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Structure and activity of the tyrosyl-tRNA synthetase: the hydrogen bond in catalysis and specificity

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The role of hydrogen bonding in specificity, binding and catalysis by the tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* has been investigated by systematic mutation of residues which form hydrogen bonds with substrates during the reaction between ATP and tyrosine to form tyrosyl adenylate.

Data on hydrogen bonding as a determinant of biological specificity are summarized thus: deletion of an hydrogen-bond donor or acceptor between the enzyme and substrate to leave an unpaired but uncharged acceptor or donor weakens binding by only 2–7 kJ mol^{-1}; but deletion to leave an unpaired but charged acceptor or donor weakens binding by some 17 kJ mol^{-1} or so.

Hydrogen bonding is found to have a profound role in catalysis by mediating the differential binding of substrates, transition states and products. The formation of tyrosyl adenylate is not catalysed by classical mechanisms of acid–base or nucleophilic catalysis but the enhancement of rate is solely a result of a combination of hydrogen bonding and electrostatic interactions which stabilize the transition state of the substrates relative to their ground states. The binding energy of ATP increases by more than 29 kJ mol^{-1} as it passes through the transition state, enhancing the rate by more than a factor of 10^5. The residues involved in differential binding are spread over the molecule, away from the seat of reaction. The catalysis is delocalized over the whole binding site and not restricted to one or two specific residues.

Some regions of the binding site are complementary in structure to the intermediate, tyrosyl adenylate. The apparent binding energies of certain side chains increase as the reaction proceeds, being weakest in the enzyme–substrate complex, stronger in the enzyme–transition-state complex and strongest in the enzyme–intermediate complex. This converts the unfavourable equilibrium constant for the formation of tyrosyl adenylate in solution to a favourable value for the enzyme-bound reagents and helps sequester the reactive tyrosyl adenylate.

1. Introduction

The distinctive feature of enzyme catalysis is the utilization of the binding energy of enzyme and substrate to provide both the specificity of catalysis and enhancement of catalytic rate: complementary interactions between enzyme and ligands give specificity of binding; complementary interactions between enzyme and transition states provide catalysis (Pauling 1946). Knowledge of how binding energy is used is crucial to the understanding of enzyme catalysis and the design of new enzymes. The relation between structure and activity in enzyme catalysis has, until now, been studied by experiments in which the structure of the substrates or ligands only has been systematically modified. However, by using the technique of site-directed mutagenesis, described by Smith (this symposium), it is now possible to study enzyme structure and activity directly by the systematic modification of the structure of the enzyme. The goals of the present study are, by using directed mutagenesis: (i) to measure empirically the

contributions of individual side chains on the enzyme to the binding energy with the substrate; and (ii) to analyse directly the effects on catalysis of small changes in enzyme structure which alter the binding energy of the enzyme and substrate. The side chains examined here are those that form hydrogen bonds with the substrates.

This study was initiated in 1982 (Winter *et al.* 1982), and earlier work has been summarized by Fersht *et al.* (1984).

Strategy

The strategy used in this study is to make small, conservative, changes in enzyme structure by replacing side chains of amino-acid residues in the active site by smaller side chains. The side chains concerned do not interact directly with the atoms involved in bond making and breaking but bind to the substrate away from the seat of the reaction. The parts of the side chains that make interactions with the substrate are removed and so the binding energy is altered. Mutant enzymes of changed, but measurable, activity are thus produced, and so it is possible to quantify the interactions. This approach and the analysis of the kinetic consequences of mutation are based upon the application by Fersht (1974, 1985) of transition-state theory to enzyme–substrate complementarity and catalysis. The equations were formulated to analyse the effects on the constants k_{cat} and K_M of the Michaelis–Menten equation, of altering the binding energy between enzyme and substrate (or transition state of the substrate). Suppose a side chain of an enzyme interacts with the substrate in the transition state to contribute a binding energy ΔG_b, relative to enzyme and substrate separately in solution. If removal of the side chain in a mutant enzyme causes just the loss of that interaction energy and no changes in the structure of the enzyme or enzyme–substrate complex, then

$$\Delta G_b = RT \ln [(k_{cat}/K_M)_{mut}/(k_{cat}/K_M)_{wt}], \quad (1)$$

where subscripts mut and wt refer to mutant and wild-type enzymes respectively, R is the gas constant and T the absolute temperature. In practice, removal of a side chain may cause additional effects because of subtle changes in the structure of the enzyme. We therefore prefer to use the term ΔG_{app}, the *apparent binding energy* of the side chain, defined by (2).

$$\Delta G_{app} = RT \ln [(k_{cat}/K_M)_{mut}/(k_{cat}/K_M)_{wt}] \quad (2)$$

ΔG_{app} contains any artefacts associated with changes of enzyme structure but, where these do not occur, $\Delta G_{app} = \Delta G_b$. Thus, the contributions of individual side chains to the overall binding energy of the enzyme and substrate may be measured by preparing a series of mutant enzymes that differ from wild type by lacking the relevant side chains. Determination of k_{cat}/K_M for each enzyme will give the apparent binding energy by application of (2).

Measurement of binding energy in the transition state by (2) is the most reliable method of detecting and quantifying interactions (Fersht 1985). The more obvious procedure of making direct measurements of enzyme–substrate dissociation constants often underestimates the full magnitudes of binding energies because phenomena such as strain, induced fit and non-productive binding cause changes in binding energy to be manifested in changes in catalytic rate constants and not changes in dissociation constants. However, these effects cancel out in k_{cat}/K_M, as do certain consequences of steady-state kinetics that obscure individual values of k_{cat} and K_M. Further, binding energies tend to be maximized in the transition state. Because strain and induced fit are manifested by binding energy being used to change rate and not

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dissociation constants, measurement of apparent binding energies in enzyme–substrate complexes may be used to detect and quantitate these phenomena.

Tyrosyl-tRNA synthetase

An enzyme chosen for a systematic study, by site-directed mutagenesis, which requires the accurate comparison of kinetic data and its relation to structure should possess the following characteristics: (i) the crystal structure should be known at high resolution; (ii) the crystal structure of enzyme–substrate or enzyme–ligand complexes should also be known either directly or from model building; (iii) a means of active-site titration must be available so that concentration of mutants may be measured with certainty – without this, accurate steady-state kinetics cannot be performed; (iv) an enzyme-bound intermediate or product should accumulate on the pathway in the experimentally accessible time range – this provides individual rate and dissociation constants from pre-steady-state kinetics. The tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* is suitable for such a study. It catalyses the amino-acylation of $tRNA^{Tyr}$ in a two-step reaction (equations (3) and (4); Fersht & Jakes (1975)).

$$E + Tyr + ATP = E \cdot Tyr\text{–}AMP + PP_i, \tag{3}$$

$$E \cdot Tyr\text{–}AMP + tRNA^{Tyr} = Tyr\text{–}tRNA^{Tyr} + AMP + E. \tag{4}$$

In the absence of pyrophosphate or $tRNA^{Tyr}$, the enzyme-bound tyrosyl adenylate is stable and may be isolated and handled in solution. The enzyme is a dimer of relative molecular mass, M_r, $2 \times 47\,500$ (Koch 1974; Irwin *et al.* 1976). The nucleotide sequence of the gene has been determined from a clone in the vector pBR322 (Winter *et al.* 1982). X-ray crystallographic studies on the native enzyme and on a truncated form prepared by mutagenesis (Waye *et al.* 1984) have been performed at 0.21 and 0.25 nm resolution respectively (Blow & Brick 1985) and the structure is described by Brick (this symposium). Most importantly, the crystal structure of the enzyme-bound tyrosyl-adenylate complex has also been solved (Rubin & Blow 1981) so that there is the rare opportunity of directly knowing the interactions of the enzyme with a substrate (figure 1). The enzyme is readily assayed by active-site titration so that accurate and reproducible steady-state kinetic measurements may be made (Fersht *et al.* 1975*a*); the partial reaction of activation (3) and the overall charging reaction are readily determined by easy assays; pre-steady-state kinetics of activation may be followed by stopped-flow fluorescence studies (Fersht *et al.* 1975*b*), and pre-steady-state kinetics of the transfer step (4) followed by rapid quenching methods (Fersht & Jakes 1975). The enzyme also has interesting properties because it is a dimer. Kinetic studies show that the two active sites interact so that only one mole of tyrosine is bound tightly per two active sites in solution (Fersht 1975) as is only one mole of $tRNA^{Tyr}$ (Jakes & Fersht 1975; Dessen *et al.* 1982) and tyrosyl adenylate.

The tyrosyl-tRNA synthetase from *B. stearothermophilus* – a thermophilic organism – has one further very important property: the enzyme is thermostable and loses no activity on prolonged incubation at 56 °C, unlike the enzyme from *Escherichia coli*, which is rapidly denatured. As the mutant tyrosyl-tRNA synthetases used in the studies are produced from the clones in *E. coli*, provided the mutation does not reduce thermostability, any background native enzyme from *E. coli* can be readily removed by heating. Thus, weakly active mutants may be studied without interference from active native enzyme.

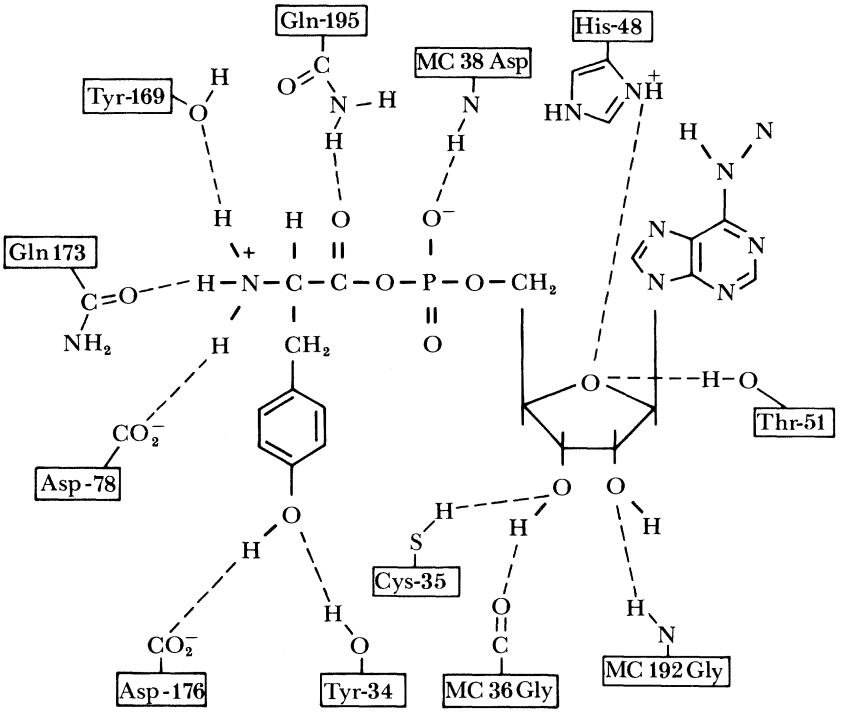

FIGURE 1. Sketch of hydrogen bonds between tyrosyl-tRNA synthetase and tyrosyl adenylate; MC represents main chain. (Courtesy of Professor D. M. Blow.)

A striking feature of the structure of the enzyme–substrate complex is the number and variety of hydrogen bonds that are made (figure 1). We have chosen to modify these first to investigate the role of hydrogen bonding in specificity and catalysis.

2. THE HYDROGEN BOND IN BINDING AND SPECIFICITY

The hydrogen bond is a ubiquitous feature of biological interactions: it has an essential role in determining the structure of proteins and nucleic acids; it is a major determinant of specificity in enzyme catalysis and in biological information transfer; and it can directly influence the rate of enzymic reactions by stabilizing ionic charges that are formed in the transition state. Hydrogen bonding in macromolecules and their complexes in aqueous solution is a complex phenomenon because water competes for the hydrogen-bonding sites (Kauzmann 1959; Klotz & Franzen 1962). The calculation of the overall energetics is consequently difficult and there is little knowledge of the energies involved (Cantor & Schimmel 1980). The absolute strengths of hydrogen bonds *in vacuo*, that is, the enthalpy changes in (5), where —H is a hydrogen-bond donor and —B is an acceptor have been calculated for use in molecular dynamics calculations.

$$\text{X—H}\cdots\text{B—Y} = \text{X—H} + \text{B—Y}. \qquad (5)$$

For example, representative values for the enthalpies of stabilization for different donors and acceptors are: water–water, -27 kJ mol^{-1}; water–CH_3SH, -13 kJ mol^{-1} for —SH = acceptor, -13.4 kJ mol^{-1} for —SH = donor; imidazolium–water, -59 kJ mol^{-1}; acetate–water,

-53 kJ mol^{-1} (Weiner *et al.* 1984). But hydrogen bonding in aqueous solution must be analysed according to (6) (Jencks 1969; Hine 1972).

$$\mathrm{E{-}H\cdots OH_2 + HOH\cdots B{-}S = E{-}H\cdots B{-}S + HOH\cdots OH_2.} \qquad (6)$$

Here, for convenience, it is assumed that the enzyme has a hydrogen bond donor, —H, and the substrate an acceptor, —B, which pair in the enzyme–substrate complex. In the free enzyme, —H is bound to a water molecule and so is —B. The number and types of hydrogen bonds are conserved in the reaction, which is thus essentially isoenthalpic (within the limits of Hine's (1972) equation). There is thus a crucial distinction between the absolute energy of a hydrogen bond as in (5) and the overall energies of hydrogen bonding in solution. It is the overall energetics that are important in the binding of enzymes and substrates, and it is the overall energetics that we can measure from site-directed mutagenesis.

The exchange nature of hydrogen bonding in aqueous solution has two important consequences. First, as discussed by Wilkinson *et al.* (1983), an —SH group, for example, is just as effective a hydrogen-bond donor–acceptor as an —OH group, despite the different absolute strengths of —SH···O and —OH···O bonds in (5): if, in (6), —H is part of an —SH group, then there is an —SH···O bond on the left-hand side and an —SH···O bond on the right in the enzyme–substrate complex. Second, the removal of one of the hydrogen-bonding groups from (6), e.g. a side chain of the enzyme, as in (7), does not necessarily lead to the loss of the absolute binding energy (enthalpy) of a hydrogen bond as defined by (5).

$$\mathrm{E\ OH_2 + HOH\cdots B{-}S = [E\ B{-}S] + HOH\cdots OH_2.} \qquad (7)$$

A crude inventory of the hydrogen bonds on each side of (7) shows that the number of bonds is, to a first approximation, conserved. An alternative way of viewing the situation is that deletion of a hydrophilic side chain means that a water molecule must be located next to a hydrophobic region of the enzyme in the space vacated. This water molecule is at a high energy because it is at an interface and not in bulk water where it can readily fulfil all its hydrogen bonding. There is no hydrogen bond from E to H_2O in the free enzyme and so this partly (or even fully) compensates for the lack of the hydrogen bond from E to —B in the enzyme–substrate complex: when the substrate binds, it displaces the high-energy water. The overall energetics of reaction (7) depend on the precise interactions made by the mutant enzyme with the water molecule and the group —B on the substrate. Therefore, deletion of the hydrogen bond in the enzyme–substrate complex does not lead to the overall loss of the absolute strength of a hydrogen bond and may, in certain circumstances, cause no loss of binding energy.

Experimental binding energies

The apparent binding energies of the side chains have been determined (Fersht *et al.* 1985*a*; table 1) and may be summarized and classified thus: (i) deletion of a side chain on the enzyme that forms a hydrogen bond with an uncharged group on the substrate weakens binding energy by only about 2–7 kJ mol^{-1}; (ii) deletion of an uncharged side chain on the enzyme that forms a hydrogen bond with a charged group on the substrate weakens binding by approximately 14–19 kJ mol^{-1} (only two relevant experiments were performed for this category, and so the range could be much wider, but the crucial point is that these energies are considerably higher than in (i)); (iii) deletion of a group that forms a 'too-long' hydrogen bond actually improves binding.

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These data, which have been discussed in detail elsewhere (Fersht *et al.* 1985*a*), may be rationalized by performing simple hydrogen bond inventories, as listed in figure 2. For example, deletion of a donor or acceptor which forms a bond with a charged acceptor or donor, to leave it unpaired in the enzyme–substrate complex results in the loss of a strong hydrogen bond on the left-hand side of the equation in figure 2 and is only partly compensated for by the gain of a weaker water–water bond on the right-hand side.

		inventory
(*a*)	$E{-}H\cdots OH_2 + HOH\cdots B{-}S = [E{-}H\cdots B{-}S] + HOH\cdots OH_2$	0
(*b*)	$E\ OH_2 + HOH\cdots B{-}S = [E\ B{-}S] + HOH\cdots OH_2$	0
(*c*)	$E\ OH_2 + HOH\cdots {}^{-}B{-}S = [E\ {}^{-}B{-}S] + HOH\cdots OH_2$	−s+w
(*d*)	$E{-}H^{+}\cdots OH_2 + HOH\ S = [E{-}H^{+}\ S] + HOH\cdots OH_2$	−s+w
(*e*)	$E{-}H\cdots OH_2 + HOH\cdots B{-}S = [E{-}H\ B{-}S] + HOH\cdots OH_2$	−1

FIGURE 2. Hydrogen bond inventory on binding substrates to wild-type and mutant enzymes. (*a*) Wild-type enzyme with proton donor —H on enzyme and acceptor B— on substrate; (*b*) removal of —H (plus side chain) from E–H; (*c*) as (*b*), but acceptor B— is charged, resulting in the loss of one strong (s) hydrogen bond and the gain of one weak (w) water–water bond: (*d*) deletion of B— from substrate when —H is charged gives qualitatively the same changes as in (*c*); (*e*) constraints in the enzyme–substrate complex which prevent a bond between —H and B— in the enzyme–substrate complex.

TABLE 1. APPARENT HYDROGEN BOND ENERGIES

amino-acid residue	side chain	apparent binding energy[a]/(kJ mol^{-1})
	deletion of bonds to uncharged residues	
Tyr-34	—OH	−2.2
Cys-35	—SH	−4.8
His-48	imidazole	−4.0
Asn-48[b]	—$CONH_2$	−3.2
Cys-51[c]	—SH	−2.0
	deletion of bonds to charged residues	
Tyr-169	—OH	−15.6
Tyr (substrate)	—OH	−22.8
	deletion of 'too-long' bonds	
Ser-35[d]	—OH	4.9
Thr-51[c]	—OH	5.9

[a] Calculated from (2).
[b] The tyrosyl-tRNA synthetase from *Bacillus caldotenax*, which is 99% homologous with the enzyme from *B. stearothermophilus* (M. D. Jones, T. Borgford, D. M. Lowe & A. R. Fersht, unpublished results), has Asn-48.
[c] The distance between the β-carbon and the ribose ring oxygen is suitable for an —SH···O bond but too long for an —OH···O.
[d] From mutation of Cys→Ser-35.

Evidence has been found that the bond between Thr-51 and the ribose ring oxygen is longer than optimal: it was found that deletion of the bond (Thr→Ala-51) leads to an increase in enzyme–transition-state affinity (Wilkinson *et al.* 1984; Carter *et al.* 1984). This is easily rationalized by the inventory at the bottom of figure 2. More direct evidence for the bond being too long has now been provided by substituting —SH for —OH in the side chain (Thr→Cys-51).

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The —SH···O< hydrogen bond is longer than the —OH···O< bond, and the mutant TyrTS(Cys-51) is found to bind ATP more tightly (by 4 kJ mol^{-1}) than does wild-type enzyme (Fersht *et al.* 1985*b*).

Do the apparent binding energies correspond to the true binding energies?

The hydrogen-bond donors–acceptors that are removed on mutagenesis are the only portions of the side chains that are in direct contact with the substrate. Model building suggests that the mutations listed in table 1 should not cause structural changes in the protein. Nevertheless, the mutation of an amino acid in a protein from a larger side chain to a smaller may have further structural consequences, which will be superimposed upon the energetics of the hydrogen bonding. Any fine-structure analysis must consider these possibilities, and attempt to rule out artefacts. The obvious experimental procedure is to crystallize the mutants and use protein crystallography to detect structural changes. Such a technique will detect changes of the order of 0.1 Å† or more, but will miss a series of smaller changes. Further, the effects of small relaxations of protein structure on binding are not known. Because kinetic measurements are exceptionally sensitive and can detect changes of a fraction of a kilojoule, we are using kinetic methods to increase the reliability of analysis. For example, application of the 'double-mutant' procedure of Carter *et al.* (1984) has shown that mutation of residues 35 and 48 does not cause structural changes that are propagated through the protein. To minimize artefacts, we have analysed many different mutants so that trends may be observed and exceptions noted. The combination of the extensive and consistent kinetic data and modelling by molecular graphics suggests that the results so far reflect the direct effects of hydrogen bonding *per se* and are not dominated by structural change. However, in some cases, as in the mutation His→Gly-48, sufficient room is left on mutation that water may penetrate the structure of the enzyme and form hydrogen bonds with the substrate. A hydrogen bond inventory of this type of process shows that, to a first approximation, the effects should be similar to the presence of an unpaired hydrogen-bond donor or acceptor when it is uncharged, but the presence of water will attenuate the effect of having a charged donor or acceptor (Fersht *et al.* 1985*a*).

Implications for biological specificity

The experimental data give the apparent binding energies of the different types of hydrogen bonds, the values that occur in practice and dictate biological specificity. Irrespective of any small structural effects that there are on deletion of side chains, a side chain that forms a hydrogen bond between the enzyme and an uncharged group on a substrate provides only 2–7 kJ mol^{-1} of binding energy relative to the absence of the side chain. This means that an unpaired, uncharged, hydrogen bonding species provides a factor of only 2.5 to 15 or so towards specificity. But, the absence of a group on the enzyme or a substrate that should form a hydrogen bond with a charged group affects binding by *ca.* 17 kJ mol^{-1} and is worth a factor of 10^3 in specificity. Thus, specificity is caused to some extent by hydrogen bonding but is best mediated by charged residues. The important driving force for high specificity in complementary interactions between biological molecules is the avoidance of unsolvated charged and unfavourable steric interactions between the two components.

† 1 Å = 10^{-1} nm = 10^{-10} m.

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3. The hydrogen bond in catalysis

Although the amino-acid residues of TyrTS which interact with the bound tyrosyl adenylate are known from the crystallographic studies, there is no immediate indication of how they catalyse the reaction. The chemical mechanism of the reaction (figure 3) involves the nucleophilic attack of the carboxylate group of tyrosine on the α-phosphoryl group of ATP to generate a pentacoordinate transition state, followed by elimination of magnesium pyro-

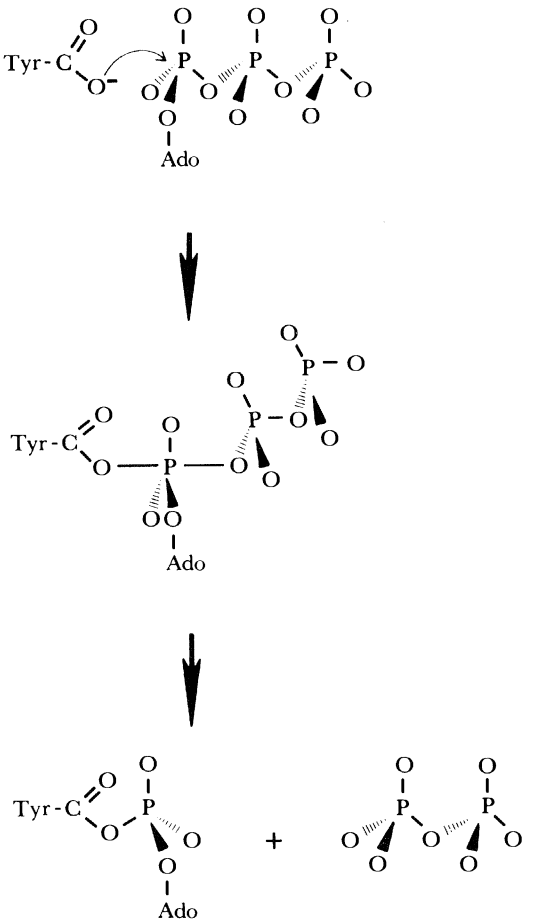


Figure 3. Minimal chemical mechanism for the formation of tyrosyl adenylate (from Leatherbarrow *et al.* 1985).

phosphate, with inversion at the α-phosphoryl group (Lowe & Tansley 1984). The classical enzymic processes of acid–base or covalent catalysis do not seem to be applicable to this reaction, as it consists of the attack of a fully ionized good nucleophile on an activated compound with a good leaving group. To date, no studies on any aminoacyl-tRNA synthetases have implicated residues involved in catalysis. It thus appears likely that the enzyme must use a strain mechanism, whereby there are groups on the enzyme that interact better with the transition state than the unaltered substrate: the concept of differential binding of substrates and transition states. The improvement in binding energy on going from substrate to transition state lowers the activation energy of the reaction, and so increases the catalytic rate.

Site-directed mutagenesis allows the idea of differential binding of transition states and substrates to be tested directly by varying the structure of the enzyme. An amino-acid side chain which interacts with the substrate can be altered to remove the interaction. If the side chain binds equally well with the substrate in both the ground and transition states, then removal should raise K_S and not affect k_{cat}. Conversely, if the side chain binds the substrate only in the transition state, then removal should leave K_S unaltered, and just lower the value of k_{cat}. Importantly, just one interaction out of many with the substrate may be changed at a time, so artefacts associated with a change of mode of binding of the substrate will be minimized. The contributions of individual interactions to differential binding energies are not known and this approach enables them to be quantified. It has been pointed out that hydrogen bonds have potential energy functions that are suitable for mediating differential binding effects (Fersht 1974).

Transition-state stabilization by improved binding of the γ-phosphoryl group of ATP is a major catalytic factor in nucleophilic attack at the α-phosphoryl group

Model building of the probable transition state in the reaction pathway was used to identify catalytic interactions with the enzyme (Leatherbarrow *et al.* 1985). The structure of the enzyme-bound tyrosyl adenylate complex is known, and so it is possible to build the transition state for the formation of tyrosyl adenylate by the addition of pyrophosphate to its α-phosphoryl moiety. It was found that the γ-phosphate group of the intermediate could be placed to interact with the side chains of Thr-40 and His-45 (figure 4). These side chains are positioned so that each can make similar polar interactions via the β-OH and $N_\varepsilon H$ groups respectively. It should

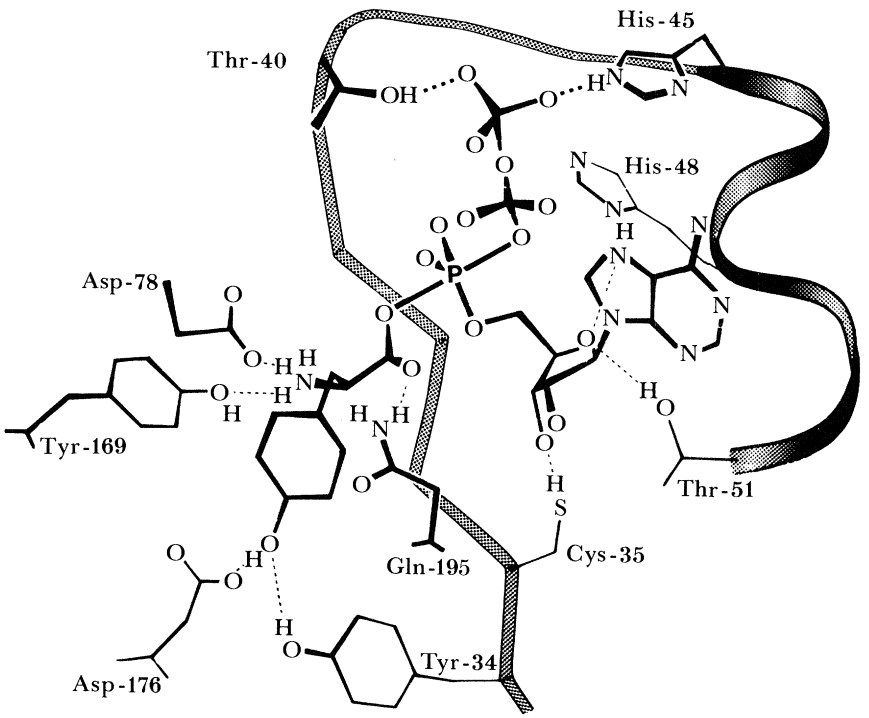
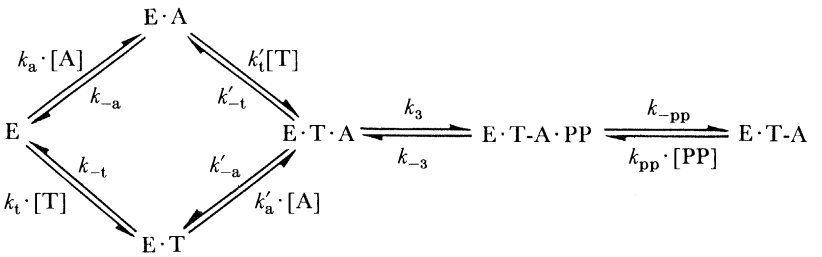


FIGURE 4. Model of pentacoordinate transition state for (or intermediate in) the formation of tyrosyl adenylate by the tyrosyl-tRNA synthetase (from Leatherbarrow *et al.* 1985).

be noted that His-45 is conserved in various aminoacyl-tRNA synthetases (Barker & Winter 1982; M. D. Jones, T. Borgford, D. M. Lowe & A. R. Fersht, unpublished data).

Mutations at positions 40 and 45 of the tyrosyl-tRNA synthetase have little effect on the dissociation constants of tyrosine, ATP and tyrosyl adenylate from the enzyme (table 2). The binding of pyrophosphate, however, is severely weakened (data not shown). The most striking feature is the large reduction of the rate constant k_3 in scheme 1. The absence of large effects



Scheme 1. Reaction scheme for the formation of tyrosyl adenylate (T-A) and pyrophosphate (PP) from tyrosine (T) and ATP (A). Rate constants are as defined in the scheme. Dissociation constant of tyrosine from $E \cdot T = K_t = k_{-t}/k_t$, dissociation constant of ATP from $E \cdot T \cdot A = K'_a = k'_a/k'_{-a}$, etc.

Table 2. Effect of mutations in γ-phosphoryl group binding site[a]

amino-acid residue at positions				
40	45	K'_a (ATP)/mM	K_t (Tyr)/μM	k_3/s^{-1}
Thr	His (wild-type)	4.7	12	38
Thr	Gly	1.2	10	0.16
Ala	His	3.8	8	5.5×10^{-3}
Ala	Gly	1.1	4.5	1.2×10^{-4}

[a] From Leatherbarrow *et al.* 1985. See scheme 1 for definition of constants and figure 4 for residues. Pre-steady-state data at 25°, pH 7.78 and 10 mM $MgCl_2$ (free).

on the binding of both substrates and enzyme-bound tyrosyl adenylate strongly indicates that it is unlikely that there is any serious structural change in the enzyme on mutation of those residues. Instead, it is indicated that there is a weakening of binding of pyrophosphate in both the transition state and E·Tyr–AMP·PP complexes. Thr-40 and His-45 thus appear to form a binding site for the γ-phosphoryl group of ATP in the transition state and for pyrophosphate in the reverse reaction, but not for ATP in the enzyme–substrate complex.

Mutation of the γ-phosphoryl binding site (Thr→Ala-40, His→Gly-45) results in an increase in the energy level of the transition-state complex, while the energy levels of the enzyme–substrate complex are unaltered (figure 5). This results in increased activation energy and consequently lowered k_3. These kinetic studies (Leatherbarrow *et al.* 1985) therefore provide direct evidence for stabilization of the transition state as a major factor in the catalytic mechanism of this enzyme.

A possible mechanism for the mediation of differential binding (figure 6) is that the enzyme takes advantage of the large change in bond angles about the α-phosphoryl group as it goes from four- to five-coordinate. The movement concomitant on change in angle is amplified at the γ-phosphoryl group. It is possible that the γ-phosphate of ATP in the E·Tyr·ATP complex does not bind between Thr-40 and His-45 but just remains solvated by water. During the

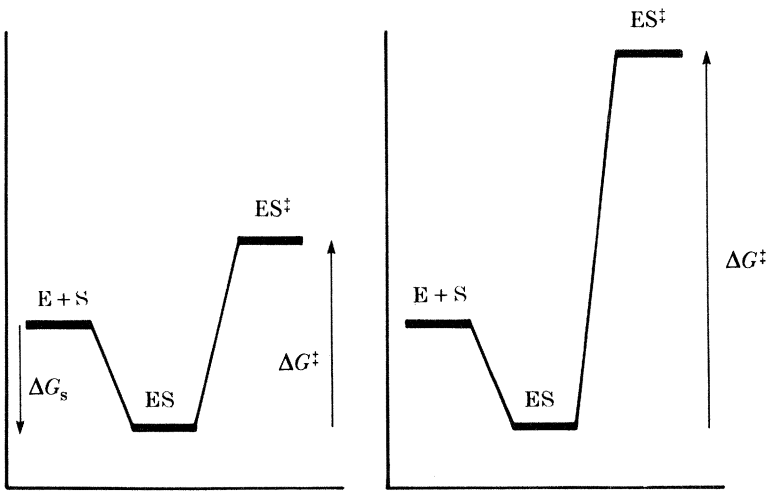


FIGURE 5. Change in Gibbs free energy on mutation of an enzyme to remove a group which binds to the transition state of the substrate but not the unreacted substrate. The free energy of activation is increased but free energy of formation of the enzyme–substrate complex (ΔG_s) is unaffected (from Leatherbarrow *et al.* 1985).

reaction, it swings into its binding site and releases the solvated water. Preliminary data on the activation energies of wild-type and TyrTS(Thr → Ala-40) support this model because both enzymes have similar enthalpies of activation, but the wild-type enzyme has a more positive entropy of activation (A. R. Fersht & J. Knill-Jones, unpublished results). Direct structural evidence on the location of bound ATP would help resolve the mechanistic possibilities. Unfortunately, it has so far proved impossible to solve the crystal structure of the enzyme–ATP complex by X-ray diffraction because of poor binding (Montheilet *et al.* 1984).

Further catalysis by differential binding of ATP far from the seat of reaction

Rapid reaction kinetics and equilibrium binding measurements have been performed on several mutant enzymes to determine all the enzyme–substrate dissociation constants and the rate constants k_3 and k_{-3} in scheme 1 (Wells & Fersht 1985 and unpublished results). Removal of side chains that bind to tyrosine (Tyr → Phe-34 and Tyr → Phe-169) does not significantly alter k_3 but increases the dissociation constant of tyrosine from the E·Tyr·ATP complex (table 3). It is thus inferred that the hydroxyl moieties of Tyr-34 and Tyr-169 interact equally well with tyrosine when it is in its unreacted form and when it is in the transition state of the reaction. The binding energies of the —OH groups of Tyr-34 and Tyr-169 with the substrate and transition state are readily calculated and are seen to be nearly identical (table 4). In contrast, removal of side chains that interact with ATP causes significant changes in k_3 but, apart from His → Gly-48, hardly affects the value of K'_S for ATP. Mutation of Cys-35 to Gly-35 or Ser-35 leads to a tenfold lowering of k_3 with no significant change in K'_a. On changing His → Gly-48, there is a mixture of effects with a fourfold lowering of k_3 and a twofold increase in K'_a. It is thus inferred that the side chains of residues 35 and 48 have lower interaction energies with ATP when it is in its unreacted form and when it is in its transition-state structure.

Catalysis of formation of tyrosyl adenylate is 'delocalized' around the binding site. Each of the side chains of Cys-35, Thr-40, His-45 and His-48 makes a contribution to rate enhancement by differential binding. It is calculated that this amounts to lowering the activation energy by some 29 kJ mol^{-1}.

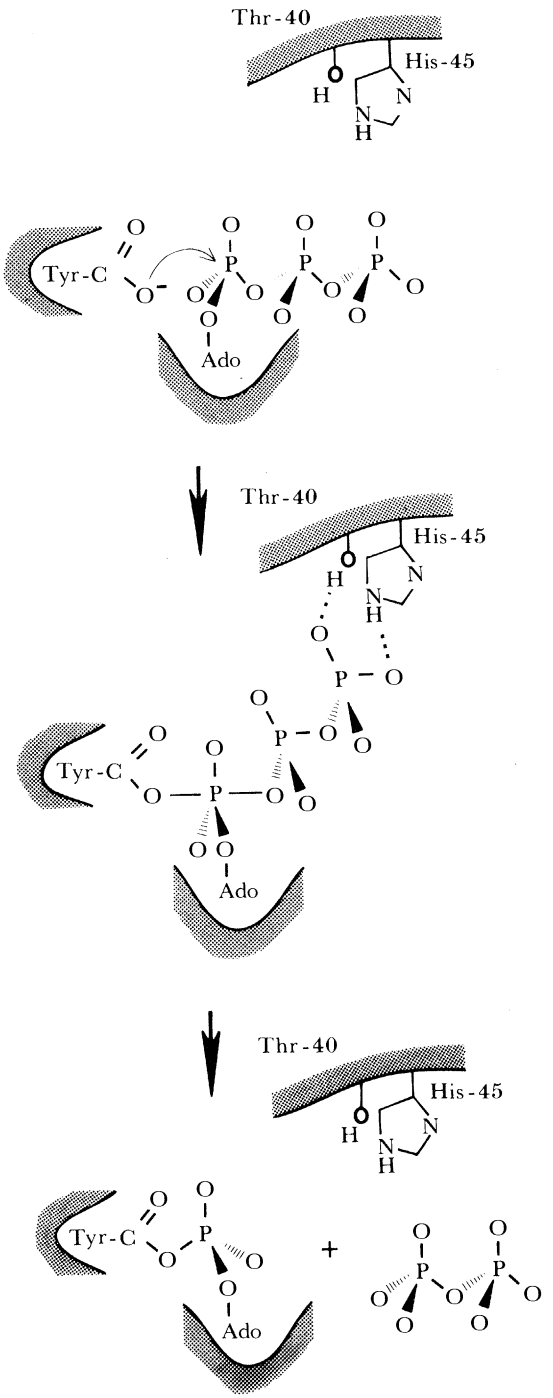


FIGURE 6. A possible mechanism of utilization of the binding energy of the γ-phosphoryl site for binding the transition state of ATP and tyrosine but not the γ-phosphoryl group of unreacted ATP (from Leatherbarrow *et al.* 1985).

Fine tuning of enzyme catalysis – differential binding of substrates, transition states and intermediates

Free-energy profiles for the reactions in scheme 1 have been calculated (figure 7; Wells & Fersht 1986). Interestingly, it was found that the apparent binding energies of the side chains of Tyr-34, Cys-35 and His-48 have their highest contributions in the E·Tyr–AMP complexes (figure 8). Most marked is the contribution of Cys-35. Removal of the side chain has no effect

Table 3. Formation of enzyme-bound tyrosyl adenylate[a]

enzyme	$k_3\ s^{-1}$	K'_a (ATP)/mM	k_t (Tyr)/μM
wild-type	38	4.7	12
Δ(321–419)[b]	34	5.2	12
	tyrosine binding-site mutants		
Tyr→Phe-34	35	4.4	29
Tyr→Phe-169[b]	35	—	1300
	ATP binding-site mutants		
Cys→Ser-35	4.7	4.8	8
Cys→Gly-35	4.0	4.5	11
His→Gly-48	9.9	9.9	22
Thr→Ala-51	75	4.7	12

[a] From Wells & Fersht (1985). Experiments performed at 25 °C at pH 7.78 (144 mM Tris–HCl) in the presence of 10 mM $MgCl_2$ (free), using stopped-flow fluorimetry and equilibrium binding methods to measure rate and equilibrium constants in scheme 1.

[b] Experiments performed on truncated enzyme (Waye *et al.* 1983).

Table 4. Apparent binding energies of side chains with substrates and transition states[a]

side chain	binding energy with substrate/(kJ mol^{-1})	binding energy with transition state/(kJ mol^{-1})
	tyrosine binding site	
Tyr-34–OH	−2.2	−2.4
Tyr-169–OH	−12.3	−12.3
	ATP binding site	
Cys-35–SH	−0.1	−5.1
His-48–imidazole	−1.8	−5.2
Thr-51–OH + γ-CH_3	0	+1.7

[a] From Wells & Fersht (1985). Values for transition-state binding differ slightly from those in table 1 as those values were derived from steady-state kinetics, and the values here from pre-steady-state kinetics.

on the dissociation constant of ATP, but raises the energy of the transition state by 5 kJ mol^{-1} and the energy of the E·Tyr–AMP complex by 6.7 kJ mol^{-1}. This implies that the structure of the enzyme encompassing Tyr-34, Cys-35 and His-48 is complementary to the structure of the intermediate, tyrosyl adenylate. The result of enzyme–intermediate complementarity is to increase radically the equilibrium constant for the formation of Tyr–AMP and PP from ATP and Tyr from a value of 2×10^{-7} in solution (C. K. Ho & A. R. Fersht, unpublished results) to 2.3 for the enzyme-bound species. The better binding of tyrosyl adenylate and pyrophosphate than tyrosine and ATP by the enzyme stabilizes the equilibrium by 40 kJ mol^{-1}. Tyr-34, Cys-35 and His-48 contribute one-third of this energy. A second reason for the enzyme–intermediate complementarity is to decrease the rate of dissociation of tyrosyl adenylate from the enzyme into solution where it would be a potent acylating agent.

Studying catalysis by reverse evolution

Mutation of residues that form favourable interactions with the substrates during the reaction is a retrograde step in evolution because less efficient enzymes are formed. Although the mutant enzymes we have constructed *in vitro* are less evolved than the wild-type enzyme, they are not necessarily its evolutionary precursors – but there has been sufficient time during evolution for

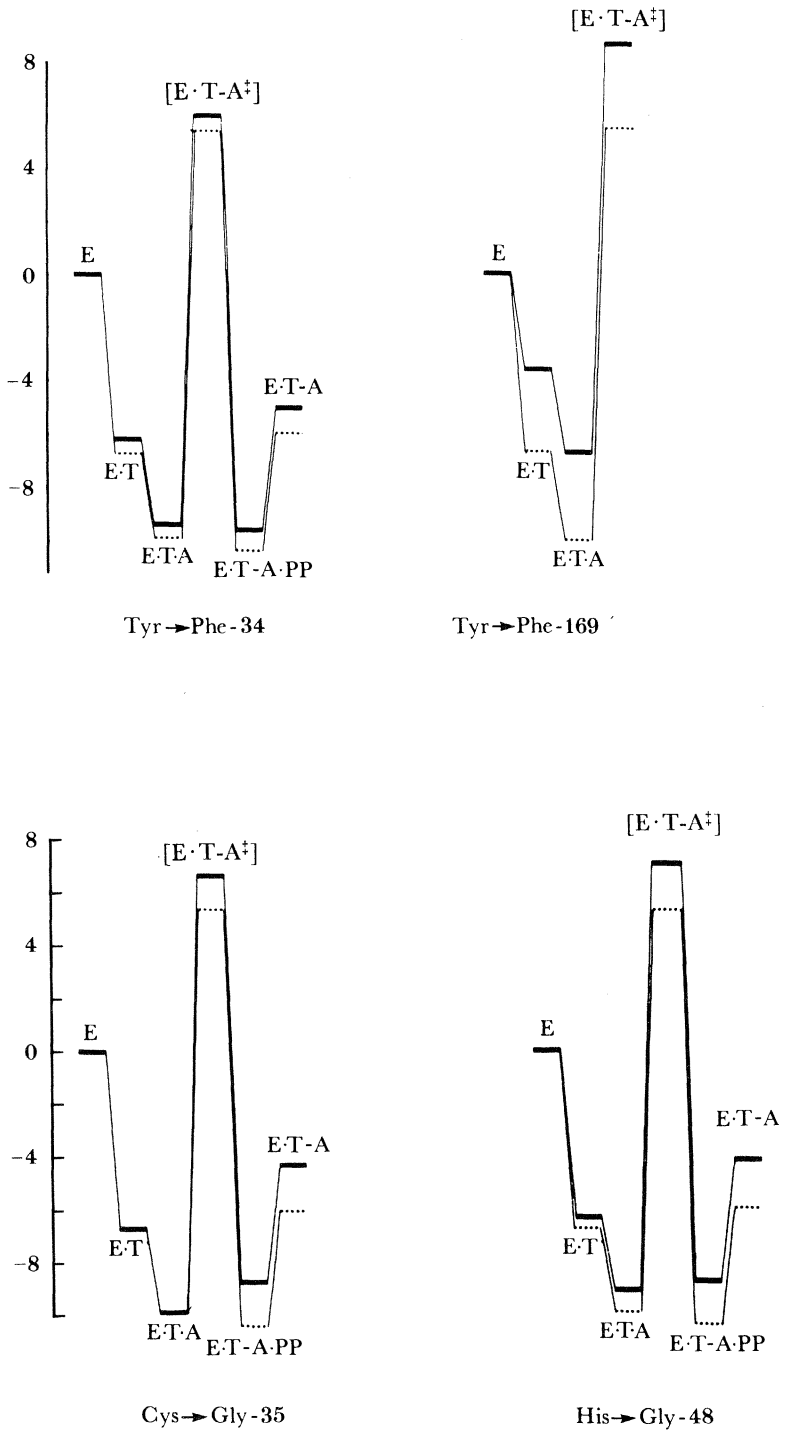


FIGURE 7. Gibbs free-energy profiles for the formation of tyrosyl adenylate and pyrophosphate as defined in scheme 1. Broken lines refer to energy levels of complexes with wild-type enzyme, and solid heavy lines to those with mutants. Standards states are 1 M for each of tyrosine, ATP and pyrophosphate. [T–A‡] is the transition state for formation of tyrosyl adenylate (Wells & Fersht 1986).

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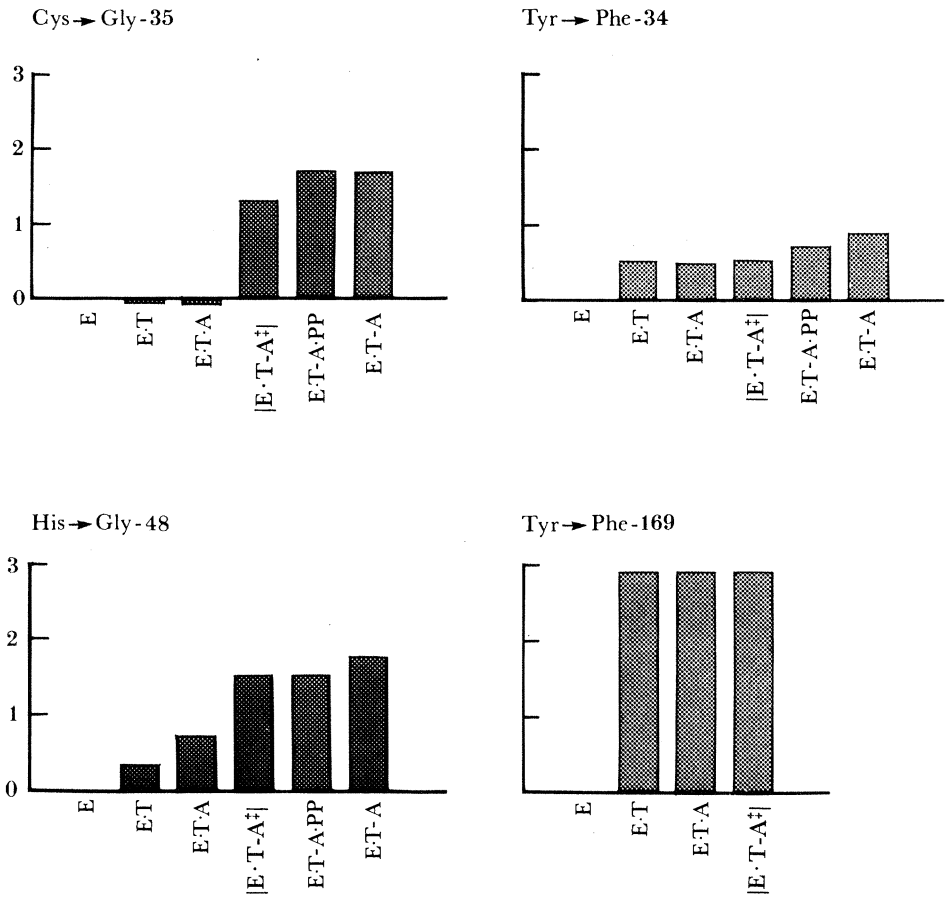

FIGURE 8. Gibbs free energies of enzyme-bound complexes of mutant enzymes relative to those of wild-type enzyme. These are the differences in energy levels in figure 7 and are equivalent to the apparent binding energies of the side chains of Tyr-35, Cys-35, His-48 and Tyr-169 with tyrosine (E·T), tyrosine+ATP (E·T·A), transition state ([E·T+A‡]), tyrosyl adenylate+pyrophosphate (E·T–A·PP) and tyrosyl adenylate (E·T–A) (Wells & Fersht 1986).

the mutants to have arisen by spontaneous mutation and to have been rejected by natural selection. Construction of such mutants, whereby favourable interactions are removed, is a form of reverse evolution. It has been seen in this study that by comparing the free-energy profiles of such mutant enzymes with wild-type enzyme, a picture is built up of how each side chain influences the course of reaction by altering the stability of each state.

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