Hydrogen bonding and biological specificity analysed by protein engineering

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The role of complementary hydrogen bonding as a determinant of biological specificity has been examined by protein engineering of the tyrosyl-tRNA synthetase. Deletion of a side chain between enzyme and substrate to leave an unpaired, uncharged hydrogen-bond donor or acceptor weakens binding energy by only 0.5–1.5 kcal mol⁻¹. But the presence of an unpaired and charged donor or acceptor weakens binding by a further ~3 kcal mol⁻¹.

The hydrogen bond is a ubiquitous feature of biological interactions: it is essential 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 influence directly the rate of enzymatic reactions by stabilizing the ionic charges 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. The calculation of the overall energies is consequently difficult and there is little knowledge of the energies involved. Here we apply an experimental approach to the problem, using site-directed mutagenesis to produce mutant enzymes that differ in their abilities to form hydrogen bonds with their substrates. The interaction energies can be calculated from kinetic data on the modified enzymes.

Our experimental system is the tyrosyl-transfer RNA synthetase from Bacillus stearothermophilus. This enzyme catalyses the aminoclaylation of tRNA Tyr in a two-step reaction: activation of the amino acid to form the enzyme-bound tyrosyl adenylate complex followed by transfer to tRNA Tyr (ref. 4).

E + Tyr + ATP = E.Tyr-AMP + PPi (1)
E.Tyr-AMP + tRNA = Tyr-tRNA + AMP (2)

The particular suitability of this enzyme (E) for obtaining accurate kinetic data and its other favourable characteristics for systematic site-directed mutagenesis have been described in detail elsewhere. The three-dimensional structure of the enzyme and its bound aminoacyl adenylate are known from X-ray crystallography. Eleven possible hydrogen bonds have been identified that may be formed between the enzyme and substrate, eight of which are from amino-acid side chains that may be mutated (Fig. 1). We are systematically mutating these residues to measure the energetics of their interactions and to test whether hydrogen bonds are actually involved. In all cases, the mutations lead to a smaller side chain that either lacks or has a modified hydrogen-bond donor or acceptor. Our initial data are consistent with hydrogen bonding being an exchange reaction whereby the hydrogyn-bond donors and acceptors on the free enzyme and free substrate break their bonds with water on forming the bonds in the enzyme-substrate complex. Here we analyse the effects of mutation of several different types of hydrogen bonds, including bonds from residues involved in determining the high specificity of the enzyme for tyrosine rather than phenylalanine (Tyr 34), bonds for simple binding of the substrate (Cys 35, His 48, Thr 51, Tyr 169) and a bond closer to the site of reaction (Gln 195). These bonds may be classified further according to charge: most are between uncharged polar residues, but some involve a charged group on either the enzyme or substrate.

Experimental observations

Our experimental results for the activation of tyrosine are listed in Table 1. The contributions of the side chains to binding were calculated from the values of kcat/KM as described previously (ref. 5, Table 2). Note that the data refer to the binding of the substrate in the transition state. Such data give, in general, the most reliable measurements of incremental binding energies as the effects of strain, nonproductive binding and induced fit do not affect kcat/KM (ref. 13). The data for the activation of phenylalanine by the wild-type enzyme and a mutant in the specificity pocket are listed in Table 3. The contributions of side chains to binding energy, discussed in more detail below, may be summarized and classified thus: (1) deletion of a side chain on the enzyme that forms a good hydrogen bond with an uncharged group on the substrate weakens binding energy by only 0.5–1.5 kcal mol⁻¹; (2) deletion of an uncharged side chain on the enzyme that forms a hydrogen bond with a charged group on the substrate weakens binding by ~3.5–4.5 kcal 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 (1); (3) deletion of a group that forms a too-long hydrogen bond actually improves binding.

Effects of removal of side chains

Our measurements on the mutated enzymes give the overall contributions of the side chains to binding. The hydrogen bond donors/acceptors that are removed on mutagenesis are the only portions of the side chains in direct contact with the substrate. But the mutation of an amino acid in a protein from a larger side chain to a smaller may have further structural consequences superimposed on the energetics of the hydrogen bonding. Any finite structure analysis must consider these possibilities and...
Hydrogen-bond strengths in *vacuo*

The stabilization energies of hydrogen bonds in *vacuo*, between compounds X-H and Y-B in equation (3), have been calculated:

$$X\cdot\cdot\cdot H \cdot\cdot\cdot Y = X\cdot H + Y\cdot B$$ (3)

(where H is a hydrogen-bond donor and B is an acceptor). Representative values for the energies of stabilization for different donors and acceptors are: water/water, $-6.4$ kcal mol$^{-1}$; water/$\text{CH}_3\text{SH}$, $-3.1$ kcal mol$^{-1}$; for $-\text{SH}$ as the acceptor, $-3.2$ kcal mol$^{-1}$; for $-\text{SH}$ as the donor; imidazolium/water, $-14$ kcal mol$^{-1}$; acetate/water, $-19.8$ kcal mol$^{-1}$. These values are much greater than those in Table 2 for the overall energies of bonding in aqueous solution.

General model for H bonding in water

Our data fit the formulation of Jencks and Hine:

$$E\cdot H\cdot\cdot\cdot O\cdot H + O\cdot H\cdot\cdot\cdot B\cdot S = E\cdot H\cdot\cdot\cdot B\cdot S + O\cdot H\cdot\cdot\cdot O\cdot H$$ (4)

For convenience, we assume that the enzyme has a hydrogen-bond donor, $-H$, and the substrate (S) an acceptor, $-B$, that pair in the enzyme-substrate complex. In the free enzyme, $-H$ and $-B$ are bound to water molecules. The number and types of hydrogen bonds are conserved in the reaction, which is thus essentially isoenthalpic (within the limits of Hine’s equation).

As for example, as discussed by Wilkinson et al., a $-SH$ group is just as effective as a $-OH$ group as a hydrogen-bond donor/acceptor, subject to the different absolute strengths of $-SH\cdot\cdot\cdot O$ and $-OH\cdot\cdot O$ bonds in equation (3); if, in equation (4), $-H$ is part of a $-SH$ group, then there is a $-SH\cdot\cdot\cdot O$ bond.

Table 1  Activation of tyrosine by tyrosyl-tRNA synthetase and its mutants

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_\text{cat}$ (s$^{-1}$)</th>
<th>$K_\text{M}$ (mM)</th>
<th>$K_\text{M}$ (mM)</th>
<th>$k_\text{cat}/K_\text{M}$ (s$^{-1}$ M$^{-1}$)</th>
<th>$k_\text{cat}/K_\text{M}$ (s$^{-1}$ M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>8.35</td>
<td>1.08</td>
<td>2.23</td>
<td>7,730</td>
<td>3.04 x 10$^4$</td>
</tr>
<tr>
<td>Tyr $\rightarrow$ Phe 34</td>
<td>6.86</td>
<td>1.2</td>
<td>4.4</td>
<td>5,720</td>
<td>1.56 x 10$^4$</td>
</tr>
<tr>
<td>Cys $\rightarrow$ Gly 35</td>
<td>2.95</td>
<td>2.6</td>
<td>2.7</td>
<td>1,130</td>
<td>1.09 x 10$^3$</td>
</tr>
<tr>
<td>Cys $\rightarrow$ Ser 35</td>
<td>2.52</td>
<td>2.4</td>
<td>2.6</td>
<td>1,050</td>
<td>9.65 x 10$^2$</td>
</tr>
<tr>
<td>His $\rightarrow$ Asn 48</td>
<td>7.90</td>
<td>1.4</td>
<td>3.8</td>
<td>5,640</td>
<td>2.08 x 10$^3$</td>
</tr>
<tr>
<td>His $\rightarrow$ Gly 48</td>
<td>2.00</td>
<td>1.3</td>
<td>3.2</td>
<td>1,540</td>
<td>6.25 x 10$^2$</td>
</tr>
<tr>
<td>Thr $\rightarrow$ Ala 51</td>
<td>8.75</td>
<td>0.54</td>
<td>2.0</td>
<td>16,200</td>
<td>4.36 x 10$^3$</td>
</tr>
<tr>
<td>Glu $\rightarrow$ Gly 195</td>
<td>0.19</td>
<td>2.5</td>
<td>100</td>
<td>76</td>
<td>1.90 x 10$^2$</td>
</tr>
<tr>
<td>Tyr $\rightarrow$ Phe 169†</td>
<td>6.05</td>
<td>1.25</td>
<td>1,030</td>
<td>4,840</td>
<td>5.85 x 10$^3$</td>
</tr>
<tr>
<td>Δ(321–419)†</td>
<td>7.50</td>
<td>1.08</td>
<td>2.4</td>
<td>6,940</td>
<td>3.12 x 10$^3$</td>
</tr>
</tbody>
</table>

Mutant enzymes were prepared, assayed and active-site titrated as described previously$^{14}$. Activation was measured by pyrophosphate exchange at 25°C, pH 7.8 in the presence of 144 mM Tris-Cl, 10 mM MgCl$_2$ and 2 mM P$_i$. Kinetic constants for variation of ATP were determined in the presence of 0.05 mM tyrosine (except where indicated otherwise) and for tyrosine in the presence of 2 mM ATP. Values of $k_\text{cat}$ are extrapolated to infinite concentration of tyrosine and ATP.

* 0.3 mM tyrosine.
† Experiments on truncated enzyme with residues 321-419 deleted (the tRNA binding domain$^{15}$).
‡ 1.7 mM tyrosine.

on the left-hand side and a $-SH\cdot\cdot\cdot O$ bond on the right in the enzyme-substrate complex. The contribution of hydrogen bonds to enzyme-substrate binding energy arises from entropy: water hydrogen bonded to the enzyme or substrate has lower entropy than bulk water and the release of hydrogen-bonded water helps drive enzyme-substrate binding$^{13,16}$. Thus, there is a crucial distinction between the absolute energy of a hydrogen bond as in equation (3) and the overall energetics 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 measure from site-directed mutagenesis.

The removal of one of the hydrogen-bonding groups from equation (4), for example a side chain of the enzyme as in equation (5),

$$E\cdot OH_2 + HOH\cdot\cdot\cdot B\cdot S = [E\cdot B\cdot S] + HOH + O_2\cdot(\text{bulk})$$

(5)

do not necessarily lead to the loss of the absolute binding energy (entropy) of a hydrogen bond as defined by equation (3). 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 fulfill all its hydrogen bonding. There is no hydrogen bond from E to H$_2$O in the free enzyme and so this partly (or fully) compensates for the lack of the hydrogen bond from E to $-OH$ in the enzyme-substrate complex. When the substrate binds, it displaces the high-energy water molecule (which returns to the bulk solvent), and the hydrophilic hydrogen-bond acceptor of the substrate occupies the ‘hydrophobic’ site. The overall energetics of the reaction in equation (5) depend on the precise interactions made by the mutant enzyme with the water molecule and the group $-B$ on the substrate. If, for example, the enzyme-bound water molecule in equation (5) is in the same orientation as that in equation (4), then equation (5) may be written as

$$E\cdot OH_2 + HOH\cdot\cdot\cdot B\cdot S = [E\cdot B\cdot S] + HOH - O_2$$

(6)

There is one hydrogen bond on the right and a similar one on the left of equation (6). This reaction is approximately isoenthalpic when $-B$ is $-OH$ and, to a first approximation, the energetics of equation (6) are the same as those of equation (4). 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. The energetics clearly depend on the precise nature of the interactions in each particular example and so we...
Table 2 Comparative binding energies of wild-type and mutant enzymes with substrates

<table>
<thead>
<tr>
<th>Comparison (Residue and position)</th>
<th>Substrate</th>
<th>ΔG (kcal mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phe 34 Tyr 34*</td>
<td>Tyr</td>
<td>0.52</td>
</tr>
<tr>
<td>Gly 35 Cys 35*</td>
<td>ATP</td>
<td>1.14</td>
</tr>
<tr>
<td>Ala 51 Cys 35</td>
<td>ATP</td>
<td>0.47</td>
</tr>
<tr>
<td>Gly 48 Asn 48</td>
<td>ATP</td>
<td>0.77</td>
</tr>
<tr>
<td>Gly 48 His 48</td>
<td>ATP</td>
<td>0.96</td>
</tr>
<tr>
<td>Ser 35 Cys 35*</td>
<td>ATP</td>
<td>1.18</td>
</tr>
<tr>
<td>Phe 160† Tyr 160†</td>
<td>Tyr</td>
<td>3.72</td>
</tr>
<tr>
<td>Gly 195 Gln 195*</td>
<td>Tyr</td>
<td>4.49</td>
</tr>
<tr>
<td>Gly 35 Ser 35</td>
<td>ATP</td>
<td>-0.04</td>
</tr>
<tr>
<td>Ala 51 Thr 51</td>
<td>ATP</td>
<td>-0.44</td>
</tr>
</tbody>
</table>

The first two columns show the residues compared. The apparent contributions (ΔG) of the side chains of different amino acids to the binding energy of the enzyme-transition state complexes were calculated by comparing the ratios of $k_{cat}/K_M$ for activation by wild-type and mutant enzymes as described previously, using the equation $ΔG = -RT \ln \left( \frac{(k_{cat}/K_M)_{wild}}{(k_{cat}/K_M)_{mut}} \right)$ (subscripts mut and wt refer to wild-type and (or) reference enzymes respectively).

* Wild-type; † truncated enzyme.

Table 3 Activation of tyrosine and phenylalanine by tyrosyl-tRNA synthetases

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activation of Tyr $k_{cat}$ (s⁻¹)</th>
<th>Activation of Phe $k_{cat}/K_M$ (s⁻¹ M⁻¹)</th>
<th>Relative specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>5.4</td>
<td>2.2</td>
<td>2.5 x 10⁶</td>
</tr>
<tr>
<td>Tyr←Phe 34</td>
<td>4.4</td>
<td>4.4</td>
<td>5x10⁶</td>
</tr>
</tbody>
</table>

Impurities of tyrosine in the phenylalanine were removed by scavenging with tetratinamethane. Conformations as described in Table 1 but data are not extrapolated to infinite concentration of ATP but given for 2 mM ATP. For activation of phenylalanine, the Phe concentration was varied between 5 and 30 mM, well below the $K_M$ value. The specificity of wild-type enzyme is close to that measured previously under different conditions.

vary from case to case. Before this study, the magnitudes of the energetic changes were unknown. Now we discuss these and show how particular examples may be rationalized and categorized.

Case A. Deletion of a side chain (–H) that interacts with an uncharged group on the substrate. Equation (5) or (6) describes this. The low contribution to specificity of a hydrogen bond between two uncharged residues is exemplified by the mutation Tyr→Phe 34 (Figs 1, 2). This is a very conservative mutation whereby a small group is deleted whose interaction is important in the recognition of tyrosine compared with phenylalanine. Yet, $K_M$ for tyrosine increases by a factor of only two (Table 1) and the specificity for tyrosine decreases by a factor of only 15 (Table 3). The apparent bond energy of 0.5 kcal mol⁻¹ is one of the lowest yet found, perhaps because the tyrosine–OH is a poorer hydrogen-bond acceptor than an aliphatic or water–OH and so the bond from Tyr 34 to the tyrosyl–OH of the substrate is relatively weak. The thiol-containing side chain from Cys 35, which donates to the 3'-OH of the ribose of ATP, contributes 1.1 kcal mol⁻¹. The tyrosyl-tRNA synthetase from Bacillus caldotenax differs from the enzyme from B. stearothermophilus by only four amino-acid replacements (M.D. Jones, unpublished data). One of these is an asparagine at position 48. The side chain of Asn 48 contributes 0.77 kcal mol⁻¹ and that of His 48 contributes slightly more binding energy at 0.96 kcal mol⁻¹.

Note that equation (5) is relatively insensitive to the nature of the side chain deleted (when –B is uncharged). For example, consider the deletion of a charged histidine side chain (–H is imidazolyl) as in the mutation His→Gly 48. Then, relative to wild-type enzyme, a strong interaction between imidazolyl and the substrate is lost in the mutant enzyme–substrate complex, but this is compensated for by a loss of a strong interaction between imidazolyl and water in the free mutant enzyme.

We have analysed several mutants as well as those listed in Table 2. In all cases, the deletion of a side chain that interacts with an uncharged group on the substrate loses between 0.5 and 1.5 kcal mol⁻¹ of binding energy.

Case B. Deletion of a side chain that interacts with a charged group on the substrate. This is exemplified by the mutation Tyr→Phe 169 (Fig. 1) whereby the hydrogen bond to the ammonium ion is lost in the enzyme–substrate complex. Equation (5) still describes the situation, but there is now the loss of a strong hydrogen bond between the charged group on the substrate and water when forming the enzyme–substrate complex and 3.7 kcal mol⁻¹ of binding energy are lost on Tyr→Phe 169. Gln 195 binds to a carboxy1 oxygen of tyrosine in the E.Tyr complex and to the carboxyl oxygen of tyrosyl adenylate (Fig. 1; refs 6, 7, 18). On mutation of Gln→Gly 195 there is a loss of binding energy of 4.5 kcal mol⁻¹ in the transition state where the oxygen atom is fractionally charged.

Case C. Deletion of a group on the substrate that interacts with a charged residue on the enzyme, described by the equation:

$$E^+\cdots O-H_2O + H OH_2S = [E^+\cdots O-H_2O + H OH_2S]$$

This has similar consequences to Case B. A strong interaction in the enzyme–water complex is lost, an example being the ‘modification’ of tyrosine to phenylalanine. Phenylalanine binds more poorly to the enzyme by ~7 kcal mol⁻¹ (Table 3). The phenylalanine either displaces the water molecule that is bound between Asp 176 and Tyr 34 in the enzyme (Fig. 2), leaving two unpaired hydrogen bonds, or, more probably, binds with a water molecule still bound between Asp 176 and Tyr 34. The water is crammed in a space which is too small, so that there are unfavourable interactions with the substrate.

A minimum estimate of the strength of the bond between Asp 176 and the tyrosine–OH can be made from the relative binding of tyrosine and phenylalanine to the mutant Tyr→Phe 34 (5.45 kcal mol⁻¹ calculated from the data in Table 3). If phenylalanine displaces the bound water molecule as in Fig. 2, then there will be the loss of the hydrogen bond and also of the dispersion energy between the enzyme and the –OH group. The latter is worth ~1-2 kcal mol⁻¹ (ref. 19) so the hydrogen bond and the substrate is lost in the mutant enzyme–substrate complex, but this is compensated for by a loss of a strong interaction between imidazolyl and water in the free mutant enzyme.

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Biological specificity

The classical view of biological specificity is that binding energy is provided by dispersion forces and the hydrophobic effect, whilst complementary hydrogen bonds and salt bridges give specificity. This study shows that, irrespective of any small structural effects that occur 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 0.5–1.5 kcal mol⁻¹ of binding energy relative to the absence of the side chain. This means that an unpaired uncharged hydrogen bond provides a factor of only 2.5–15 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 (as in Cases B and C) affects binding by ~4 kcal mol⁻¹ and is worth a factor of 1,000 in specificity. Thus, specificity is caused to some extent by hydrogen bonding but is best mediated by charged residues.

In principle, the same reasoning may be applied to the contribution of hydrogen bonding in base pairing during DNA replication or transcription. There is, however, one important difference when analysing, for example, G-T and A-C mispairing. All the cases discussed here concern the removal of groups so that no unfavourable steric interference is set up between the enzyme and substrate. But, if in base mispairing, G-T takes up the same overall geometry as an A-T base pair and A-C the same as a G-C pair, unfavourable steric and electrostatic interactions will be set up between >NH groups. These effects will compound the loss of hydrogen bonds in the mispairs. In general, unfavourable steric interactions are an important element in specificity, as van der Waals’ repulsion energies are such a strong function of interatomic distance. High specificity by steric repulsion is the basis of double-sieve sorting of amino acids by the aminoaetyl-rRNA synthetases. Thus, two important components of biological specificity are the avoidance of uncharged groups and unfavourable steric interactions between enzyme and substrate.

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New quasars with \( z = 3.4 \) and 3.7 and the surface density of very high redshift quasars

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It has been suggested that there is a sharp decline in the space density of quasars beyond redshifts \( z \sim 3.5 \). We describe here the discovery on a UKST (UK Schmidt telescope) low-dispersion IIIaF objective prism plate of a QSO (quasi-stellar object) with \( z = 3.7 \), which is the second highest redshift QSO known and, moreover, lies in the same region in which a QSO with \( z = 3.61 \) has already been reported. We also note a QSO with \( z = 3.4 \). Before these observations, only seven QSOs with \( z \geq 3.4 \) were known throughout the whole sky. When compared with the results of a search for QSOs with \( 2.7 < z < 3.3 \) on the same plate, these observations do not support suggestions of a \( z \sim 3.5 \) cut-off in the QSO distribution, but rather support the suggestion of a steady decline from \( z = 2 \). We show here that deep searches over small areas of sky are unlikely to be successful in discovering QSOs at \( z > 4 \) and that such searches must be carried out over very large areas, even if only to a moderate limiting magnitude of \( m(R) \sim 19 \).

The UKST, in combination with the 44-arc min objective prism and IIIaF plates, has proved a powerful tool for the detection of QSOs by their Lyα emission up to \( z = 3.3 \), a limit set by Lyα then passing beyond the red limit of the IIIaF response at \( 3,500 \) Å. This particular prism has usually been considered to have too low a dispersion to be used effectively with a IIIaF emulsion, which has a red limit at \( 6,800 \) Å and can, in principle, detect QSOs up to \( z = 4.5 \). However, the discovery of the \( z = 3.52 \) QSO1159 ± 123 (ref. 3) has shown that UKST IIIaF prism plates can be used to find high-z QSOs; the selection problems caused by irregularities in the spectral response of the