# Probing the Limits of Protein–Amino Acid Side Chain Recognition with the Aminoacyl-tRNA Synthetases. Discrimination against Phenylalanine by Tyrosyl-tRNA Synthetases†

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ABSTRACT: The specificity of the tyrosyl-tRNA synthetases from *Escherichia coli* and *Bacillus stearothermophilus* for tyrosine compared with phenylalanine has been determined by using samples of phenylalanine which have been scrupulously freed from tyrosine by either chemical or enzymic scavenging procedures. Both kinetic measurements and product analyses give a value of $1 \times 10^5$–$2 \times 10^5$ for the preferential activation of tyrosine. Combined with the known ratio of phenylalanine to tyrosine in rapidly growing *E. coli*, an error rate of about $\sim 5/10^4$ is calculated for the misactivation of phenylalanine. Since we find no evidence for an editing mechanism and this error rate is similar to observed rates in protein synthesis, the tyrosyl-tRNA synthetases appear to have adequate amino acid selection by simple preferential binding of the correct substrate. The incremental binding energy of the phenolic hydroxyl group of tyrosine is ~7 kcal/mol, a value presumed close to the maximum possible because of the evolutionary pressure on tyrosyl-tRNA synthetases for maximum specificity. A summary of high incremental binding energies determined from experiments on aminoacyl-tRNA synthetases is presented.

The selection of amino acids during protein biosynthesis is extremely precise. The recognition of the correct amino acid takes place during the aminoacylation of transfer ribonucleic acid (tRNA) catalyzed by the aminoacyl-tRNA synthetases. This is a two-step reaction, the amino acid first being activated by formation of aminoacyl adenylate and then transferred to the tRNA (eq 1). In some cases [for example, cysteine

$$E^{AA} + AA + ATP \xrightarrow[PP_i]{} E^{AA}\cdot AA\text{–}AMP \xrightarrow{tRNA^{AA}} AA\text{–}tRNA^{AA} \quad (1)$$

(Fersht & Dingwall, 1979a)], the selection of amino acid is relatively easy since the cognate amino acid is so different from its competitors that it is bound far more tightly to the aminoacyl-tRNA synthetase. In other examples, such as the rejection of valine by the isoleucyl-tRNA synthetase (Baldwin & Berg, 1966), threonine and $\alpha$-aminobutyrate by the valyl-tRNA synthetase (Fersht & Dingwall, 1979a,b), and homocysteine by the methionyl-tRNA synthetase (Fersht & Dingwall, 1979c), the differences in binding energy between the correct and incorrect substrates are inadequate for sufficiently precise selection, and the selectivity is enhanced by editing or proofreading: the aminoacyl-tRNA synthetases have in addition to their synthetic activity a hydrolytic activity which preferentially destroys incorrectly synthesized intermediates or products. In all cases, however, the data indicate that the preferential binding of the correct substrate has been optimized during evolution.

The selective pressure on the aminoacyl-tRNA synthetases to bind the distinctive portions of their cognate substrates as tightly as possible affords an experimental opportunity to explore the limits of protein–amino acid side chain interactions. However, because the binding energies of small groups have been found to be far higher than initially expected by Pauling (1959), the cognate substrate is far more reactive than its competitors. This sensitivity to trace impurities of the cognate amino acid in samples of noncognate has obscured many earlier studies. To overcome this problem, we have introduced the use of enzymic and chemical methods to scavenge these trace impurities (Fersht & Dingwall, 1979c,d). Either the inherent ability of the aminoacyl-tRNA synthetase to convert its cognate amino acid to the hydroxamate (eq 2) or a specific chemical reaction is used to remove the residual traces of the more reactive cognate substrate. In this study we apply both

$$E^{AA} + AA + ATP \xrightarrow[PP_i]{} E^{AA}\cdot AA\text{–}AMP \xrightarrow{H_2NOH} AA\text{–}NHOH \quad (2)$$

of these procedures to the tyrosyl-tRNA synthetases from *Escherichia coli* and *Bacillus stearothermophilus* to assess the need for an editing mechanism for the rejection of phenylalanine and measure the intrinsic binding energy of the phenolic hydroxyl group. We present a summary of the maximum intrinsic binding energies with proteins of nonionic groups in aminoacyl side chains, calculated from our accumulated data on studies of the amino acid activating enzymes.

## Materials and Methods

*Materials.* The preparations of tyrosyl-tRNA synthetases were those previously used in this laboratory and are described elsewhere (Mulvey & Fersht, 1977). Amino acids were obtained from British Drug House and twice recrystallized from ethanol–water before use. Zerolit 225 ion-exchange resin was obtained from Hopkins and Williams.

*Removal of Tyrosine from Phenylalanine by Treatment with Tetranitromethane.* To a solution of phenylalanine (1 g) in ammonium bicarbonate (25 mL, 0.5 M, pH 7.7) was added a solution of tetranitromethane (1 mL) in ethanol (25 mL). After standing at room temperature overnight, the solution was lyophilized, dissolved in water, and applied to a column of Zerolit 225 ($H^+$ form). After the solution was washed with water to remove the nitroformate, the phenylalanine was eluted with 3% $NH_4OH$ and recrystallized from ethanol–water. The spectrum revealed no absorbance corresponding to nitrotyrosine.

*Treatment of Phenylalanine with Hydroxylamine (Fersht & Dingwall, 1979d).* A reaction mixture (25 mL) containing phenylalanine (120 mM), adenosine 5′-triphosphate (ATP)

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Table I: Activation of Samples of Phenylalanine (60 mM) by Tyrosyl-tRNA Synthetases[a]

| method of treatment of Phe | length of treatment (h) | $k^b$ ($s^{-1}$) *E. coli*[c] | $k^b$ ($s^{-1}$) *B. stearothermophilus*[d] |
|---|---|---|---|
| none | | 9.5 | 2.7 |
| $H_2NOH$[e] | 0.5 | 7.3 | 1.9 |
| | 1 | 4.5 | 1.4 |
| | 1.6 | 2.5 | 0.80 |
| | 2.85 | 2.4 | 0.84 |
| tetranitromethane | 24 | 2.4 | 0.81 |

[a] 25 °C, pH 7.8, 144 mM Tris-HCl, 2 mM ATP, 2 mM [$^{32}$P]pyrophosphate, and 14 mM mercaptoethanol. [b] $v/[E]$ (per mole of dimer). [c] [E] = 72 mM ($M_r$ = 95K). [d] [E] = 363 nM ($M_r$ = 95K). [e] Pretreatment with $H_2NOH$, ATP, and enzyme from *E. coli* to convert tyrosine to its hydroxamate.

(10 mM), $MgCl_2$ (10 mM), phenylmethanesulfonyl fluoride (0.1 mM), and inorganic pyrophosphatase (5 units) was incubated at 37 °C with the tyrosyl-tRNA synthetase from *E. coli* (0.1 μM) to convert any residual tyrosine to its hydroxamate. Samples (5 mL) were taken periodically, quenched with glacial acetic acid (1.5 mL), and chromatographed on SP-Sephadex as described previously. The resultant phenylalanine was desalted on Zerolit 225 as described above.

*Kinetic Procedures.* Pyrophosphate exchange and ATP–pyrophosphatase activities were measured at 25 °C and pH 7.78 in a standard buffer containing Tris-HCl (144 mM), $MgCl_2$ (10 mM), mercaptoethanol (14 mM), and phenylmethanesulfonyl fluoride (0.1 mM) by conventional procedures (Baldwin & Berg, 1966; Fersht & Kaethner, 1976) unless noted.

*Direct Measurement of Competition between Phenylalanine and Tyrosine from Extent of E·[$^{14}$C]Tyr–AMP Formation.* To a solution (98 μL) containing tyrosyl-tRNA synthetase (0.1 μM enzyme from *E. coli*, 0.05 μM enzyme from *B. stearothermophilus*), [$^{14}$C]Tyr (0.96 μM, 531 mCi/mmol), inorganic pyrophosphatase (0.5 unit), and various concentrations of phenylalanine (10–70 mM) in the standard buffer at 25 °C was added ATP (2 μL, 100 mM). After 1.0 and 2.0 min, samples (40 μL) were withdrawn, filtered through a presoaked Schleicher and Schüll BA85 nitrocellulose disk, and washed with 3.0 mL of cold standard buffer. The E·[$^{14}$C]Tyr–AMP which was absorbed to the disk was measured, after drying, by scintillation counting. The sample of phenylalanine used was that which had been treated for 2.85 h with $H_2NOH$–ATP–enzyme before purification.

*Competition between Phenylalanine and Tyrosine for Aminoacylation of $tRNA^{Tyr}$.* To a solution (49 μL) containing $tRNA^{Tyr}$ (from *E. coli*, 0.05 μM, tyrosine acceptance = 900 pmol/$A_{260}$), ATP (1 mM), [$^{14}$C]Tyr (190 nM, 531 mCi/mmol), inorganic pyrophosphatase (0.5 unit), and various concentrations of phenylalanine (as above) in the standard buffer at 25 °C was added tyrosyl-tRNA synthetase (1 μL, 4 μM). A sample (40 μL) was withdrawn after 10.0 min and quenched with 5% trichloroacetic acid, and the precipitate was collected on a nitrocellulose disk.

## Results

*Activation of Phenylalanine by Tyrosyl-tRNA Synthetases.* It is seen in Table I that recrystallized samples of phenylalanine at a concentration of 60 mM stimulate pyrophosphate exchange with both enzymes. After scavenging the phenylalanine with hydroxylamine to remove tyrosine or any other amino acid that is activated and forms a hydroxamate, the activity drops, according to the length of the scavenging treatment, reaching a minimum level after 1.6 h. The same lowered activities are found with samples of phenylalanine which were pretreated with tetranitromethane, a reagent specific for tyrosine. The identical results with the two different procedures suggest that the bulk of the original activity is attributable to impurities of tyrosine and that they may be removed completely (i.e., below background) by the methods.

Table II: Comparison of Activation of Phenylalanine and Tyrosine by Tyrosyl-tRNA Synthetase[a]

| source of enzyme | amino acid | $k_{cat}$ ($s^{-1}$) | $K_M$ (μM) | $k_{cat}/K_M$ ($s^{-1}$ $M^{-1}$) |
|---|---|---|---|---|
| *E. coli*[b] | Tyr[c] | 21 | 2.8 | $7.8 \times 10^6$ |
| | Phe[d] | >3 | $>5 \times 10^4$ | 59 |
| *B. stearothermophilus*[e] | Tyr[c] | 2.2 | 2.3 | $9.8 \times 10^5$ |
| | Phe[d] | >0.5 | $>5 \times 10^4$ | 9.8 |

[a] 25 °C, pH 7.8, 72 mM Tris-HCl, 1 mM ATP, 2 mM [$^{32}$P]pyrophosphate, and 7 mM mercaptoethanol. [b] 56 nm ($M_r$ = 95K). [c] 0.5–400 μM. [d] 0–50 mM. [e] 170 nm ($M_r$ = 95K).

At constant concentration of enzyme, the rate of activation of phenylalanine increases linearly with increasing concentration of the phenylalanine to at least 50 mM. This shows that the residual activity is not due to traces of phenylalanyl-tRNA synthetases since these would exhibit low values for $K_M$. The activity is unlikely to be caused by trace impurities of any other noncognate aminoacyl-tRNA synthetases since (a) the rates are so high and (b) similar activities are found with different preparations of the same enzyme.

*Activation of Tyrosine.* Although the tyrosyl-tRNA synthetases are symmetric dimers (Irwin et al., 1976), the binding of tyrosine exhibits negative cooperativity. In the concentration range up to 1 mM, only 1 mol of tyrosine binds per mol of enzyme dimer (Fersht et al., 1975; Jakes & Fersht, 1975; Bosshard et al., 1975). The binding of the second mole of tyrosine may be seen, however, in the presence of the other ligands: 2 mol of tyrosyl adenylate is formed and bound in the presence of ATP though at vastly different rates (Mulvey & Fersht, 1977); biphasic kinetics are observed in the aminoacylation reaction, the second mole of tyrosine appearing to bind some 1–2 orders of magnitude less strongly (Jakes & Fersht, 1975). In the present study, however, there is no indication of any cooperativity of tyrosine binding in the pyrophosphate exchange reaction. As listed in Table II, from 0.5 to 400 μM tyrosine there is simple Michaelis–Menten kinetics. This is consistent with the earlier observation that under the conditions of the pyrophosphate exchange measurements, only 1 mol of [$^{14}$C]tyrosine is bound to the dimer [as determined from equilibrium dialysis measurements (Fersht et al., 1975)].

*Specificity of Tyrosyl-tRNA Synthetases for Tyrosine.* When two substrates A and B compete for the active site of an enzyme, their relative rate of reaction is given by eq 3.

$$v_A/v_B = [A](k_{cat}/K_M)_A/[B](k_{cat}/K_M)_B \quad (3)$$

There is a potential complication in applying this relationship to the tyrosyl-tRNA synthetases since the dimeric enzyme contains two incipiently active sites and the binding at both must be considered. Although the presence of interacting active sites cannot enhance specificity in a mixture of competing substrates (Fersht, 1975, 1977), this could cause complications in experiments on individual substrates if 2 (or more) mol is bound. In this case, the value of $k_{cat}/K_M$ could contain contributions from the binding of more than 1 mol of substrate. There is direct evidence, however, from the equilibrium dialysis experiments (Fersht et al., 1975) that this does not happen

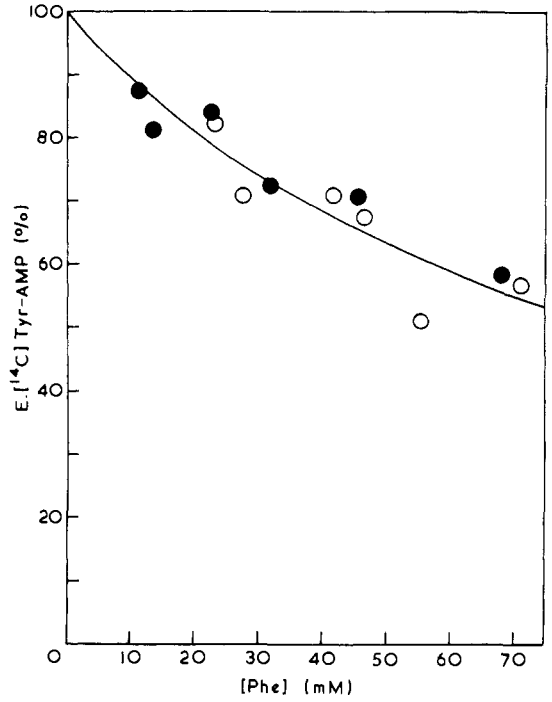


FIGURE 1: Suppression of extent of formation of enzyme-bound tyrosyl adenylate by phenylalanine. Tyrosyl-tRNA synthetase from *E. coli* [(○) 0.1 μM] or *B. stearothermophilus* [(●) 0.05 μM], [$^{14}$C]Tyr (0.96 μM), phenylalanine, ATP, and inorganic pyrophosphatase were incubated in the standard buffer. The curve is calculated for a specificity of $10^5$ for tyrosine relative to phenylalanine.

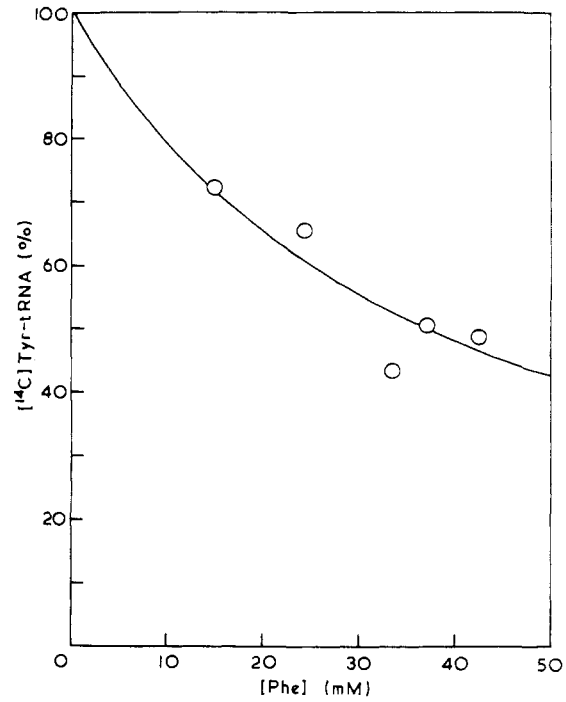


FIGURE 2: Suppression of extent of formation of [$^{14}$C]Tyr-tRNA$^{Tyr}$ (*E. coli*) by phenylalanine. Tyrosyl-tRNA synthetase (*E. coli*, 0.08 μM), tRNA$^{Tyr}$ (0.05 μM), [$^{14}$C]Tyr (0.18 μM), phenylalanine, ATP, and inorganic pyrophosphatase were incubated in the standard buffer. The curve is calculated for a specificity of $2 \times 10^5$ for tyrosine relative to phenylalanine.

here. Nevertheless, to check that the separate measurements of $k_{cat}/K_M$ for the activation of phenylalanine and tyrosine do give the specificities in a mixture of the two, we also determined the specificities directly from the inhibition of the extent of formation of enzyme-bound [$^{14}$C]Tyr–AMP by added phenylalanine (Figure 1). The E·Tyr–AMP complexes may be detected quantitatively by nitrocellulose disk filtration of reaction mixtures containing the tyrosyl-tRNA synthetases, [$^{14}$C]Tyr, and ATP (Mulvey & Fersht, 1977). Addition of increasing concentrations of phenylalanine to reaction mixtures containing either of the tyrosyl-tRNA synthetases, ATP, inorganic pyrophosphatase, and 0.86 μM [$^{14}$C]Tyr (free) leads to a progressive decrease in the amount of E·[$^{14}$C]Tyr–AMP formed. This is caused not by a decrease in the rate of formation but in the *extent*, since samples taken after 2.0 min of incubation are on average only 2–3% higher than those assayed after 1.0 min, consistent with earlier results on the rate of tyrosyl adenylate formation (Mulvey & Fersht, 1977). That is, the decrease in E·Tyr–AMP is caused by the accumulation of E·Phe–AMP. The data in Figure 1 are consistent with tyrosine being $(1.0 \pm 0.1) \times 10^5$ times more reactive than phenylalanine (i.e., at a concentration of 0.86 μM tyrosine and 86 mM phenylalanine, there is a 50:50 mixture of E·Tyr–AMP and E·Phe–AMP complexes). This is in excellent agreement with the specificities, calculated from the values of $k_{cat}/K_M$ in Table II, of $1 \times 10^5$ and $1.3 \times 10^5$ for the enzymes from *B. stearothermophilus* and *E. coli*, respectively.

It could be argued that the apparent pyrophosphate exchange activity in the presence of phenylalanine and the apparent formation of E·Phe–AMP are caused by an impurity of tyrosine at a level of 1 part in $10^5$ in the phenylalanine, which is inert. In this case, the convergence of the $H_2NOH$–ATP and the tetranitromethane procedures in Table I is a fortuitous coincidence, both treatments being incomplete by the same extent. However, the experiment in Figure 2 shows that the level of tyrosine in the phenylalanine is no greater than 5 parts in $10^6$. Plotted in Figure 2 are the results of measuring the extent of aminoacylation of tRNA$^{Tyr}$ (0.05 μM) in the presence of tyrosyl-tRNA synthetase (0.08 μM), [$^{14}$C]Tyr (0.19 μM), phenylalanine (0–43 mM), ATP, etc. The lowering of the extent of aminoacylation at higher concentrations of phenylalanine is due to either the formation of Phe-tRNA$^{Tyr}$ or an impurity of tyrosine diluting the specific activity of the [$^{14}$C]Tyr. The concentration of phenylalanine at which the extent is lowered by 50% is 38 mM, i.e., $2 \times 10^5$ times higher than the concentration of [$^{14}$C]Tyr. Thus, the *upper limit* of tyrosine in the phenylalanine is $1/(2 \times 10^5)$.

It should be noted that eq 3 cannot be applied to this experiment to measure the specificity for tyrosine vs. phenylalanine. This is because the concentration of enzyme is greater than that of the tRNA so there is less than one turnover of the enzyme. The enzyme still catalyzes the formation of E$^{Tyr}$·Phe–AMP and E$^{Tyr}$·Tyr–AMP in proportions governed by eq 3, but the transfer step to tRNA$^{Tyr}$ may provide a further selectivity: there is insufficient tRNA to accept all of the enzyme-bound aminoacyl adenylate which is formed so the more rapidly reacting species dominates in the final product. The calculated and measured specificity of $10^5$ for tyrosine vs. phenylalanine in amino acid activation is thus reliable certainly within a factor of two, and probably to better than this.

*Search for an Editing Mechanism.* The ATP–pyrophosphatase activity of the tyrosyl-tRNA synthetase from *E. coli* (0.5 μM) in the presence of tRNA$^{Tyr}$ (2.5 μM), phenylalanine (tetranitromethane-treated, 50 mM), [γ-$^{32}$P]ATP (2 mM), and inorganic pyrophosphatase (5 units/mL) in the standard buffer was assayed by the procedure of Baldwin & Berg (1966) and Fersht & Kaethner (1976) to detect if the misactivated phenylalanyl adenylate was indirectly or directly hydrolyzed by an editing process. There was not detectable

release of $^{32}P$ from the [$\gamma$-$^{32}P$]ATP above background over 45 min (<30 $\mu$M), setting an upper limit of 0.02 $s^{-1}$ for the ATP–pyrophosphatase activity, <1% of the expected rate of activation of phenylalanine under these conditions. This apparent lack of activity cannot be caused by an impurity of tyrosine in the phenylalanine being preferentially transferred to $tRNA^{Tyr}$, forming the relatively stable Tyr-$tRNA^{Tyr}$ [$k_{hydrolysis} = 5 \times 10^{-4}$ $s^{-1}$ (Jakes & Fersht, 1975)], since the upper limit of the concentration of tyrosine is $(5 \times 10^{-6}) \times$ 50 mM, i.e., 0.25 $\mu$M compared with 2.5 $\mu$M $tRNA^{Tyr}$.

## Discussion

The available data on the accuracy of protein synthesis indicate an error rate of $\sim 1$ in $10^4$ per residue (Loftfield & Vanderjagt, 1972; Popp et al., 1976; Edelman & Gallant, 1977). It is our experience that commercial samples of amino acids often contain impurities of other amino acids in the region of 0.1%, an order of magnitude above the error rate, and that it is difficult to remove these impurities by conventional recrystallization. This is seen to be true in the present study for phenylalanine with traces of tyrosine. On scavenging recrystallized, commercially obtained, phenylalanine to remove tyrosine by both chemical and enzymic procedures, the stimulation of the pyrophosphate exchange reaction with the tyrosyl-tRNA synthetase drops fourfold to give a specificity of $10^5$ for tyrosine vs. phenylalanine. Thus, if in an equimolar mixture of phenylalanine and tyrosine, all of the phenylalanine that is misactivated by the tyrosyl-tRNA synthetase is successfully transferred to $tRNA^{Tyr}$ and misincorporated into protein, the error rate would be only $1/10^5$, a value well below observed error rates. However, it has been reported by Raunio & Rosenqvist (1970) that the content of phenylalanine in *E. coli* is about 60–70-fold higher than that of tyrosine during rapid growth. This biases the error rate for the misincorporation of phenylalanine (according to eq 3) to $5/10^4$, a value similar to that of $3/10^4$ found by Loftfield & Vanderjagt (1972) for the misincorporation of valine for isoleucine. It seems, therefore, that even without an editing mechanism for the removal of misactivated phenylalanine, the tyrosyl-tRNA synthetase has a selectivity for tyrosine which is just adequate for its accurate selection during protein synthesis. Consistent with this is the lack of evidence for an editing mechanism from the search for ATP consumption in the presence of the tyrosyl-tRNA synthetase, $tRNA^{Tyr}$, and phenylalanine. As with the cysteinyl-tRNA synthetase (Fersht & Dingwall, 1979a), there appears to be adequate selection of the cognate amino acid by simple preferential binding. It is of interest that both these enzymes are rare examples of aminoacyl-tRNA synthetases which are not rigorously specific for the 2′- or 3′-hydroxyl of the accepting adenosine of the tRNA (Cramer et al., 1975).

*Incremental Binding Energy of the Phenolic Hydroxyl Group of Tyrosine.* The incremental binding energy of a particular group on a substrate molecule is the contribution that group makes to the Gibbs free energy of transfer of the molecule from aqueous solution to the binding pocket of the protein. The incremental binding energy of a group R on a substrate RS (relative to the hydrogen on the smaller substrate HS) may be measured by comparing the values of $k_{cat}/K_M$ for the enzyme-catalyzed reactions using the relationship (Fersht, 1977):

$$\Delta G_B = RT \ln (k_{cat}/K_M)_{RS}/(k_{cat}/K_M)_{HS} \qquad (4)$$

(where $R$ is the gas constant and $T$ the absolute temperature).

There are two difficulties in applying eq 4. The first is theoretical since eq 4 ignores the contribution of the electronic inductive effects of R on the reaction. Fortunately, for the activation of the amino acids, this is a negligible effect since all the amino acids have very similar $pK_a$ values for the $\alpha$-carboxyl groups and the type of reaction (the nucleophilic attack of a carboxylate ion on a phosphate diester) has low Brønsted $\beta$ values (Khan & Kirby, 1970). The second is experimental because of problems of purity of reagents. In the determination of whether or not the rate of misactivation of a noncognate amino acid is sufficiently low to be tolerable during protein synthesis, it has only to be purified to an extent necessary to give a sufficiently low rate of reaction. That is, only an upper limit of its rate of activation is required. To use the rates of activation quantitatively to calculate binding energies by eq 4, we must consider possible artifacts caused by impurities in both the preparations of enzymes and substrates. Various possibilities have been eliminated in this study. (a) The presence of phenylalanyl-tRNA synthetase in the tyrosyl-tRNA synthetase was eliminated since the $K_M$ for phenylalanine is far too high. (b) The presence of tyrosine in the phenylalanine is unlikely since both chemical and enzymic scavenging procedures give the same results; the experiment involving a possible isotopic dilution of [$^{14}C$]Tyr with any contaminating unlabeled tyrosine sets an upper limit of contaminant at 50% of that required to give the observed pyrophosphate exchange activity. (c) The presence of an aminoacyl-tRNA synthetase noncognate to phenylalanine which activates phenylalanine at an appreciable rate cannot be eliminated by the high value of $K_M$ for phenylalanine as in (a) but is extremely unlikely since (1) the rates are far too high to be expected from low levels of enzymic impurities and (2) the ratio of rates of activation of phenylalanine and tyrosine are found to be constant with different batches of the same enzyme and enzymes from two different organisms. (d) The presence of a further noncognate amino acid in the phenylalanine which is rapidly activated by the tyrosyl-tRNA synthetase and is resistant to enzymic scavenging with hydroxylamine has not been ruled out experimentally but is exceedingly unlikely, there being no obvious candidates.

Application of eq 4 gives values of –6.82 and –6.97 kcal/mol for the binding energy of the phenolic hydroxyl group of tyrosine to the tyrosyl-tRNA synthetases from *B. stearothermophilus* and *E. coli*, respectively. The direct measurements of the competition of phenylalanine and tyrosine in Figure 1 also give direct measurements of the binding energy of the hydroxyl group utilized in the reaction, that is, $-RT \ln 10^5$ or –6.82 kcal/mol. With allowance for a possible error of a factor of 2 for any impurities of tyrosine in the phenylalanine, the binding energy is in the range –6.8 to 7.2 kcal/mol.

*Summary of Incremental Binding Energies Determined from Experiments with the Aminoacyl-tRNA Synthetases.* The observed values of incremental binding energies vary in practice according to the particular degree of the fit of the group with the enzyme. For example, at one extreme, the binding of tyrosine to the phenylalanyl-tRNA synthetase is inhibited by the hydroxyl group, there being no binding pocket for this. With chymotrypsin, which is equally specific for tyrosine and phenylalanine derivatives, there is no net binding energy. With the tyrosyl-tRNA synthetase, there has been evolutionary pressure toward perfect fit, and the binding energy tends to its maximum value, the *intrinsic* binding energy (Jencks, 1975).

In the past, incremental binding energies of aminoacyl side chains and, in particular, those of the hydrophobic class have been measured either from chemical partition studies, using water organic solvents and immiscible organic solvents, or from

Table III: Incremental Binding Energies of Side Chains (Relative to Glycine)[a]

| side chain | binding energy (kcal/mol) enzymic | hydrophobic[b] |
|---|---|---|
| valine | 9.6[c] | 1.5 |
| alanine | 3.1[c] | 0.5 |
| α-aminobutyrate | 6.5[c] | 1.0[d] |
| methionine | 10–11[e] | 1.3 |
| norleucine | 7–8[e] | 2.6 |

[a] Contribution to Gibbs free energy of transfer from enzyme or organic solvent to water. [b] Nozaki & Tanford (1971). [c] Experiments with valyl-tRNA synthetase. [d] Estimated by interpolation. [e] Experiments with methionyl-tRNA synthetase.

Table IV: Summary of Incremental Group Binding Energies (Relative to $H_2O$)[a]

| binding cavity constructed from | binding cavity occupied by | unfavorable energy (kcal/mol) |
|---|---|---|
| $CH_3$- | H- | 3.4[b] |
| -S- | H- | 5.4[c] |
| $CH_3S$- | H- | 8.0[c] |
| HS- | H- | 9.1[d] |
| HO- | H- | 7.0[e] |
| $CH_3$- | HO- | 3.5[f] |
| -S- | $-CH_2-$ | 3.1[c] |
| HS- | $CH_3$- | ≥7.6[d] |
| HS- | HO- | ≥11[d] |
| H- | $CH_3$- | ≥7[g] |

[a] Determined at 25 °C from pyrophosphate exchange kinetics on aminoacyl-tRNA synthetases. [b] A spread of data from 3 to 3.8 kcal/mol found from comparing valine and isoleucine with the isoleucyl-tRNA synthetase and α-aminobutyrate and valine with valyl-tRNA synthetases (Loftfield & Eigner, 1966; Owens & Bell, 1970; Fersht & Dingwall, 1979b). [c] Binding to methionyl-tRNA synthetase (Fersht & Dingwall, 1979c). [d] Binding to the cysteinyl-tRNA synthetase (Fersht & Dingwall, 1979a). [e] This study. [f] Binding of threonine vs. valine to valyl-tRNA synthetase (Fersht & Dingwall, 1979d). [g] Binding of isoleucine vs. valine to valyl-tRNA synthetase (Fersht & Dingwall, 1979d).

solubility studies. Listed in Table III are some values (relative to glycine) for the transfer from water to an organic solvent compared with the transfer to valyl- or methionyl-tRNA synthetases. It is seen that the binding energies for the purely hydrocarbon side chains are 6.2–6.5 times higher with the enzyme, corresponding to a factor of ~100 in the equilibrium constant per methylene group involved. The value for methionine is even higher.

Various physical reasons for the tighter binding to proteins than to hydrophobic solvents have been discussed at length (Jencks, 1975; Page, 1976; Fersht, 1977; Fersht & Dingwall, 1979a). These involve (a) higher dispersion energies with proteins, which are more densely packed than liquids, and (b) the existence of a preformed cavity in the binding pocket of an enzyme [it has been calculated from the scaled particle theory of liquids that the creation of a cavity in benzene of the size of a $CH_2$ group costs about 3.6 kcal/mol (Pierotti, 1965)]. Recent data, however, suggest a possible additional factor in the binding of sulfur-containing side chains, the specific ligation of a metal ion. Pororske et al. (1979) have shown that the methionyl-tRNA synthetase from *E. coli* contains zinc, and Sigel et al. (1979) have found that neutral sulfur weakly ligates with $Zn^{2+}$.

Listed in Table IV is a summary of the incremental binding energies for various groups determined so far from experiments on the aminoacyl-tRNA synthetases. These are experimentally determined values and refer directly to equilibrium constants in solution. Whatever the physical basis, they are far higher than those obtained from simple chemical studies and indicate fundamental differences between noncovalent interactions of substrates with enzymes and with organic solvents. The precise stereochemical description of how these high binding energies arise awaits the solution of the crystal structures of aminoacyl-tRNA synthetases and their complexes with amino acids by X-ray diffraction methods.

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