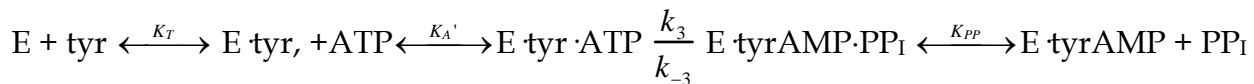


## Tyrosyl-tRNA Synthetase

The reaction is:



$E \cdot \text{tyrAMP} + \text{tRNA} \rightarrow E \cdot \text{tyrAMP} \cdot \text{tRNA} \rightarrow E + \text{tyr-tRNA} + \text{AMP}$ . The  $V_m$  for the second reaction is considerably less than that for the first reaction. When tRNA is absent the enzyme forms an equimolar concentration of bound tyrAMP, which hydrolyzes only slowly (the enzyme stabilizes this labile intermediate by some 9 kcal/mole: the equilibrium constant for its formation in solution is  $3.5 \times 10^{-7}$ , but on the enzyme is 2.3). This reaction may be used to titrate enzyme concentration:  $E[^{14}\text{C}]\text{tyrAMP}$  is collected on a nitrocellulose filter and counted. Or,  $\gamma\text{-}^{32}\text{P-ATP}$  is used as substrate at 2-3x the concentration of enzyme, residual charcoal-adsorbable  $[^{32}\text{P}]\text{-ATP}$  collected at points in time and the decrease extrapolated back to time zero [draw this]; this is done over a fairly long period of time, 7 min with the present enzyme, 60 min with valyl-tRNA synthetase, because the release of aminoacyl-AMP is fairly slow; the rapid decrease =  $[E]$ . This is important for accurate determination of total enzyme concentration and therefore of  $k_{\text{cat}} = V_m/[E]_t$ .

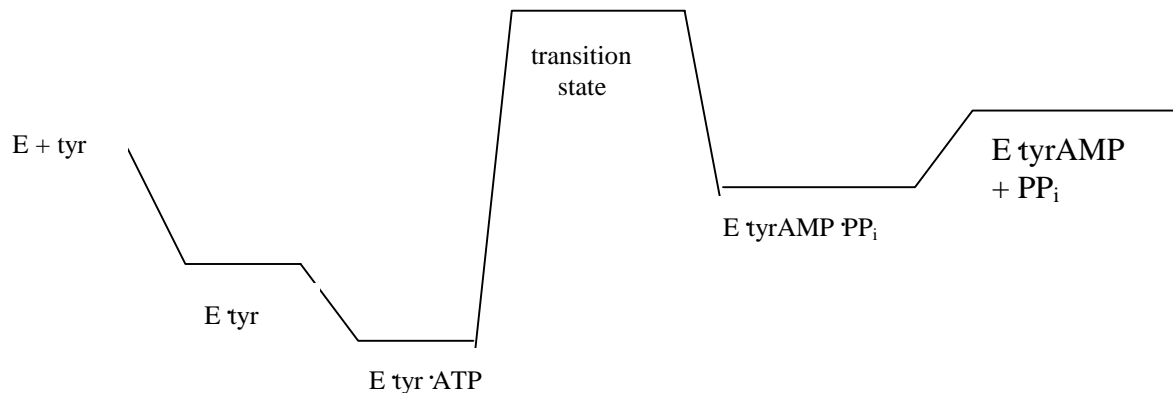
It is most easily assayed catalytically by exchange of  $^{32}\text{PP}_i$  into ATP, which is isolated by adsorption on charcoal and counted; the back reaction is rapid in presence of added  $\text{PP}_i$ , the slow step is reformation of  $E \cdot \text{tyrAMP}$ . It may also be assayed by charging of tRNA with  $[^{14}\text{C}]\text{-tyrosine}$ ; the tRNA is acid-precipitable (but of course this is a more expensive assay, because of the need for tRNA!). The exchange assay is however not good for quantitative kinetics, because it includes the reaction in both directions, and  $K_m$ s are therefore complex.  $k_{\text{cat}}$  for exchange =  $k_3 k_{-3} / (k_3 + k_{-3})$ , where  $k_{-3} = k_{-3}[\text{PP}_i] / K_{\text{pp}} + [\text{PP}_i]$ . Instead, advantage is taken of the fact that formation of enzyme-bound tyrosyl adenylate quenches protein fluorescence by 8% as compared to  $E \cdot \text{tyr}$ ; this may be followed by stopped-flow fluorescence - essentially comparable to following an absorbance change, but fluorescence, and over a period of 5 to 100 msec so that a single reaction is observed (pyrophosphatase is included in the reaction to hydrolyze  $\text{PP}_i$  produced and prevent the back reaction).  $K_{A'}$  (the  $K_d$  for ATP from the  $E \cdot \text{tyr} \cdot \text{ATP}$  complex) and  $k_3$  are determined from the rate of this reaction and its dependence on  $[\text{ATP}]$ ; note that when the  $[\text{ATP}]$  is  $\ll$  than  $K_{A'}$  the slope of  $k_{\text{obs}}$  vs  $[\text{ATP}]$  is essentially  $k_{\text{cat}}/K_{A'}$ . The binding of tyrosine is determined by equilibrium dialysis, though it could also be determined by fluorescence change, since binding tyrosine causes an 8% decrease in fluorescence before that due to binding ATP; but the interest is in determining the amount of tyrosine bound and thence the dissociation constant for tyrosine,  $K_T$ . The observed rate constant of binding is the sum of an on rate,  $2.4 \times 10^6 [\text{tyr}] \text{s}^{-1}$ , and an off rate,  $24.4 \text{ s}^{-1}$ . This on rate is actually much slower than expected if limited by diffusion, which would give a rate of  $10^8 \text{ s}^{-1}$  or greater; it suggests a conformational change in the enzyme on binding of tyrosine, though this has not been indicated by the X-ray crystallography. The binding of ATP is much weaker,  $K_A$  on the order of 2 mM, which is around the physiological concentration, while  $K_T$  is 10  $\mu\text{M}$ . One can also measure the rate of the reverse reaction, breakdown of enzyme-bound tyrosyl adenylate upon mixing with  $\text{PP}_i$ , by **increase** of fluorescence. This gives  $k_{-3}$ , and  $K_{\text{pp}}$ , the dissociation constant ( $\cong K_m$ ) for pyrophosphate, when  $\text{PP}_i$  is varied.

The order of binding of tyrosine and ATP is actually random, in the sense that both can combine with free enzyme, though the binding of ATP to free enzyme is too weak for a dissociation constant to be measured. However, the structure derived by X-ray crystallography suggests that ATP covers the tyrosine site so that tyrosine cannot bind to E·ATP, and the reaction is thus best described as ordered, tyrosine binding first, then ATP, with binding rapid compared to catalysis. When the rate of formation of E·tyrAMP is being measured by stopped-flow fluorescence the enzyme is generally pre-mixed with saturating tyrosine (0.2 mM), then mixed with ATP to start the reaction.

Other key points about this enzyme are: the three-dimensional structure has been determined by X-ray crystallography, although only the structure of the first 319 amino acids is seen, that of the last 100 being too disordered to give a clear picture. The first 319 are responsible for forming tyrosyl adenylate, the last 100 being needed only for binding tRNA and transferring tyrosine to it. A mutant with the last 100 a.a. deleted has been constructed; it is identical to the wild-type enzyme in forming tyrosyl adenylate, but unable to bind or acylate tRNA. Its crystal structure is very similar to that of the corresponding domain of the wild type enzyme; also, heterodimers can be formed, incorporating one native and one mutant subunit. When one subunit has a mutation suppressing tyrAMP formation *and* is shortened, it has half normal activity in formation of tyrAMP (exchange assay), as expected for one functional subunit, but essentially **no** activity in forming tyr-tRNA. The conclusion is that tyrAMP is formed on one subunit, but transferred to tRNA bound to the **other** subunit; the doubly mutant subunit can neither form tyrAMP for transfer to tRNA bound to the normal subunit, nor bind tRNA to receive tyrosine **from** the normal subunit.

Also, tyrosyl-tRNA synthetase, and probably most bacterial tRNA synthetases, show what is called half-of-the-sites reactivity: the dimeric enzyme bears only one equivalent of tyrAMP at a time. It appears that the enzyme, though a symmetrical dimer in the crystal, is asymmetric in solution; any given dimer knows which subunit will form tyrAMP and transfer it to tRNA bound on the other subunit. Most enzymes with half-of-the-sites reactivity either alternate in catalyzing the reaction (flip-flop' mechanism) or are mnemonic enzymes, i.e. binding of substrate, or catalysis itself, is what generates the asymmetry; but in this enzyme the asymmetry is preexistent, since in all heterodimers half of each kind forms tyrAMP and half doesn't. Some tRNA synthetases are monomeric, in the sense that they are a single polypeptide with mol. wt. double that of dimeric tyrosyl-tRNA synthetase, but are functionally dimeric, i.e. have a 50,000 dalton sequence repeated, with two active sites, but the same sort of half-of-the-sites reactivity. Many aminoacyl tRNA synthetases have editing mechanisms for removing the wrong amino acid if by chance they form the adenylate - see Fersht pp. 354-358 - but tyrosyl-tRNA synthetase is specific enough not to have to, this 'half-of-the-sites activity' does not serve this function.

The enzyme under study, tyrosyl-tRNA synthetase from *Bacillus stearothermophilus*, has been cloned in M13 and many specific single amino acid mutations prepared. This enzyme is heat-stable; the similar *E. coli* enzyme is removed by heating at 56°. Enough enzyme (10-20 mg) can be produced from one liter of *E. coli* containing the mutant gene in M13 to carry out several thousand assays or 30 ten-point equilibrium dialysis experiments. For each mutant,  $K_T$ ,  $K_A'$ ,  $k_3$ ,  $k_{-3}$  and  $K_{pp}$  are determined as described above and combined to give equilibrium constants for the various complexes, and thence  $\Delta G$  (relative to enzyme and free substrates) as follows:  $\Delta G_{E.tyr} = -RT \ln K_T$ ,  $\Delta G_{E.tyr.ATP} = -RT \ln K_T K_A'$ ,  $\Delta G^*$  of the transition state =  $RT \ln k_T/h - RT \ln(k_3/K_A' K_T)$ ,  $\Delta G_{E.tyrAM.PPPi} = -RT \ln(k_3/k_{-3} K_A' K_T)$ ,  $\Delta G_{E.tyrAMP} = -RT \ln(k_3 K_{pp}/k_{-3} K_A' K_T)$ . The energy levels of the intermediates can then be plotted as shown, and compared with those of the wild type enzyme.



$\Delta G_{\text{mut}}$  from the wild type energy level can be calculated from  $\Delta G_{\text{mut}} = -RT \ln(K_{\text{mut}}/K_{\text{wt}})$  (K is any one of the above combinations) and shown as bar graph; a bar above the base line corresponds to a positive  $\Delta G_{\text{mut}}$  and a less active enzyme, a bar below the base line corresponds to a negative  $\Delta G_{\text{mut}}$  and a more active enzyme (with respect to that particular complex).

The  $\Delta G_{\text{mut}}$  of one intermediate (or the logarithm of the relevant ratio of rate and equilibrium constants) may then be plotted vs the same for a later intermediate (each individual point in such a plot represents the two values for one mutant; the  $\Delta G$  values get larger as the mutant is less active), showing a **linear free energy relationship**,  $\Delta G_1 = \text{constant} + m\Delta G_2$ , similar to the Brønsted plot for general base catalysis,  $\log k = \beta \log K_a + \text{constant}$ , where a value of  $\beta$  close to 1 indicates that proton transfer to the base is nearly complete in the transition state, a value close to 0 indicates little proton transfer in the transition state. In the present case, a plot of  $\Delta G^*$ , or  $\log k_3$ , vs  $\Delta G_{\text{E.tyrAMP.PP}_i}$  or  $\log k_3/k_{-3}$  has a slope of 0.79, indicating that 79% of the change in binding energy (affected by the mutations) in the product complex is realized in the transition state. A plot of  $\Delta G_{\text{E.tyrAMP.PP}_i}$  vs.  $\Delta G_{\text{E.tyrAMP}}$  has a slope of 0.9, i.e. there is little change in effectiveness of binding between the product ternary complex and the E tyrAMP product binary complex. When the interactions are important only in the earlier complex, as for instance those with the  $\beta$ - and  $\gamma$ -phosphates of ATP in the transition state vs E tyrAMP, the slope  $m$  will be **much greater than 1** and the plot can be almost vertical. For a discussion of this see Kirsch, Protein Engineering 1:148-150 (1987), and for Fersht's own discussion see Nature 322: 284-6 (1986). One of the nice points is that if the point for a mutant lies on such a plot, it indicates that the mutation only affects the process under study directly, not by more far-reaching changes in protein structure, which would put the point off the line. For instance, the plot described above was of values for  $\Delta G^*$  and  $\Delta G_{\text{E.tyrAMP.PP}_i}$  of 13 mutants mainly affecting the ribose-binding site.

The enzyme-substrate interactions seen in the X-ray crystallographic structure of the E tyrAMP complex are shown in the handout. Gln<sup>173</sup>, asp<sup>78</sup> and tyr<sup>169</sup> H-bond to the  $\alpha$ -amino group of the tyrosine, asp<sup>176</sup> and tyr<sup>34</sup> to the phenolic OH, Cys<sup>35</sup> to the ribose 2'-OH of the AMP, thr<sup>51</sup> and his<sup>48</sup> to the ring oxygen of the ribose. Site-directed mutation of any one of these to a smaller, non-H-bonding residue decreased the  $\Delta G$  of formation of the E tyrAMP complex, by 0.5 to 1.5 kcal/mol for an H bond involving an uncharged acceptor, by 3 to 6 kcal/mol for a charged acceptor, though this overstates the binding

energy of the latter type of bond. For instance, mutation of any of asp<sup>78</sup>, tyr<sup>169</sup> and gln<sup>173</sup> weakens binding of tyrosine by 3 kcal/mole; asp<sup>78</sup> and gln<sup>173</sup> are in H-bonding networks and changing them has further effects on the binding of ATP and PP<sub>i</sub>, while changing tyr<sup>169</sup> has no such effects. Cys<sup>35</sup>, thr<sup>51</sup> and his<sup>48</sup> contribute nothing to binding of ATP in the E tyr·ATP complex, but contribute significantly in the transition state (i.e.  $\Delta G^*$  is larger when these are mutated), indicating that the transition state is significantly stabilized by forming such interactions. They contribute even more to the E tyrAMP complex, which would normally contradict the idea that complementarity is maximal in the transition state; but enzyme-product complementarity may be important either to shift an otherwise unfavorable equilibrium or to stabilize an unstable intermediate; both are true here. The phosphate-carboxylate anhydride bond of tyr-AMP has the highest free energy of hydrolysis known, 16.7 kcal/mole, yet the specific binding interactions make the formation of the E tyr-AMP complex from tyrosine + ATP have a favorable equilibrium constant, as mentioned earlier. The rate of hydrolysis is decreased from 0.076 s<sup>-1</sup> for the free compound to 5.14 x 10<sup>-5</sup> s<sup>-1</sup> for the enzyme-bound compound. The reaction is direct attack by OH<sup>-</sup>, as shown by its pH dependence. The mutant his<sup>48</sup> to gly shows an anomalously high rate of hydrolysis, explained as allowing OH<sup>-</sup> easier access to the tyr carbonyl. The **rate** constant of dissociation of tyrAMP from the enzyme,  $k_{-ta}$ , can be determined by forming the complex with [<sup>14</sup>C]-tyr and chasing with cold tyr, measuring the rate of loss of radioactivity from the enzyme; the equilibrium constant of dissociation,  $K_{ta}$ , can be calculated from the equilibrium constant of formation of the E tyr-AMP complex and that of free tyr-AMP. A plot of log  $k_{ta}$  vs. log  $K_{ta}$  for mutants in the tyr-AMP binding site has a slope  $\beta = 1.0$ , indicating that binding interactions act entirely through changes in the off rate, which is hardly surprising.

The X-ray structure does not indicate where the pyrophosphate, and the  $\beta$  and  $\gamma$ -phosphates of ATP, bind, but mutation of his<sup>45</sup>, thr<sup>40</sup>, arg<sup>86</sup> and lys<sup>230</sup> all affect the  $\Delta G^*$  of the transition state and the  $\Delta G$  of the E tyrAMP·PP<sub>i</sub> product complex, but not of any prior complex, suggesting that they form interactions in the transition state which persist in the product complex; lys<sup>82</sup> and lys<sup>233</sup> also bind to ATP in the E tyr·ATP complex, though lys<sup>82</sup> improves its interaction in the transition state. Lys<sup>230</sup> and lys<sup>233</sup> are on a mobile loop of the backbone, and their side chain positions cannot be seen at all the the X-ray structure; the backbone position in the free enzyme would not allow them to come within 8 Å of the pyrophosphate, the backbone must move in forming the transition state complex! Why do this, since it must take at least some energy? It further stabilizes the transition state, but if the lysines were in that position in 'free enzyme' the ATP wouldn't be able to get through them to bind; so this 'induced fit' takes place only when the ATP has already bound. The conformation in which this change has occurred would be a transient, high-energy intermediate just prior to the transition state of formation of E tyr-AMP·PP<sub>i</sub>. Mutations of lys<sup>230</sup> and lys<sup>233</sup> affect  $K_{ta}$ , the calculated dissociation constant of E tyr-AMP, but not  $k_{ta}$ , the off rate; so they would have to affect  $k_{ta}$  the on rate, indicating that this conformational change would be partially rate-limiting for the binding reaction, while the other mutations do not affect  $k_{ta}$  because they do not affect the conformational change. Mutation of lys<sup>233</sup> to alanine decreases affinity of the enzyme for ATP and introduces positive cooperativity (sigmoidal dependence of rate on ATP concentration; binding of ATP to either subunit enhances its binding to the other, and the loop from one subunit is coupled to the active site in the other subunit). Binding of ATP is strengthened, the cooperativity is lost and M-M kinetics restored at 0.5 M NaCl or by mutating either lys<sup>230</sup> or thr<sup>234</sup> to alanine.

Thr<sup>234</sup>, also on that loop and part of a conserved sequence (almost always thr or ser), is important in stabilization of the transition state, as shown by a 500-fold decrease in  $k_3$  - increase in activation energy of 2.7 kcal/mole - when it is mutated to ala. Mutation to ser has little effect. The hydroxyl probably interacts in some way with the Mg<sup>++</sup> required in the reaction, as shown by a further destabilization of the transition state by 1.7 kcal/mole on substituting Cd<sup>++</sup> for Mg<sup>++</sup>, not seen with the wild-type enzyme. Other mutations in the loop have only small effects, but the overall effect of the loop, as seen in a mutant where it is deleted, is to destabilize the ground-state E-tyr-ATP complex and stabilize the transition state. The loop is involved at the expense of ATP binding energy. It occurs to me - Fersht is too cautious - that the previously mentioned asymmetry of the enzyme in solution - not seen in the crystal structure - could be due to the lys<sup>230</sup>-thr<sup>234</sup> loop being in different positions in the two subunits, thus selecting which subunit ATP could bind to.

The conclusion is that the enzyme catalyzes just by optimal binding in the transition state, without any chemical catalysis such as general acid or general base. Tyrosine binds through the interactions mentioned, then ATP binds, with few specific interactions that have been identified, but including some interaction with lys<sup>82</sup> and lys<sup>233</sup>. In the transition state these improve their interactions, and arg<sup>86</sup>, thr<sup>40</sup>, his<sup>45</sup> and lys<sup>230</sup> also interact with the  $\beta$ - and  $\gamma$ -phosphates of ATP, pulling them away from the  $\alpha$ -phosphate and facilitating the attack of the tyrosine carboxyl on its other side. Cys<sup>35</sup>, his<sup>48</sup> and thr<sup>51</sup> also form interactions with the ribose 2' and ring oxygens. In the product ternary complex, the interactions with the pyrophosphate are improved further, but PPi then dissociates, and the lys<sup>230</sup>/lys<sup>233</sup> loop moves away from the tyrosyl adenylate, which is further stabilized by lys<sup>82</sup> binding to the  $\alpha$ -phosphate via a water molecule. A further interaction, recently investigated by mutation, is that of glu<sup>235</sup>, presumably binding the Mg<sup>++</sup> which is coordinated to the  $\beta$ ,  $\gamma$  phosphates of the ATP; changing this to ala puts the dissociation constant of ATP out of sight.

The *E. coli* enzyme differs from that of *Bacillus stearothermophilus* at only one active site residue, thr<sup>51</sup>, which is proline in the *E. coli* enzyme, alanine in *Bacillus caldotenax*, which differs from *B. stearothermophilus* at only three other positions. Mutating thr<sup>51</sup> in the *B. stearothermophilus* enzyme to ala, cys and pro results in successively lower  $K_M$  for ATP;  $k_{cat}$  also decreases, but less so (these are  $k_{cat}$  and  $K_m$  in the aminoacylation of tRNA; similar but less consistent trends are seen in the exchange assay, in which for some reason they didn't try the proline mutant). Thus different mutants are the most active at different ATP concentrations, the wild-type at the highest [ATP] since it has the highest  $k_{cat}$  though the highest  $K_m$ , the others are optimal at successively lower [ATP] It suggests that the cytoplasmic [ATP] is lower in *E. coli* than in *B. stearothermophilus*! In terms of the mechanism, the mutants use too much binding energy in merely binding ATP and have less available to stabilize the transition state.