BL/CH401 Lecture 17

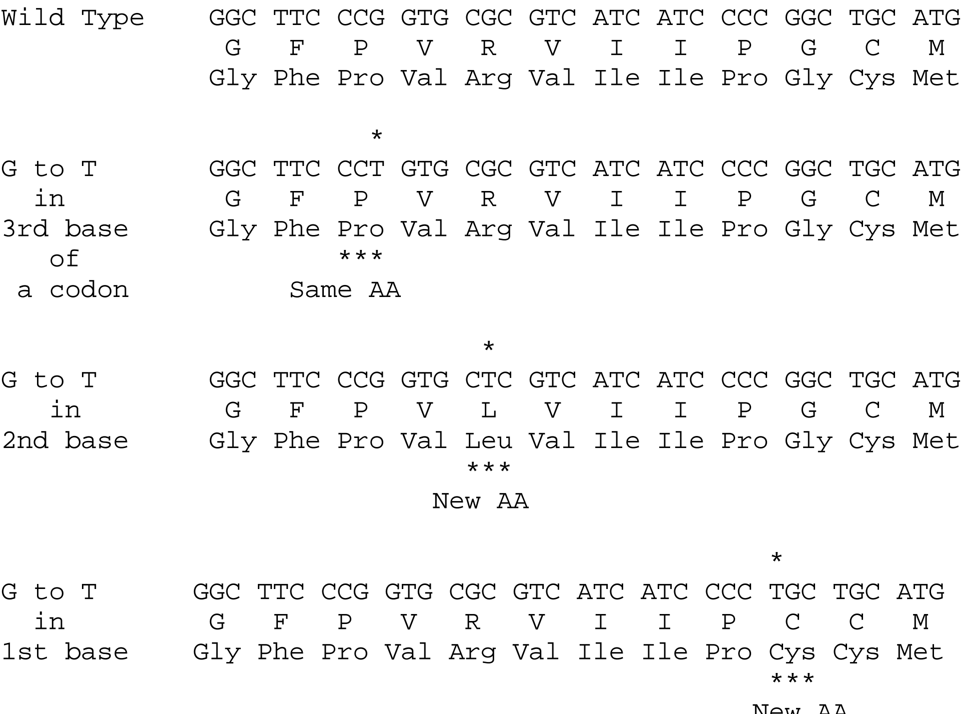
Mutagenesis & Enzyme Catalysis via Transition State

Part I. Introduction

I want to introduce the concept of site-directed mutagenesis and how this method is used to make mutant proteins for studying the biochemistry to an enzyme or protein. You must first recall that the AA sequence of a protein is encoded in the DNA sequence. So to make a mutant of the protein, one must change the DNA sequence and then express the protein in its mutant form. Overall, the process is to alter the nucleotide sequence of the DNA in vitro (ie outside the cell and actually in the lab) and I will not describe how one does that. Then the DNA is put into a bacterial cell to express the protein. Of course, the protein must be purified from the bacterial extract before it can be studied. Usually when mutants are made, they are studied in comparison to the natural or wild-type protein, as it is called. In Fig. 1 below, are shown examples of a mutant protein made by changing a single nucleotide base in the sequence of the DNA. In some cases, a base change does not lead to a new AA in the sequence since the genetic code is redundant (see lecture 2 for more information on the genetic code).

Examples of the effects of changing a nucleotide or base in a DNA sequence encoding a protein:

Here a single base is changed (always G to T)



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Wild Type     GGC TTC CCG GTG CGC GTC ATC ATC CCC GGC TGC ATG
               G   F   P   V   R   V   I   I   P   G   C   M
              Gly Phe Pro Val Arg Val Ile Ile Pro Gly Cys Met

                          *
G to T        GGC TTC CCT GTG CGC GTC ATC ATC CCC GGC TGC ATG
  in           G   F   P   V   R   V   I   I   P   G   C   M
3rd base      Gly Phe Pro Val Arg Val Ile Ile Pro Gly Cys Met
   of                 ***
 a codon            Same AA

                                 *
G to T        GGC TTC CCG GTG CTC GTC ATC ATC CCC GGC TGC ATG
   in          G   F   P   V   L   V   I   I   P   G   C   M
2nd base      Gly Phe Pro Val Leu Val Ile Ile Pro Gly Cys Met
                                ***
                              New AA

                                                     *
G to T       GGC TTC CCG GTG CGC GTC ATC ATC CCC TGC TGC ATG
  in          G   F   P   V   R   V   I   I   P   C   C   M
1st base     Gly Phe Pro Val Arg Val Ile Ile Pro Cys Cys Met
                                                    ***
                                                   New AA
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Figure 1. Mutagenesis of a protein by changing a single base.

This is a very general example and the mutations are made at random. When carrying out site-directed mutagenesis, a very specific site in the protein and an individual AA is selected for mutagenesis. That is what makes this method so important to understand...using the method the

investigator can actually choose the AA he/she wants to change and then change it to any of the other 19 AA's encoded by the codons in the genetic code. This process is very controlled and leads then to a way to gain much greater insight into protein structure and function because the impact of different AA side chains at the same site in a protein/enzyme can be studied and the importance of an individual AA side chain can be understood in some detail. Ultimately, it can be expected that this type of experiment will allow the investigator to actually design new functionality into old enzymes and even design new enzymes.In this lecture, I want to apply the concept of site-directed mutagenesis to the problem of understanding how an enzyme works as a catalysts. As it turns out, this new method of generating mutant proteins, where we make only the specific mutants we want and we think will have the greatest impact on enzyme functionality without changing the general shape of the enzyme or protein, is probably the only way to prove that certain types of hypothesis for how enzyme's work can be challenged and proved right or wrong.

Part II. Transition-State Theory of Enzyme Catalysis

I want to return to a question I have ask before in this class: How do enzymes catalyze reactions? We have addressed that in several ways but we have the same fundamental answer to start from - Like all catalysts, enzymes decrease the energy required to a reaction started. But this answer does not tell us at the molecular level how the enzyme does it and it is at this level that we have worked at finding the answer, which was partly achieved by understanding that enzymes can store energy from the binding of the substrates and use it later to make catalysis more efficient. That is part of the reason why we think enzymes have large structures. We also addressed the question by looking at the enzyme mechanism and saw how AA side chains assist with bond rearrangements. Now we want to apply another theory of chemistry to enzyme catalysis. This theory is the one called transition-state theory. Starting with the transition-state theory of chemical reactions, Linus Pauling suggested that enzyme catalysis can also work by this mechanism (he made this suggestion about 1940 or so). In this concept, an intermediate form exists between the substrates and products in an enzyme catalyzed reaction and this intermediate is called the transition-state intermediate, just as it is in chemistry.

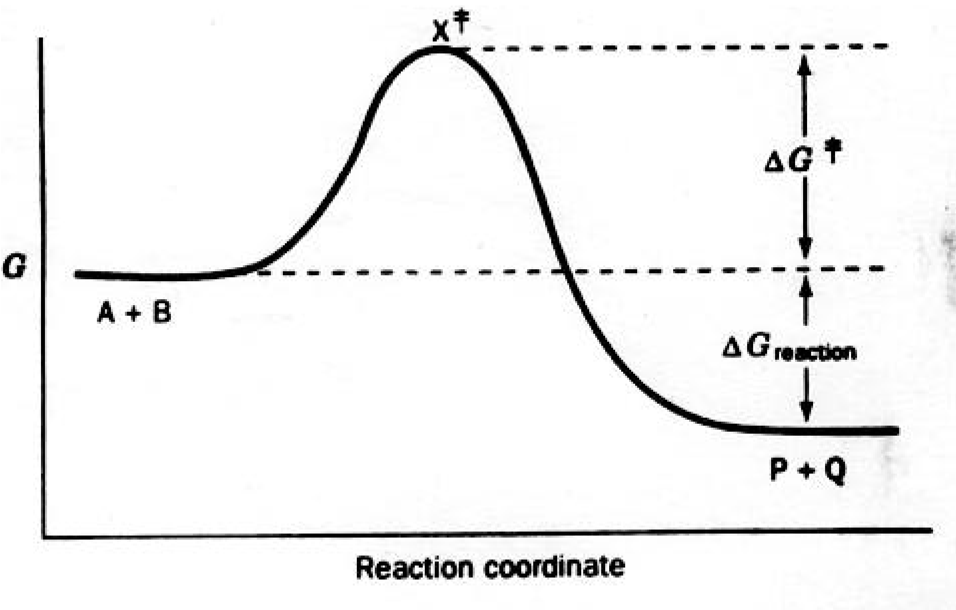

Figure 2. Diagram illustrating the concept of the transition-state intermediate in a chemical reaction.

In the above diagram, A + B are substrates which must get over the energy hump in the reaction pathway which is measured by the 'G' on the y-axis that is called "free energy". On the way to forming products P + Q, the reaction goes through an intermediate which is neither the substrates nor the products and is 'intermediate' between them - so it is called an intermediate and since it is going through the transition from substrates to products it is called the transition-state intermediate. For an enzyme and in enzyme mechanisms, this suggests a new step in the process of catalysis:

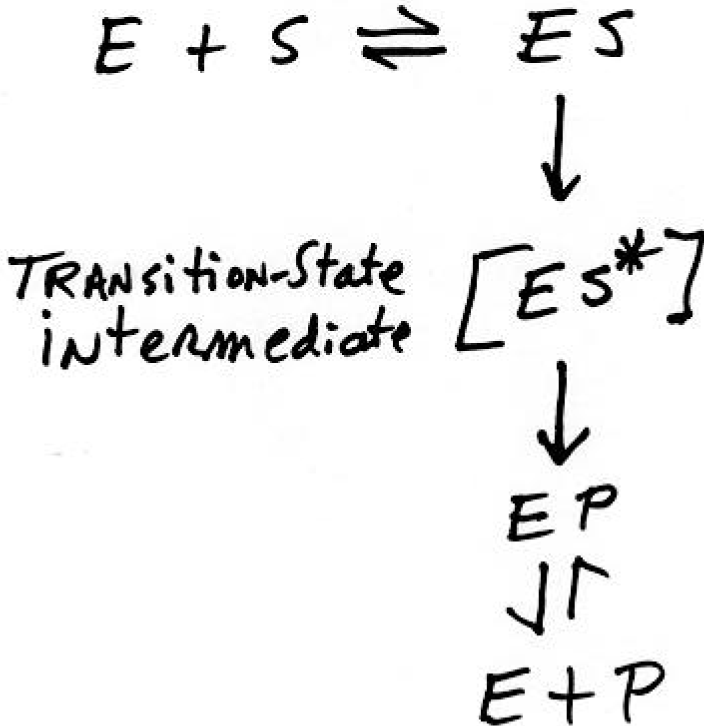

Figure 3. The standard enzyme mechanism with the addition of the transition-state intermediate in the process.

So after the ES complex forms and before product P is made, S becomes the transition-state intermediate, which we will call S*Éso ES* must form before EP does. For example, the transition state in the trypsin catalyzed hydrolysis of a peptide bond will be between the key serine's side chain hydroxyl and the carbonyl group of the peptide bond being hydrolyzed. We have also called this the tetrahedral intermediate since the carbon has a tetrahedral shape in this intermediate:

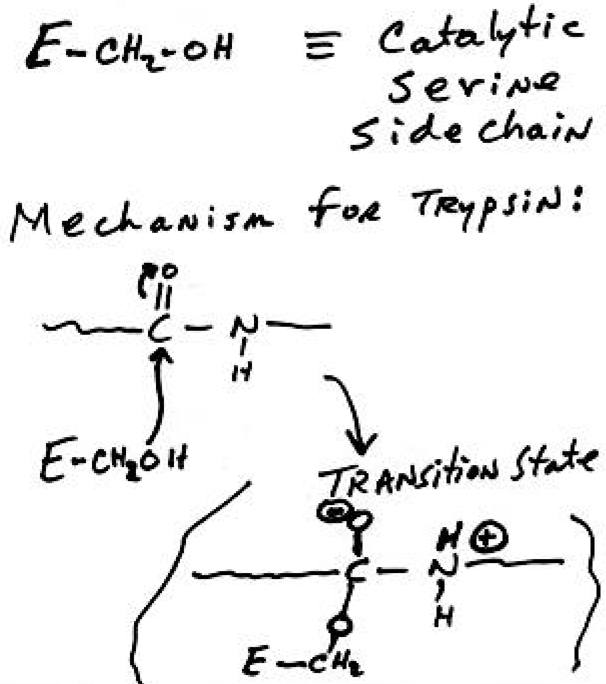

Figure 4. The transition-state intermediate in the trypsin catalyzed hydrolysis of a peptide bond.

This leads to the idea that the enzyme will have a higher affinity for the transition-state intermediate than the substrate and will bind the transition-state intermediate more tightly than substrate. For catalysis to be efficient the enzyme must bind the transition-state more tightly than the substrate or it will simply be a substrate binding protein and not a catalysts. That's why the Ks for the substrate binding to the enzyme is not the same as the Km for the substrate since the Km involves the process of making product, which must go through the transition-state intermediate, as well as the binding of the substrate.

Part III. Transition-State Analogs as Very Good Enzyme Inhibitors

One way to try to prove that the transition-state intermediate exists in an enzyme catalyzed reaction and is part of the enzyme catalyzed mechanism, is to design inhibitors of an enzyme that look like what you think the transition-state intermediate for the enzyme catalyzed reaction looks like. Furthermore, to prove the idea that the enzyme binds the transition-state intermediate more tightly than it does the substrate, we should compare these transition-state analogs (the transition-state like inhibitor molecule) to competitive inhibitors of the enzyme, which of course look like the substrate chemically, and see which is the stronger inhibitor. An example of this type of experiment is found in the studies of lysozyme:

Lysozyme:

H_2O

hydeolyzes polysacchrides

Transition-State may be carbonium ion

which is planar

∴ a planar sugar like

a lactone is better inhibitor than

than chair shape.

Figure 5. Lysozyme and its transition-state analog as an inhibitor. In this enzyme, the transition-state intermediate is thought to be a planar carbonium ion and so a transition-state analog which should be a very good inhibitor would be a sugar with a planar carbon at the reactive center, which is found in a lactone form of the sugar. The lactone sugar is a much better inhibitor than a sugar with the 'chair' shaped reactive center like is found in the substrate or competitive inhibitors. In general, it has been found that transition-state analogs are the best inhibitors of enzymes - they often have Ki's 1000 times or more smaller than competitive inhibitors (smaller Ki indicates tighter binding to the enzyme's active site).

Another way to address the issue of the importance of the transition-state intermediate in enzyme catalysis is to design a protein which will strongly bind a molecule which represents the transition-state intermediate in a reaction you wish to make a catalysis for. So if the protein binds the transition-state analog very well, then it should be a catalyst for the reaction having that transition-state intermediate. This has been done by using antibodies to make the catalyst like an enzyme or in other words make a 'catalytic antibody'. Since an animal can be immunized or induced to make an antibody against essentially any chemical, if the animal is immunized with the transition state analog, will it be an enzyme for the reaction with that transition-state? For example, phosphate esters are tetrahedral in shape and could be a transition-state analog for an enzyme that hydrolyzes carbon-based esters. To test this hypothesis, an animal was immunized with a phosphate ester containing protein and then the antibodies were isolated from the animal's serum. These purified antibodies indeed did have esterase activity and so a catalytic antibody can be made by immunization with the transition-state analog.

Part IV. Transition-State in Enzymes & Biotechnology

Another and perhaps the most convincing proof of the existence of the transition-state intermediate in the enzyme catalyzed reaction, was devised by a Professor in England, Allan Fersht, who used biotechnology to approach this question. His method involved site-directed mutagenesis. First, his research group identified the key amino acids at the active site of an enzyme for which they not only had the 3-D structure but also a clone. The cloned gene was expressed in a bacterium and so they could carry on site-directed mutagenesis on the specific AA side chains at the active site and determine what the impact of changing these AA's has on the catalytic properties of an enzyme by study both the natural or wild-type enzyme in comparison with the mutant forms of the enzyme.

The enzyme they were studying is called Tyrosine tRNA synthetase. Tyr tRNA synthetase (or Tyr RS for short) is involved in protein synthesis where the amino acid are attached to the tRNA prior to binding to the ribosome where the tRNA binds to the mRNA and the amino acid is joined to the growing chain of the polypeptide which is being made on the ribosome. Tyr RS catalyzes the following reaction in two steps:

$$\text{Tyr} + \text{ATP} \rightarrow \text{Tyr-AMP} + \text{PP}_i$$

$$\text{Tyr-AMP} + \text{tRNA} \rightarrow \text{Tyr-tRNA} + \text{AMP}$$

Figure 6. Reactions catalyzed by Tyrosine tRNA Synthetase during the coupling of Tyr to its specific tRNA.

The first of these reactions was selected by Fersht for particular study and it is called the activation step where Tyr's carboxyl group is activated to be reactive using ATP to form a mixed anhydride. For this step, Fersht hypothesized that the reaction would have the following transition-state intermediate:

Tyr tRNA Synthetase
Transition-State Mechanism

≡ ATP
(Ado is Adenosine)

Figure 7. The reaction where Tyr is activated by ATP in the first step catalyzed by Try RS and the possible transition-state intermediate in the reaction.

Fersht imagined that enzyme must have specific AA side chains in the enzyme's active site for stabilizing this transition-state intermediate and he set out to determine which amino acids side chains in the active site these were and to do site-directed mutagenesis to make mutants of the Tyr RS with different AAs in their places in the active site. This allowed him to develop a very elegant proof of the concept that an enzyme must have a high affinity for its transition-state intermediate.

Part V. Site-Directed Mutagenesis of an Enzyme

But first, before Fersht could study the part of the active site involved in stabilizing the transition-state intermediate, he needed to establish which amino acid side chains were involved in binding the substrate and catalyzing bond rearrangements. This is pretty much like what I have already described for the serine proteases and a number of other enzymes in this course. But Fersht went one step further in his studies, he made a series of mutants of these active site AA residues and studied what impact changing key AAs involved in substrate binding and other aspects of catalysis. First, he hypothesized what AA side chains in the active site were doing what:

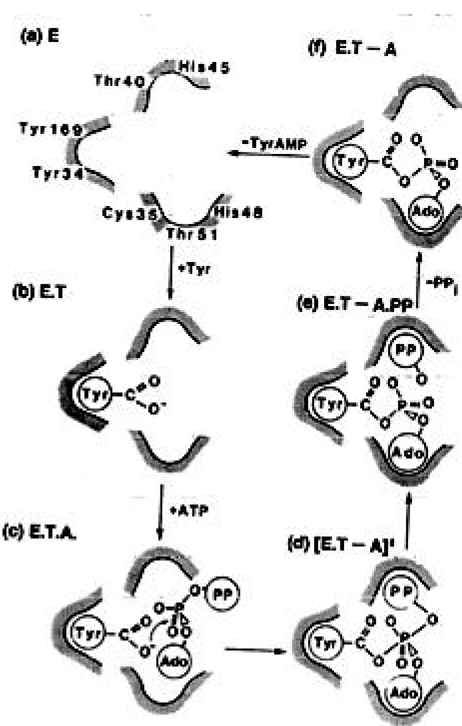

Figure 8. Hypothesis for the catalytic mechanism of Tyr tRNA Synthetase and the role of key amino acid side chains at the active site.

In this model, 7 amino acid side chains were identified at the active site: 2 Tyrosine residues (Tyr34 and Tyr169) were suggested to be involved in binding the substrate Tyr; Cys35, His48 and Thr51 were suggested to be involved in binding the adenosine part of the ATP substrate; and finally, Thr40 and His45 were expected to be involved with stabilizing the transition-state intermediate. In the catalytic mechanism, the resting enzyme ready to start catalysis is shown in part a of Fig. 8 with the AA side chains list above shown surrounding three pockets in the active site. First, the Tyr binds to its binding site in part b; next ATP binds to its binding site and the reaction starts by Tyr attacking the alpha-phosphate of the ATP as shown in part c. This leads to the transition-state intermediate, which is shown in part d with the terminal two phosphates of ATP held in the special pocket of the active site where Thr40 and His45 are located. Next, the transition-state intermediate breaks down into products as shown in part e. Finally, the products leave the active site, with the pyrophosphate (PP) leaving first followed by the activated Tyr-AMP which is shown in part f and finally going back to part a where the resting enzyme is ready to start the cycle over again.

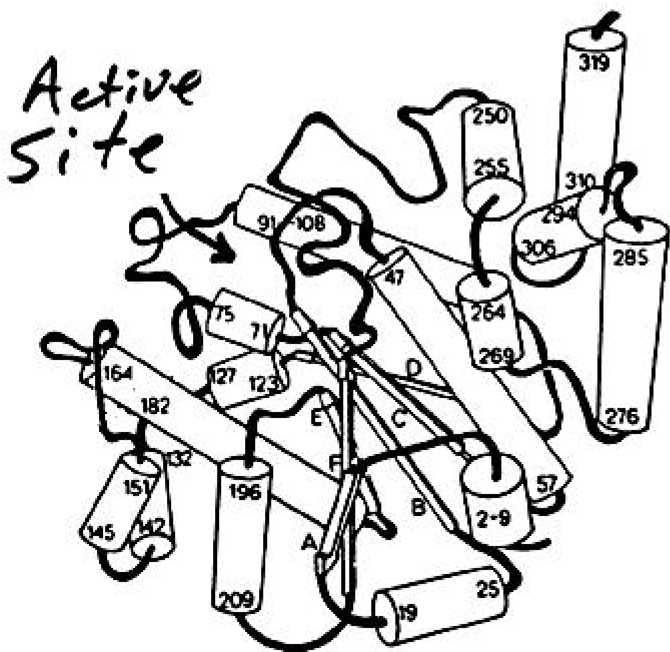

Figure 9. The 3-D structure of Tyr tRNA Synthetase with the region of the active site identified.

The active site of the enzyme was analyzed by modeling where the product of the reaction was fitted to all the possible hydrogen bonding groups available in the active site. This showed them which amino acids to target for site-directed mutagenesis to determine if altering them to other amino acids would impact the catalytic functionality of the enzyme.

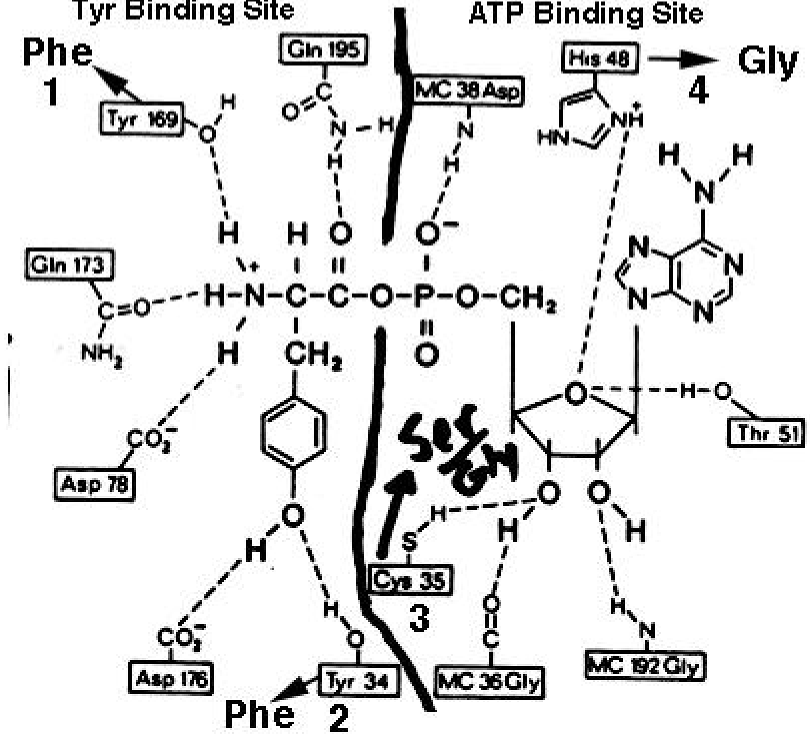

Figure 10. Model of the Active Site of Tyr tRNA Synthetase with the Product, Tyr-AMP, bound.

In this model, I have selected 4 of the residues Fersht's group studied by site-directed mutagenesis and identified the residues which these were changed to. 1. Tyr169 was changed to Phe so that it lost the ability to make a hydrogen bond with the substrate Tyr, but retained its ability to function structurally. 2. Tyr34 was changed to Phe for the same reason as Tyr169. 3. Cys35 to Ser which gives it similar chemistry but the OH on Ser is smaller than the SH on Cys and Cys35 was also changed to Gly which is completely removing the side chain. 4. His48 was changed to Gly also which eliminates its side chain. Then they analyzed the kinetic properties of the mutant forms generated by these changes in comparison to the natural or wild-type enzyme.

	Enzyme	k_3 (s^{-1})	%	K_s ATP (mM)	K_s Tyr (μM)	
	Wild type	38		4.7	12	
	Tyrosine binding-site mutants					
1	Tyr→Phe34	35		4.4	29	~2X
2	Tyr→Phe169	35		4.6	1,320	~110X
	ATP binding-site mutants					
3	Cys→Ser35	4.7	13	4.8	8	Little
3	Cys→Gly35	4.0	10	4.5	11	Effect
4	His→Gly48	9.9	26	9.9	23	Here

Figure 11. Kinetic Properties of Mutants of AA Residues involved in Substrate binding in Tyr RS.

When the 2 Tyr residues in the Tyr substrate binding site were changed to Phe, little impact was found on the k3 or catalytic constant (which you remember is sort of like the Vmax) but Ks for Tyr was increased by 2-fold when in the Tyr34Phe mutant and by over 100-fold Tyr169Phe mutant. For the Cys35 mutants, the impact was mainly on the catalytic rate constant and there was little impact on the Ks for ATP, which was also true for the His48Gly mutant. So with any of these mutants, only a small change was found in kinetic properties of the enzyme but it is sufficient to show that these AA side chains are important in catalysis. The magnitude of these changes in kinetic properties will become important next when we look at mutants in the other active site residues - so let's keep in mind that the biggest change was in the rate constant by about a factor of 10 when Cys35 was changed to Gly.

Part VI. Transition-State Intermediate Binding in an Enzyme

Well now let us look at the results for changing the side chains of amino acids thought to be involved in stabilizing the transition-state intermediate. First let's review the hypothesis:

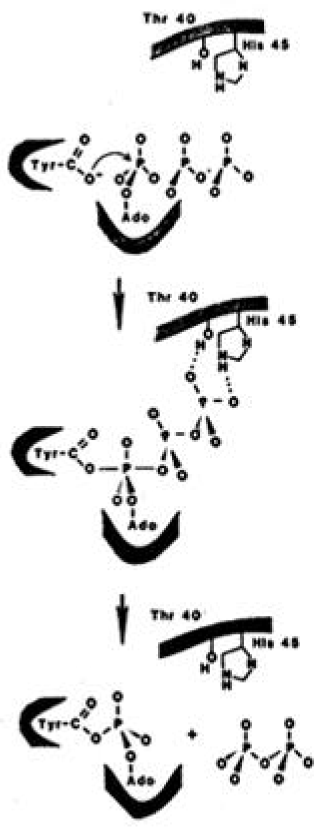

Figure 12. Hypothesis for the formation of the transition-state intermediate and the roles of Thr40 and His45 in this process.

The 2 amino acid side chains can form hydrogen/ionic bonds with the pyrophosphate group of the transition-state intermediate and hold them in position during this phase of the catalytic mechanism.

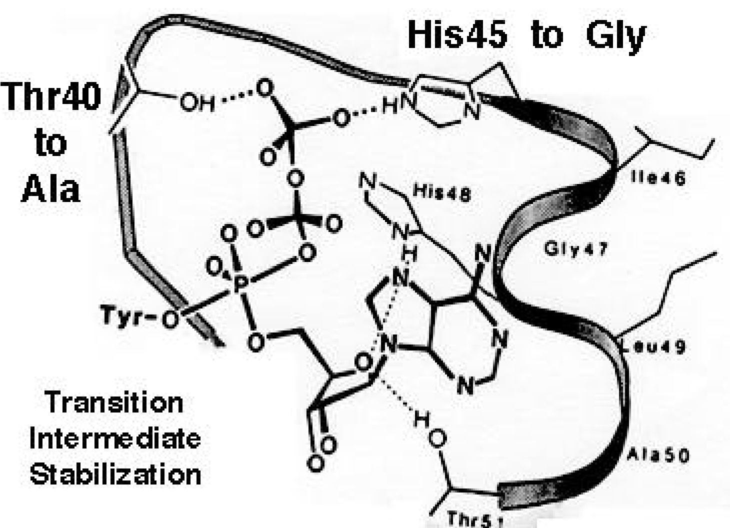

Figure 13. A more detailed model of the role of Thr40 and His45 in stabilizing the transition-

state intermediate.

To test if these 2 amino acid side chains have a special role in catalysis via transition-state intermediate stabilization, Thr40 was changed to Ala by site directed mutagenesis and His45 was changed to Gly. The idea is that neither of these changes in side chain would disturb the overall structure of Tyr tRNA synthetase nor change the position of any other groups in the active. Fersht proved this by preparing crystals of these mutants and showing that they had the same 3-D structure as the natural or wild-type enzyme. Then he studied the kinetics of these mutants.

Enzyme	k_3 (s^{-1})	K_S Tyr (μM)	K_S ATP (mM)	Fold Decrease in Catalytic Rate
1 TyrTS	38	12	4.7	
2 TyrTS(His→Gly	0.16	10	1.2	240
3 TyrTS(Thr→Ala	0.0055	8.0	3.8	7000
4 TyrTS(Thr→Ala;His→Gly	0.0012	4.5	1.1	32000

Little Impact on Binding Constants

Figure 14. Kinetics of the AA Residues involved in Transition-State Stabilization.

From the results shown in Fig. 14, it is clear that the Thr40Ala and His45Gly mutants were much poorer catalysts than the wild type with the Thr40Ala mutant having a 7000-fold lower catalytic rate constant than the wild-type. When the double mutant was made with both Thr40Ala and His45Gly, the catalytic rate constant was 32,000-fold less than wild-type. In addition, there was little impact of these mutations on the Ks binding constants for the substrates Tyr and ATP; in fact, Tyr was bound more tightly to the enzyme in the mutants than the wild-type, which suggests that taking away the transition-state intermediate stabilizing groups (ie Thr40 and His45) actually does lead to an enzyme form which binds substrate more tightly and is less catalytically active.

In summary, these experiments have shown a way to very precisely and elegantly prove that the stabilization of the transition-state intermediate in an enzyme is critically important to its functionality.

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