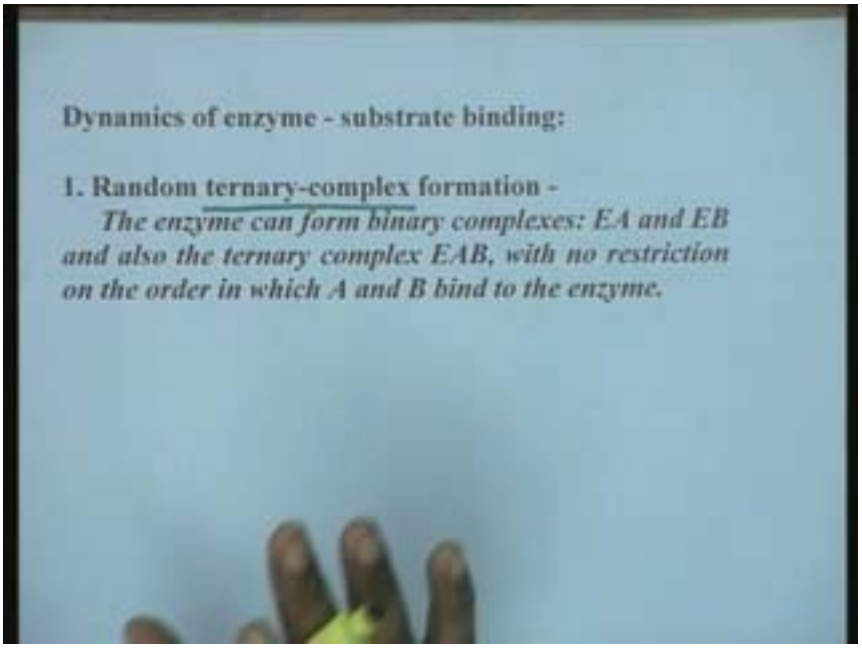
Therefore by a double reciprocal plot at different concentrations of B and plotting the 1/B versus the slope and intercept one can determine the values of the four kinetic parameters that are involved in a bi-substrate reaction that is K_A, K_B, K'_A and V_m. These are the four kinetic parameters unlike in the case of a single substrate reaction where we are concerned only with two kinetic parameters K_m and V_m. One can determine experimentally, the various kinetic parameters in the case of a bi-substrate reaction.

You will notice that while looking at the generalized expression of the bi-substrate reaction rate expression we have not really bothered about the dynamics of enzyme substrate interaction. We have just kept it in a black box some interaction takes place by which the products are formed and some general rate expression have been arrived at which involves certain kinetic parameters which have been considered in analogy to single substrate reaction because the kinetic analysis of enzyme catalyzed reactions have been so much dominated by Michaelis Menten kinetics that any new mechanism or any proposed mechanism must be interpreted in the light of those K_m and V_m as parameters and that is what we have done.

We must also look at the possible kind of interactions that are likely to take place and almost all the interactions between enzyme and substrate for bi-substrate reactions have been classified into some general forms. The first form, the first complex formation can be considered as the random ternary complex formation.

When we are talking of enzyme and two substrates there is a quite logical choice that we might end up instead of binary complex, a ternary complex where E, A and B all the three are combined together before a product formation takes place. When I talk of ternary complex formation I am referring to a ternary complex formation before the product is formed.

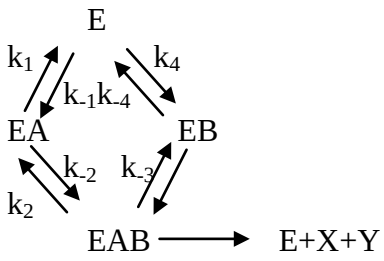
[Refer Slide Time: 23:57]



In the case of random ternary complex formation the enzyme can form binary complex EA and EB to start with and also the ternary complex EAB with no restriction on the order in which A and B bind to the enzyme. It can start by binding first with the A and then B will hook on to that or it can start with EB and then A will hook on to that or in other words that you will notice just like in the case of the non competitive inhibition the

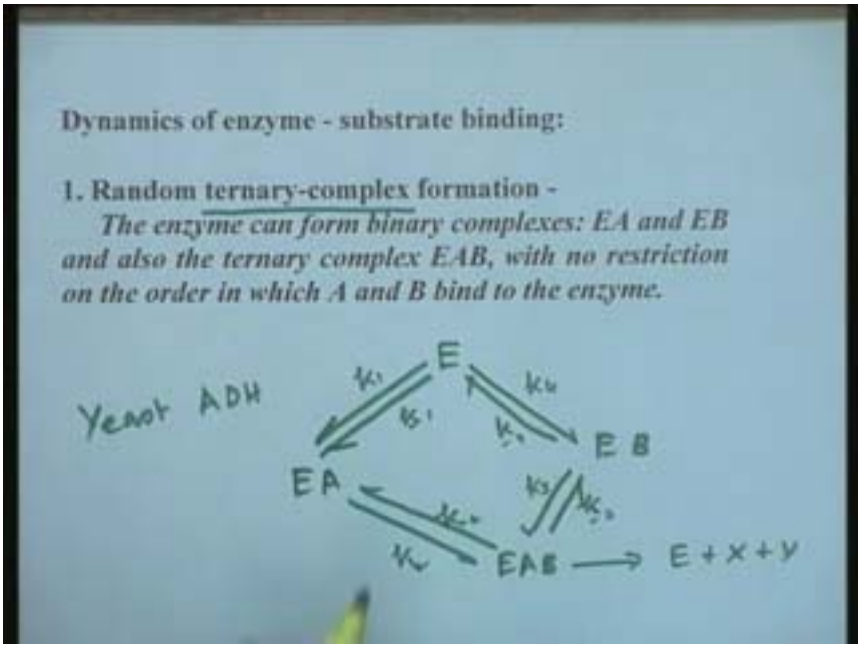
A and B both have separate binding sites on the enzyme molecule and therefore they don't interfere with each other and randomly they can bind and once the ternary complex formation has taken place the product formation will begin.

You can express it in a mechanistic way the enzyme going to EA or EB and EAB and this four this will go in cyclic form. EAB then goes to form your E plus X plus Y.



There will be rate constant at this state k_1, k_{-1}, k_2, k_{-2}, k_3, k_{-3}, k_4 and k_{-4}. Either reaction can go in this direction or in this direction there is no constraint on the order of the ternary complex formation. Typical example is yeast alcohol dehydrogenase which follows such a kind of mechanism.

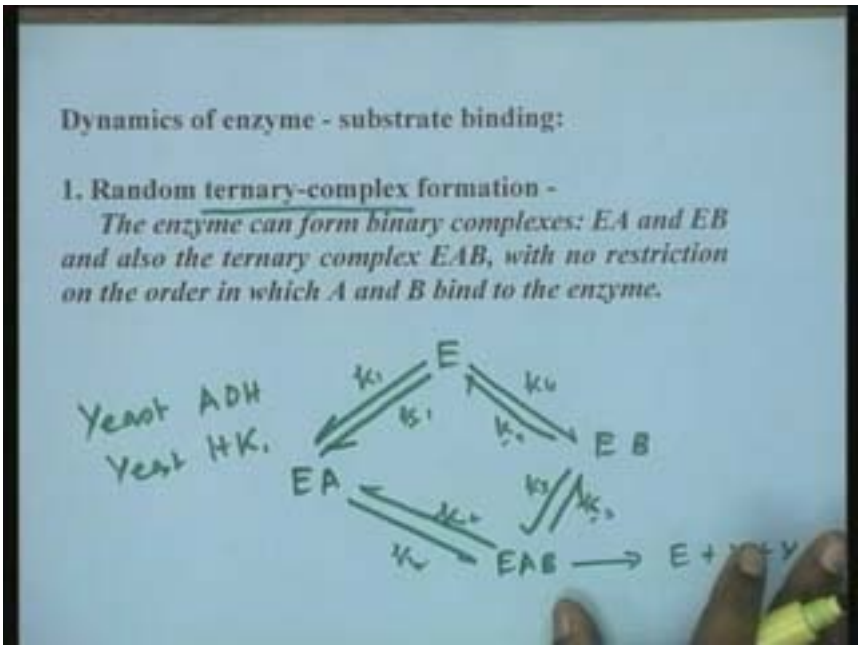
[Refer Slide Time: 26:02]



Another typical feature is that the kinetic parameters of the same enzyme which catalyses the same reaction from two different sources are different. For example yeast alcohol dehydrogenase follows a random ternary complex formation as a part of the dynamics of their enzyme substrate interaction whereas the same enzyme alcohol dehydrogenase from the mammalian cells doesn't follow this kind of mechanism; it follows an ordered

mechanism where it can bind only to one of the substrate first before the second substrate can bind and that's why the kinetic parameter will automatically turn out to be different. One of the reasons in such cases is based on the different reaction mechanisms that are involved. Similarly yeast hexokinase also follows this kind of mechanism where the ternary complex formation is obtained.

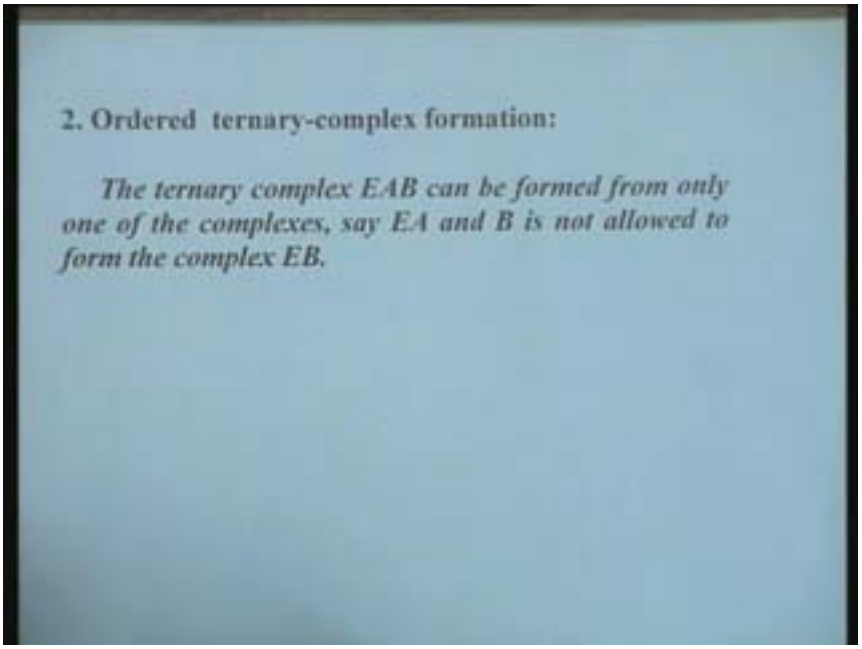
[Refer Slide Time: 27:07]



They may be equal to in some cases or they may not be equal to because ultimately the multiplication of the two dissociation constant in reaching to EAB will be same. In specific cases they may be equal also. But we have just put the different rate constants.

The second case again with respect to ternary complex formation is ordered ternary complex formation. Here the ternary complex EAB can be formed from only one of the complex EA. I have taken hypothetically EA it could be EB also and only one of the binary complex has to trigger the enzyme substrate interaction and then the other substrate will interact with the EA complex.

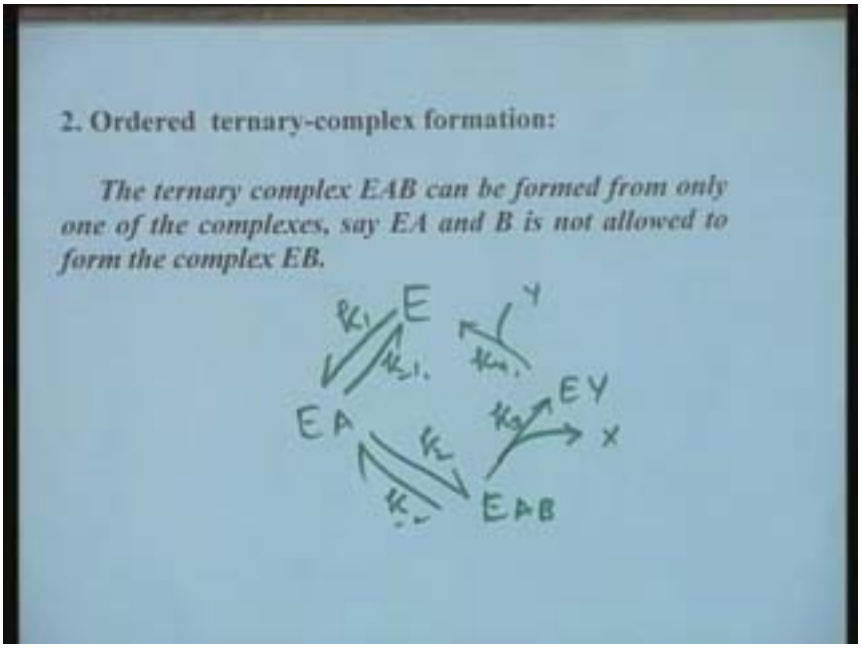
[Refer Slide Time: 28:14]



In other words if you write down the reaction mechanism EA this is k_1, k_{-1} and then this will go to EAB and this is k_2 and k_{-2}.

The reaction cannot proceed in this direction that is B cannot bind to the free E and the reaction has to proceed in an anticlockwise fashion that means after E has bound to A, B will bind to EA, EAB will be formed and ultimately this will form EY releasing one of the products let us say X and then this will release another product Y and they will be let us say k_3, k_4.

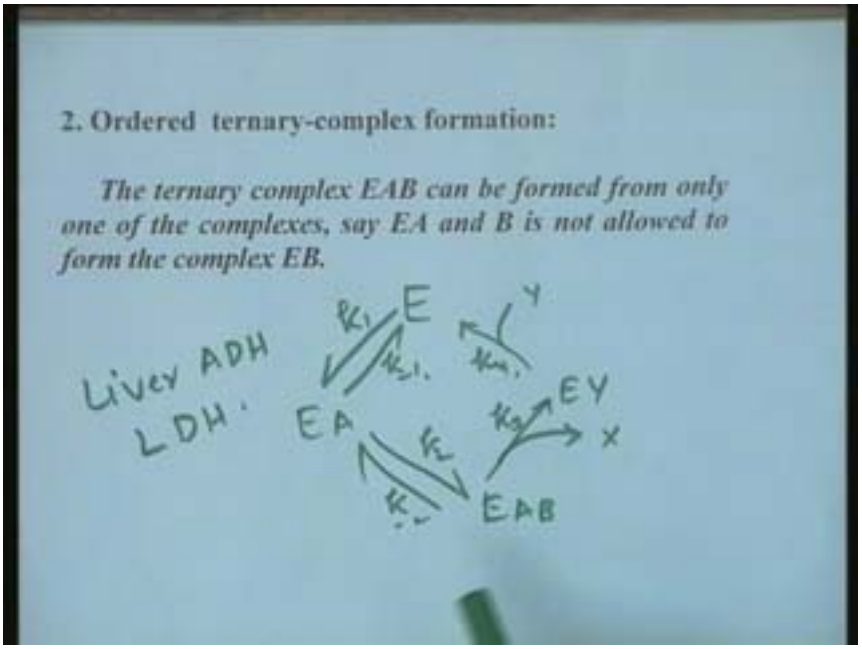
[Refer Slide Time: 29:10]



But the product formation will take place only after the ternary complex formation has begun. The process in this direction is not feasible.

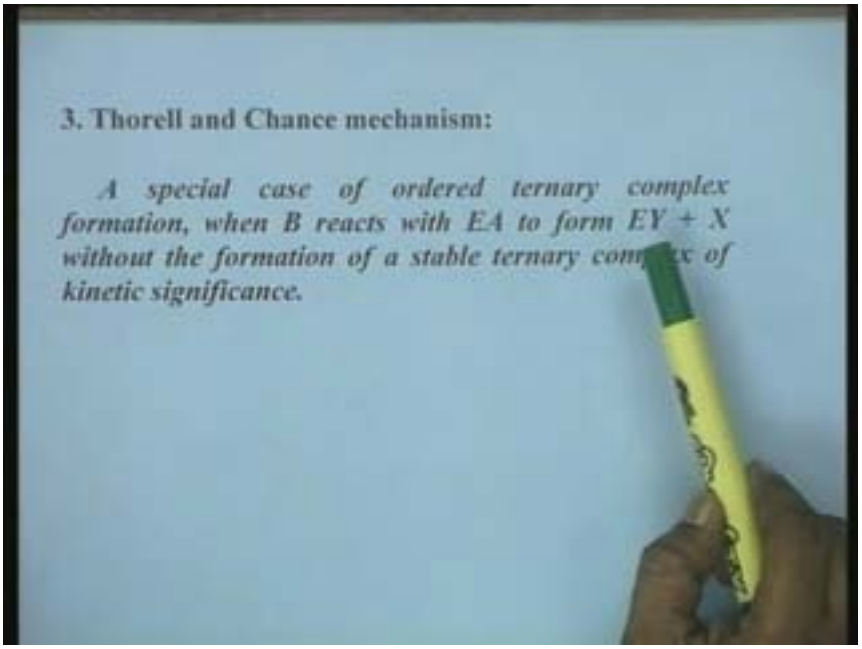
A typical example of the ordered ternary complex formation as I mentioned is liver alcohol dehydrogenase which is obtained in the mammalian systems and also lactate dehydrogenase is another example of ordered ternary complex formation. You can see that the same alcohol dehydrogenase can follow different mechanisms.

[Refer Slide Time: 29:44]



A special case of ordered ternary complex formation is Thorell and Chance mechanism because in many of the enzyme catalyzed reactions, Thorell and Chance observed that ternary complex is not a very stable entity and it exists only for a short period of time. A special case of ordered ternary complex formation when B reacts with EA; the order is important. We can only react with enzyme only when A has bound to it to form and one of the products is released and the modified enzyme substrate complex is generated.

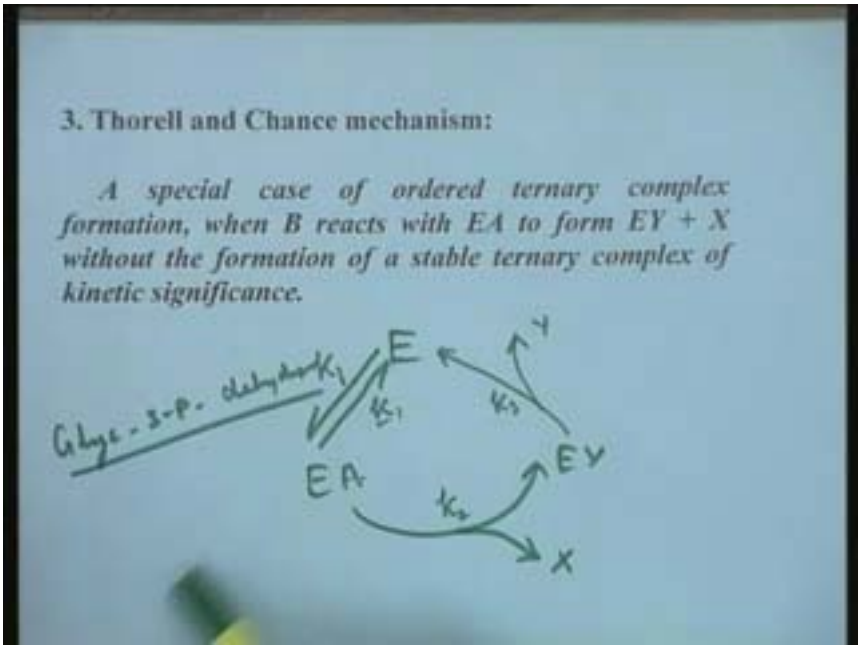
[Refer Slide Time: 30:32]



That means a modified enzyme substrate complex is generated and a first product has been released without the formation of a stable ternary complex of kinetic significance. That means the half life of the ternary complex is very, very small in this scheme. Although they never said that the ternary complex formation doesn't take place, they agreed that ternary complex formation is taking place but the half life of the ternary complex is very small and before that the second substrate can interact with the EA to form the first product.

Or in other words you can have E again going to EA and then this EA can then convert into EY releasing a product X and then this EY can give you back E releasing another product Y and you can have k_1, k_{-1}, k_2 and k_3. Such a kind of a system where although the second substrate B reacts with EA to give you the first product and the ternary complex is not a very stable entity is classified into the Thorell and Chance mechanism. A typical example of such a reaction is glyceraldehyde-3-phosphate dehydrogenase which you also come across in the case of glycolysis.

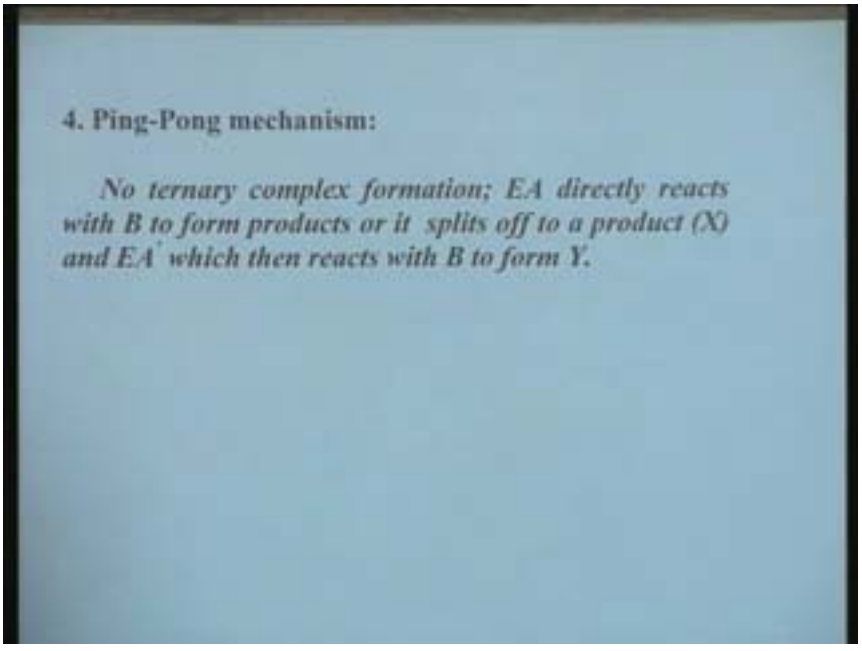
[Refer Slide Time: 32:17]



But it is only a special case of an ordered mechanism where ordered ternary complex formation is not denied. One can also consider either as a special case of ternary complex formation or a special case of binary complex formation. But binary complex cannot lead to a product. The second substrate has to interact with the binary complex before the first product can arrive at but the stable intermediates are only EA and EY.

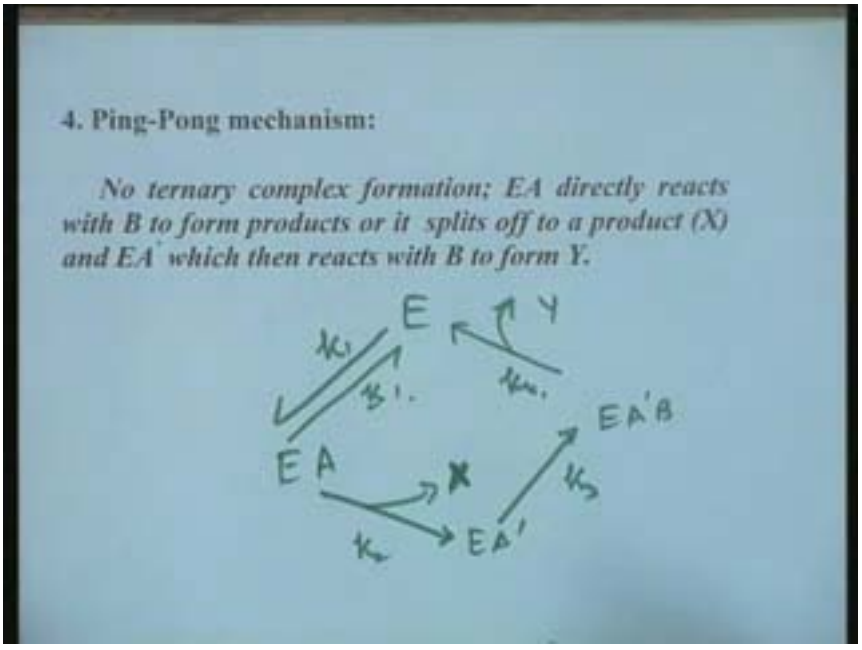
The fourth mechanism which represents the binary complex formation is Ping-Pong mechanism.

[Refer Slide Time: 33:02]



You will also notice that in many books it is written as Ping-Pong bi-bi mechanism. The term bi-bi refers to substrate and product. Bi-bi means two substrates and two products. In case of many of the ligases the situation may be bi-uni. That means the product may be one the substrates may be two. All those cases are clubbed under Ping-Pong mechanism and in this case of Ping-Pong mechanism the basic hypothesis is that no ternary complex is formed. As soon as the first substrate binds to the enzyme it can react to form the B to form a product or it can split off to a product E. You will notice that the two things like Thorell and Chance mechanism and the Ping-Pong mechanism in some way might show analogy. That means as soon as the EA of the first binary complex is formed the interaction with the second substrate forms a product, first product or it splits off to a product and a modified enzyme substrate complex which then reacts with B to form Y. Therefore E reacts with A to form EA with k_1 and k_{-1}. Right at this stage itself the product will be formed which is X and this will be EA' and this is k_2. EA' then interacts with B to give you EA'B and then this gives you a product again which is Y with k_3 and k_4.

[Refer Slide Time: 35:04]

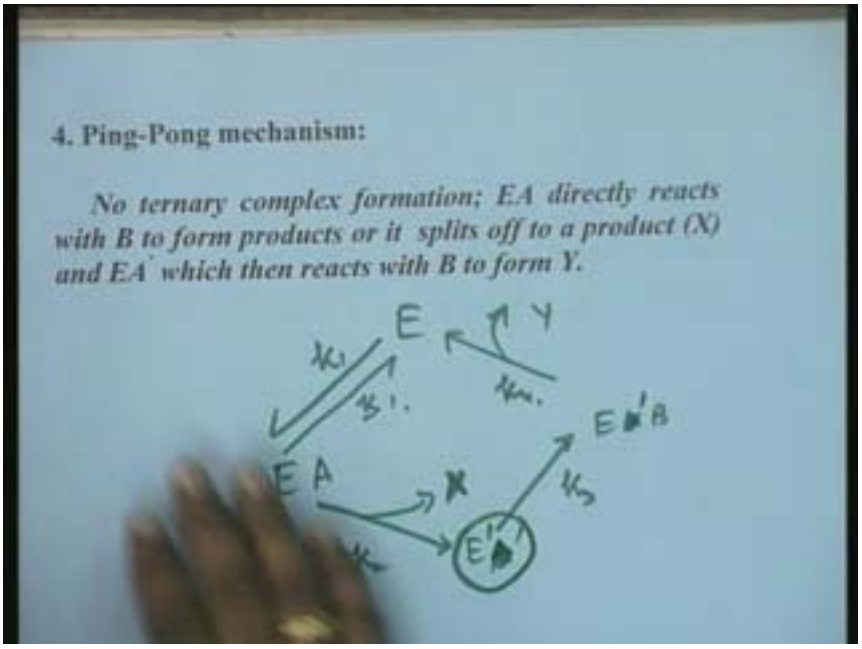


As soon as the first binary subjects complex is formed one of the product is released. Then the next substrate interacts and the next product is released. So that is what happens in such cases and a large number of transferases, most of the bulk of tranferases fall in the category of ping pong reaction mechanism excepting those of the yeast source and most of the mammalian hexokinases form under the category of ping pong. They lead to the classification of ping pong mechanism.

When we look into these mechanisms and develop steady state rate expressions specifically you will end up in quite complex situations. EA' is hypothetical in the sense it may not be a binary complex. It will be more clear it is E', the enzyme in the modified

form. The first substrate has reacted and released the product. Enzyme is modified and second substrate attacks and then releases another product. That would be a better interpretation. Whereas in the case of Thorell and Chance mechanism this will be a binary complex, this will not be a modified enzyme.

[Refer Slide Time: 36:40]

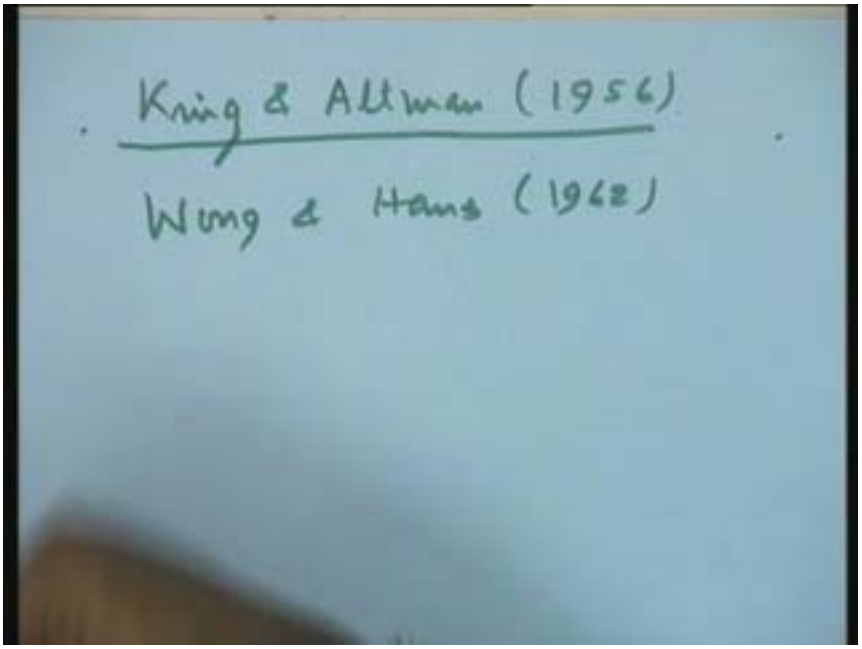


When we look at these reaction mechanisms with the increased order of complexity between the dynamics of enzyme substrate interaction, the rate expression will also be quite complex if you put them in terms of individual rate constants. Each of the reaction mechanism involves particularly the random ternary complex formation will involve nine rate constants four reversible and one irreversible step, nine rate constant and the expression will be quite complex. In practice to derive expression may be quite complex and we are talking only a binary substrate complex formation that might be enzymatic reaction which may involve three substrates and system will be still more complex.

Before I go to give you the rate expression for these different mechanisms I like to introduce you to a simplified methodology for writing a rate expression under a steady state hypothesis. Of course the steady state hypothesis is valid. The method was proposed by King and Altmen way back in 1956. They analyzed number of reaction mechanisms and using matrix algebra they wrote down rate expression for different kinetic mechanisms and all steady state expressions can be solved out.

Ultimately making some generalizations from those matrix algebra, Wong and Hans in 1962 proposed certain set of simplified rules almost like empirical rules.

[Refer Slide Time: 38:41]



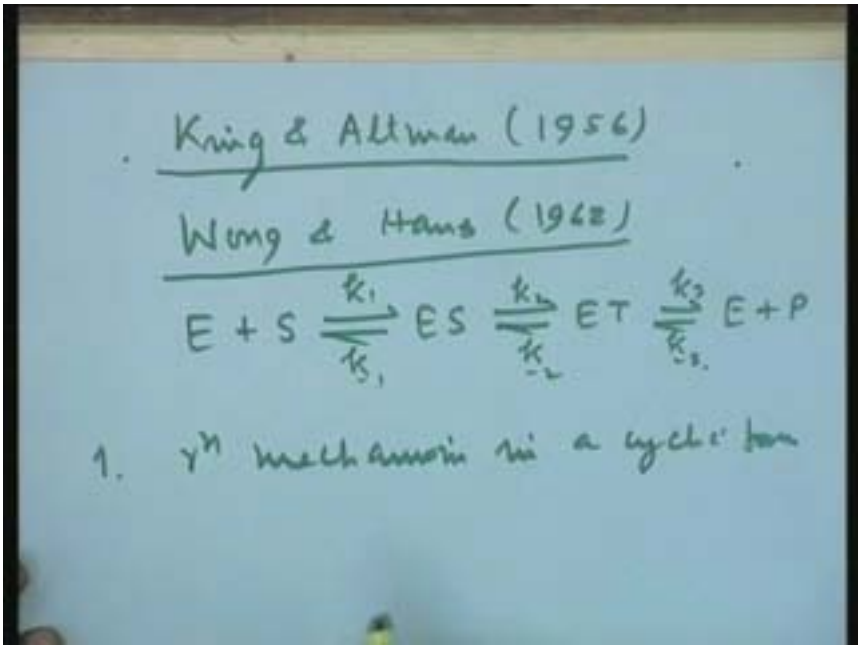
Although they are based on hard core mathematics matrix algebra he generalized certain rules so that by observation of the reaction mechanism as long as you can write a reaction mechanism one can arrive at a rate expression in a simplistic way. I will try to introduce you with that kind of a system taking a simple example so that, that can be applied to our complex binding substrate system to arrive at the actual rate expressions.

The kind of system we will take for simplicity will be a two enzyme substrate complex formation ES going to ET going to E plus P.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ET \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E+P$$

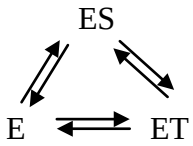
We can take a reversible system k_1, k_{-1}, k_2, k_{-2}, k_3, k_{-3}. To illustrate the procedure followed I chose this reaction mechanism. The very first step in such a procedure as proposed by Wong and Hans is that we need to write any reaction mechanism in a cyclic form.

[Refer Slide Time: 40:14]



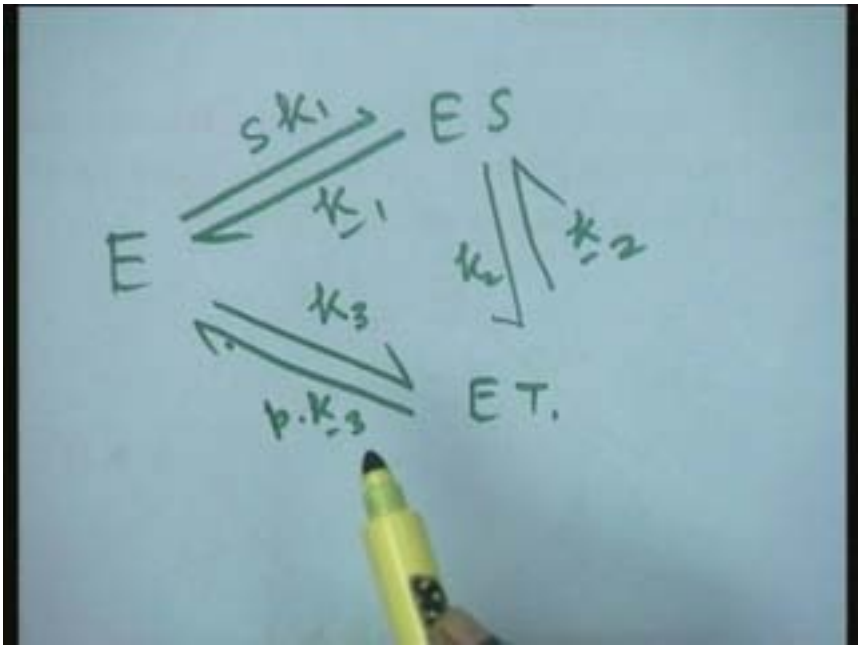
Cyclic form means you will have inter conversions of the enzyme substrate complexes. For example here as you notice enzyme species can exist in the form of E, ES and ET in three forms. So the whole reaction mechanism can be interpreted in the form of a triangle with three vertices, each vertex representing one of the enzyme species.

Therefore you can write such a reaction mechanism as E, ES and ET. Now show the inter convention of each of the enzyme species say for example E ES, ES ET and ET E.



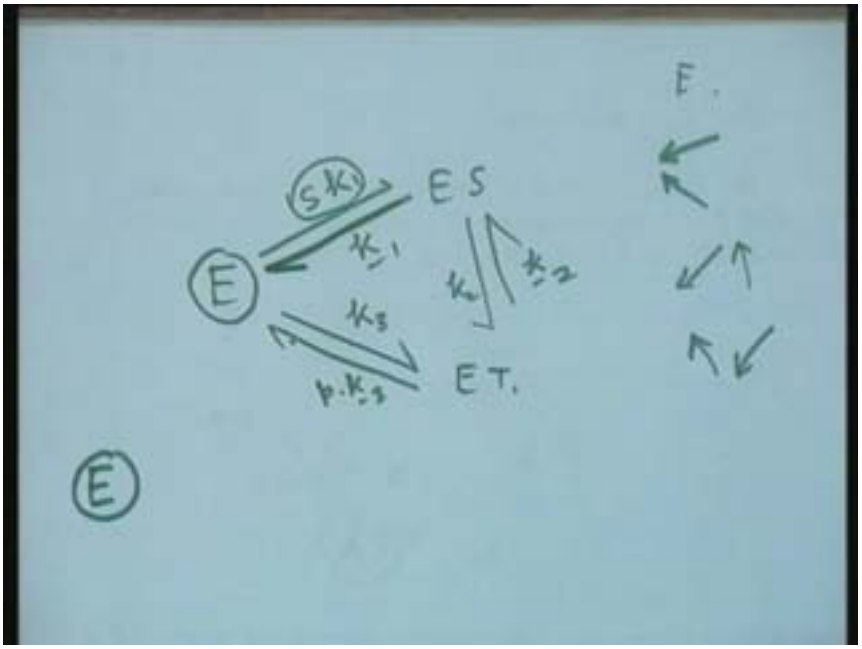
Let us say this is your Sk_1, k_{-1}, k_2, k_{-2}, k_3 and p_{k-3}. We write the rate constant for all these steps in the cyclic form making them in the kind of a first order rate constant. For example wherever there is substrate involved in binding over enzyme the rate constant can be written as Sk_1. Where there is no substrate involved only simple rate constant can be written and similarly wherever ET is going to form the product in the reverse direction, the product concentration will be incorporated

[Refer Slide Time: 42:19]



The inter conversions of the enzyme forms involved in each step must be described by a term called Kappa. The conversion of the rate constant or interpreting each of these steps in terms of first order rate constant term is called Kappa. Then we consider each enzyme species in sequence. For example we take E and consider all the possible pathways by which E is formed in this cyclic manner. E can be formed by all the possible pathway involving n-1 steps. That means if there are three enzyme species number of species are n and n-1 steps will be involved in each pathway. We identify that E can be formed by this and this. Similarly you can form this and this. Write all the possible pathways which are feasible to form and this is for E.

[Refer Slide Time: 44:10]



What I am trying to explain is that for each of the enzyme species write down all the path ways involving n-1 steps. That means if it is a three enzyme species system, if there are three enzyme species in the system then each path way must consist of two steps which can form the enzyme species E or any enzyme species which we are considering.

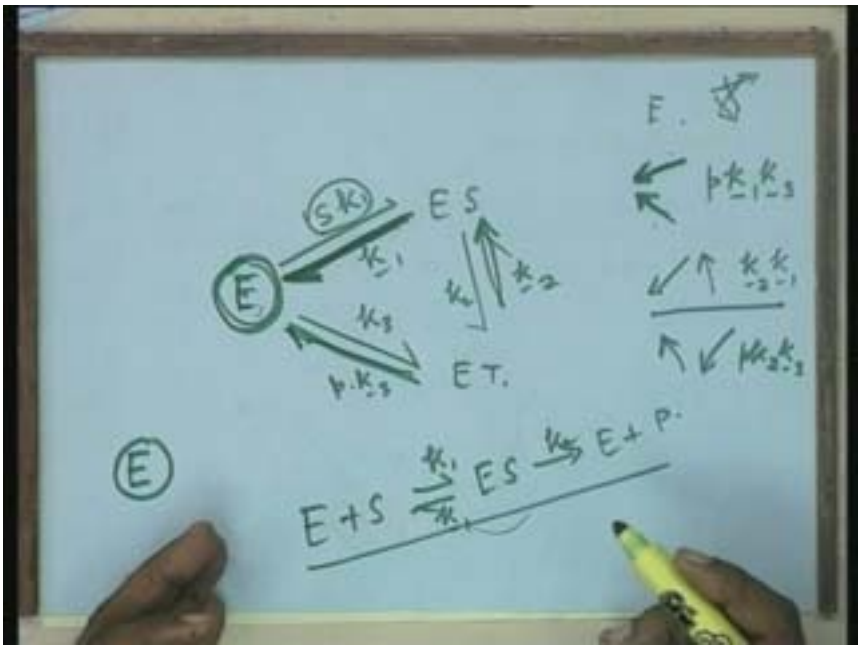
One of the considerations in such a system is that all the path ways that converges at a particular enzyme species are permissible. But the path way which leaves the enzyme species in both directions is not permissible. That is a system like this will not be permissible if it happens. Are you clear? For each of the enzyme species we work out the path way by which these species may be synthesized. Each path way must consist of n-1 steps where n is the number of enzyme species present and the product of the kappas of the steps in each pathway is determined. You can write the kappas here and you can have the kappa product. For example what is the kappa product for this pathway? pk_{-1}, k_{-3}. This pathway is represented by this and this. So the product of this Kappa is this. In this case it is k_{-2} and $k_{-1}$of this one and the third path way will be pk_2 k_{-3}.

We are talking of E. In this expression we are writing for the formation of the enzyme species E. These arrows represent this and this. sk_1 does not form E. sk_1 form ES. k_{-2} forms E by ES. What we mentioned is that we are considering only pathways which have two steps n-1 steps and any steps which lead to formation of E involving two steps will be considered. First pathway involves two steps this one and this one. They are only the steps which are involved in formation of E. They are just generalized rules. Otherwise you have to go to the matrix algebra to solve the simultaneous equations and you will arrive at the same thing. Wong and Hans simplified the whole matrix algebra did by King and Altmen and formed certain rules. He said that you form a cyclic reaction mechanism and write down the pathway for each of the enzyme species or for example we can take any simpler case let us take probably the simplest case E plus P.

$$E + S \rightleftharpoons ES \longrightarrow E + P$$

k_1, k_{-1} and k_2. I try to write this one using the same you will arrive at for the same expression as Michaelis Menten equation. For such simple system writing by observation will look to be more difficult than solving the steady state equation but if you have let us say more than one enzyme substrate complex it may look to be simpler to write by observation.

[Refer Slide Time: 49:06]



'n' represents the number of enzyme species involved in the mechanism, whereas in the case of what I showed we have three enzyme species E, ES and ET. It will be expressed in the cyclic form by a triangle each enzyme species represented on the vertex of the triangle and each of the path way which should be considered for the formation of enzyme species must have n-1 steps. This is how you define a pathway. It's not a pathway in the sense of metabolic pathway. It's the number of steps involved in formation of that each having n-1 step. So I think we will stop at this point.