L-Asn, 70-47-3; L-Ala, 56-41-7; L-Pro, 147-85-3.

References

Albery, W. J., & Knowles, J. R. (1977) *Angew. Chem., Int. Ed. Engl. 16*, 285–293.

Brown, K. A., Vrielink, A., & Blow, D. M. B. (1986) *Biochem. Soc. Trans. 14*, 1228–1229.

Estell, D. A., Graycar, T. P., Miller, J. U., Powers, D. B., Burniv, J. P., Ng, P. G., & Wells, J. A. (1985) *Science (Washington, D.C.) 233*, 659–663.

Fersht, A. R., Shi, J. P., Wilkinson, A. J., Blow, D. M., Carter, P., Waye, M. M. Y., & Winter, G. P. (1984) *Angew. Chem., Int. Ed. Engl. 23*, 467–473.

Fersht, A. R., Shi, J. P., Knill-Jones, J., Lowe, D. M., Wilkinson, A. J., Blow, D. M., Brick, P., Carter, P., Waye, M. M. Y., & Winter, G. (1985) *Nature (London) 314*, 235–238.

Fersht, A. R., Leatherbarrow, R. J., & Wells, T. N. C. (1986a) *Nature (London) 322*, 284–286.

Fersht, A. R., Leatherbarrow, R. J., & Wells, T. N. C. (1986b) *Trends Biochem. Sci. (Pers. Ed.) 11*, 321–325.

Foster, R. J. (1961) *J. Biol. Chem. 236*, 2461–2466.

Ho, C. K., & Fersht, A. R. (1986) *Biochemistry 25*, 1891–1897.

Jencks, W. P. (1985) *Chem. Rev. 85*, 511–517.

Landis, B. H., & Berliner, L. J. (1980) *J. Am. Chem. Soc. 102*, 5354–5358.

Leatherbarrow, R. J., Fersht, A. R., & Winter, G. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7840–7844.

Lowe, D. M., Fersht, A. R., Wilkinson, A. J., Carter, P., & Winter, G. (1985) *Biochemistry 24*, 1506–1509.

Lowe, D. M., Winter, G., & Fersht, A. R. (1987) *Biochemistry* (following paper in this issue).

Wells, T. N. C., & Fersht, A. R. (1986) *Biochemistry 25*, 1881–1886.

Winter, G., Fersht, A. R., Wilkinson, A. J., Zoller, M., & Smith, M. (1982) *Nature (London) 299*, 756–758.

Wolfenden, R. (1972) *Acc. Chem. Res. 5*, 10–18.

Structure–Activity Relationships in Engineered Proteins: Characterization of Disruptive Deletions in the α-Ammonium Group Binding Site of Tyrosyl-tRNA Synthetase†

Denise M. Lowe,‡,§ Greg Winter,‖ and Alan R. Fersht*,‡

Department of Chemistry, Imperial College of Science and Technology, London SW7 2AY, U.K., and Medical Research Council Laboratory of Molecular Biology, Cambridge CB2 2QH, U.K.

Received January 21, 1987; Revised Manuscript Received April 27, 1987

ABSTRACT: Residues Asp-78 and Gln-173 of the tyrosyl-tRNA synthetase of *Bacillus stearothermophilus* form part of the binding site for tyrosine by making hydrogen bonds with the α-ammonium group. Asp-38 is close enough to the group to make an important electrostatic contribution. Unlike other residues in the active site that have been studied by site-directed mutagenesis, Asp-38, Asp-78, and Gln-173 are part of hydrogen-bonded networks. Each of these residues has been mutated to an alanine, and the resultant mutants have been studied by kinetics to construct the difference energy diagrams for the formation of tyrosyl adenylate. In each example, the binding of tyrosine is weakened by about 2.5 kcal mol^{-1}. But, unlike previous mutants, the dissociation of the second substrate, in this case ATP, is also seriously affected, being weakened by some 2 kcal mol^{-1} for TyrTS(Ala-78) and TyrTS(Ala-173). The energy of the transition state for the formation of tyrosyl adenylate is raised by 7.8 kcal mol^{-1} for the former and 4.5 kcal mol^{-1} for the latter mutant. Addition of these mutants to linear free energy plots constructed for the nondisruptive mutants in the accompanying study [Fersht, A. R., Leatherbarrow, R. J., & Wells, T. N. C. (1987) *Biochemistry* (preceding paper in this issue)] reveals large deviations of the data for TyrTS(Ala-38) and TyrTS(Ala-78) from the regression line. These thus belong to a different class of mutations from previous nondisruptive examples. This observation combined with the structural evidence and difference energy diagrams strongly suggests that the mutations Asp → Ala-38 and Asp → Ala-78 are disruptive in nature.

Side chains at the active site of the tyrosyl-tRNA synthetase that may possibly form hydrogen bonds with the substrates are being systematically mutated in order to analyze their contributions to catalysis (Winter et al., 1982). The mutations so far have all been in the class that may be described as nondisruptive deletions (Fersht et al., 1987). Certain residues in the binding pocket for the α-amino group of tyrosine, however, form more extensive interactions in the protein. Examination of the crystal structure of the enzyme-bound tyrosine complex (Blow & Brick, 1985; Brick & Blow, 1987) reveals that residues Tyr-169, Gln-173, and Asp-78 make hydrogen bonds with the α-ammonium group of tyrosine (Figure 1). In addition, the aliphatic portion of the side chain of Gln-173 forms one side of the binding site for the aromatic ring of the substrate tyrosine, making van der Waals' interactions. The carboxylate of Asp-38 is close enough to make an electrostatic interaction with the α-ammonium group but does not appear to make a direct hydrogen bond with it. It

† This work was funded by the Medical Research Council of the U.K.

‡ Imperial College of Science and Technology.

§ Present address: Department of Biochemical Virology, The Wellcome Research Laboratories, Beckenham, Kent BR3 3BS, U.K.

‖ Medical Research Council Laboratory of Molecular Biology.

0006-2960/87/0426-6038$01.50/0 © 1987 American Chemical Society

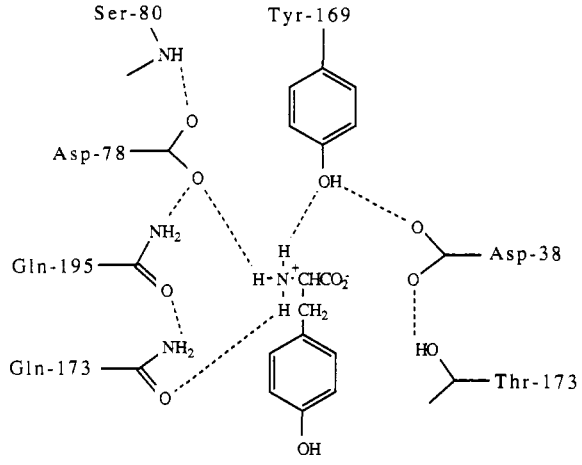

FIGURE 1: Direct and indirect interactions of side chains with the α-ammonium group of tyrosine [modified from Brick and Blow (1987)].

does, however, appear to participate in a network of hydrogen-bond interactions within the protein in this region, in particular with the hydroxyl group of Tyr-169, which, in turn, makes a hydrogen bond with the substrate α-ammonium ion. Tyr-169 may be mutated in an apparently nondisruptive manner (Tyr → Phe-169, Fersht et al., 1985; Wells & Fersht, 1985, 1986). The side-chain hydroxyl group of Tyr-169 has an apparent contribution of about 2.8 kcal mol^{-1} to the binding energy of tyrosine throughout the reaction. This value is at the lower end of the range being found for a hydrogen bond between an uncharged residue and a charged group on the substrate (Fersht et al., 1985; unpublished data from this laboratory).

In this study, we have mutated Gln-173, Asp-78, and Asp-38 to alanine residues to study the effects caused by the complete removal of any possible hydrogen-bonding or electrostatic interactions as it is not possible to devize a mutation that removes just a single interaction. Pre-steady-state kinetics of both the forward and reverse reactions of tyrosine activation catalyzed by the mutants were performed to construct the free energy profiles for each mutant. Difference energy diagrams were constructed to quantitate the apparent contribution from each residue to binding in the experimentally accessible stages of the reaction. These are compared with the "well-behaved" examples of nondisruptive deletion in the accompanying paper (Fersht et al., 1987).

Experimental Procedures

Reagents were obtained from Sigma (London) and radiochemicals from Amersham International.

Mutant Constructions. Mutant proteins were constructed from the TyrTS[1] gene cloned in the phage M13mp93 as described previously (Carter et al., 1984). The following synthetic primers were used to direct the mutations: Gln → Ala-173, 5′-GTATGCCG*C*CAGCATCAT-3′; Asp → Ala-78, 5′-CGCTCGGC*G*CGCCGATCAA-3′; Asp → Ala-38, 5′-CCGTCGGC*G*CAAACCCG-3′ (where an asterisk indicates a mismatched base). The mutations Asp → Ala-78 and Asp → Ala-38 were made on a truncated TyrTS (ΔTyrTS) lacking the tRNA-binding domain (residues 319–419; Waye et al., 1983) as the M13 clones expressing the full-length mutant TyrTS appear to be unstable.

[1] Abbreviations: TryTS, tyrosyl-tRNA synthetase; Tris, tris(hydroxymethyl)aminomethane; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; T, Tyr; A, ATP; T-A, tyrosyl adenylate; Na_2DodSO_4, sodium dodecyl sulfate.

Purification of Tyrosyl-tRNA Synthetase. Preparations of enzymes were purified as described previously (Lowe et al., 1985) and were judged to be greater than 95% homogeneous by Na_2DodSO_4–polyacrylamide gel electrophoresis.

Kinetic Procedures. Experiments were performed at 25 °C in a standard buffer containing 144 mM Tris-HCl (pH 7.78), 10 mM $MgCl_2$ (free), 10 mM 2-mercaptoethanol, and 0.1 mM phenylmethanesulfonyl fluoride. Additional $MgCl_2$ was added where necessary to compensate for its complexing with ATP and pyrophosphate. For the mutant ΔAsp→Ala-78, the buffer used was 128 mM Bis-Tris-HCl, pH 6.0, because of the increased stability of the enzyme-bound tyrosyl adenylate intermediate at the lower pH.

The concentration of enzymes was determined by active site titration with nitrocellulose discs (Wilkinson et al., 1983). Because the mutants under study had raised dissociation constants for tyrosine and ATP, it was necessary to perform the titrations at high substrate concentrations and extrapolate to saturating concentrations to obtain the true concentration of the active enzyme. The titration was also time dependent for ΔTyrTS(Ala-78) (see below).

The kinetic constants for the pre-steady-state formation and pyrophosphorolysis of enzyme-bound tyrosyl adenylate were determined by monitoring the formation and breakdown of this intermediate.

The rate and dissociation constants are defined for the scheme described in the accompanying paper (K_t = dissociation constant of tyrosine from the E·Tyr complex, K_a = that of ATP from the E·ATP complex, K'_a = that of ATP from the E·Tyr·ATP complex, etc.; k_3 and k_{-3} are the first-order rate constants for the formaton of E·Tyr-AMP·PP_i and its pyrophosphorolysis, respectively).

Formation of Tyrosyl Adenylate. The forward reaction for TyrTS(Ala-173) was followed by monitoring changes in protein fluorescence in a stopped-flow fluorometer (Wells & Fersht, 1986). Experiments were performed in the presence of 0.2 and 1 mM tyrosine and a final enzyme concentration of 1 μM. The ATP concentration was varied between 2 and 40 mM.

The forward rate constant for ΔTyrTS(Asp→Ala-78) was sufficiently low to allow a single turnover of the enzyme to be followed with [^{14}C]tyrosine and manual sampling. The amount of enzyme-bound tyrosyl adenylate was determined at various times by filtration through nitrocellulose discs (Wilkinson et al., 1983). A complete time course was monitored over about 30 min, and the observed rate was determined at various substrate concentrations from the best fit first-order exponential to the data. Experiments were performed by varying the tyrosine concentration between 40 and 400 μM at 10 mM ATP and by varying the ATP concentration between 5 and 33 mM at 0.2 mM tyrosine. Because the enzyme-bound tyrosyl adenylate intermediate of ΔTyrTS-(Ala-78) breaks down at a significant rate relative to its formation, the observed time courses do not converge at a stoichiometry of 1 mol of bound tyrosyl adenylate/mol of enzyme but reach lower end points, which increase as the substrate concentration is raised. The true concentration of active enzyme was determined by extrapolating these end points to infinite substrate concentration. This value was then used to convert the observed rates into true rate constants, from which the values of k_3 and the dissociation constants could be determined from Michaelis–Menten kinetics.

Pyrophosphorolysis of Tyrosyl Adenylate. Enzyme-bound adenylate was formed in situ in the absence of inorganic pyrophosphatase, and the excess ATP and tyrosine and pyro-

phosphate were removed by rapid-centrifuge gel filtration (Penefsky, 1977) at 4 °C in 20 mM Bis-Tris-HCl, pH 6.0, buffer. A dilute pH 6.0 buffer was used because the mutants Gln→Ala-173, ΔAsp→Ala-38, and ΔAsp→Ala-78 all form unstable enzyme-tyrosyl adenylate complexes [half-lives at 25 °C of 16 (pH 7.8), 7 (pH 7.8), and 8 min (pH 6.0), respectively]. The reverse rate constant for TyrTS(Ala-173) was measured by monitoring the fluorescence increase on mixing pyrophosphate, dissolved in 2× concentrated Tris, pH 7.8, buffer, with the enzyme–tyrosyl adenylate complex at 25 °C (Wells & Fersht, 1986). For ΔTyrTS(Ala-78) and -(Ala-38), the enzyme–tyrosyl adenylate complex was prepared with [^{14}C]tyrosine, and the breakdown of the complex in the presence of pyrophosphate was monitored by taking samples at various times for filtration through nitrocellulose discs (Wilkinson et al., 1983).

Dissociation Constant of Tyrosine. The dissociation constant K_t of tyrosine from the complex with TyrTS(Ala-173) was determined from the kinetics of pyrophosphate exchange by following the loss of label from [γ-^{32}P]ATP at various tyrosine concentrations at a constant concentration of ATP, which was well below the value of K_m for ATP (Wells & Fersht, 1986). K_t for ΔTyrTS(Ala-38) was taken to be the value of K_m for tyrosine in the steady-state kinetics of the pyrophosphate exchange reaction (Calendar & Berg, 1966). Under the conditions of this reaction (2 mM pyrophosphate, 2 mM ATP), the rate constant for the reverse reaction, pyrophosphorolysis, is at least 10-fold greater than that for the forward reaction rate, and hence, $K_m \approx K_t$. K_t for ΔTyrTS-(Ala-78) was obtained directly from pre-steady-state kinetics at subsaturating concentrations of ATP.

The free energy profiles for the mutant enzymes were calculated with the equations described by Wells and Fersht (1986) (Table III) and the difference energy profiles constructed accordingly. In some cases, insufficient data were available to calculate the complete profile because the values of dissociation constants for certain substrates from the mutant enzymes are too high to allow the calculation of k_3, the rate constant for the formation of E·Tyr-AMP·PP$_2$ from E·Tyr·ATP, or k_{-3} the first-order rate constant in the reverse direction. However, the inability to measure k_3 or k_{-3} never precludes the calculation of the energy level of the transition state for the reaction because the term k_3/K'_aK_t may be measured directly without determining the individual rate and equilibrium constants: at low concentrations of tyrosine ($\ll K_t$) and ATP ($\ll K'_a$, the dissociation constant of ATP from the E·Tyr·ATP complex), the first-order rate constant for the formation of tyrosyl adenylate (k_{obsd}) is given by $k_{obsd} = [Tyr][ATP]k_3/K'_aK_t$. Similarly, the energy level of the E·T-A complex can always be calculated, even when K_{pp}, the dissociation constant of PP$_i$ from E·Tyr-AMP·PP$_i$, is much greater than the solubility of magnesium pyrophosphate. This is because k_{-3}/K_{pp} is readily calculated from the pyrophosphorolysis reaction at low concentrations of pyrophosphate where the observed rate constant is proportional to $[PP]k_{-3}/K_{pp}$, and the energy level of E·T-A is calculated from $(k_3/K'_aK_t)/(k_{-3}/K_{pp})$. The energy levels of the transition state and E·T-A are thus known with high precision in all cases.

Results

TyrTS(Gln→Ala-173). Mutation of Gln-173 to Ala-173 removes the potential hydrogen bond between the side-chain carbonyl group and the α-ammonium group of tyrosine, as well as van der Waals' interactions between the rest of the side chain and the tyrosine ring. This results in a 58-fold increase in the dissociation constant for tyrosine but also has a large effect on K_a (Table I). Because of these large effects on substrate binding, it was not possible to saturate the enzyme with either substrate during a stopped-flow experiment to obtain an accurate determination of k_3 and K'_a. The range given in Table I was assessed from the observed forward rate constants at subsaturating concentrations of tyrosine and ATP—see Experimental Procedures. Taking into account steady-state measurements of the pyrophosphate exchange reaction, it seems likely that the upper estimates of k_3 are nearer the true value so that the mutation, Gln → Ala-173, has had little effect on the forward rate constant. In the reverse reaction k_{-3} has increased 2.4-fold, and K_{pp} is increased 2.8-fold so that overall there is very little effect on the specificity constant for the reverse reaction ($k_{-3}K_{pp}$), but the equilibrium constant (K_{eq}) between E·Tyr-AMP·PP$_i$ and E·Tyr·ATP is decreased (Table II).

Table I: Rate and Dissociation Constants for the Formation of Enzyme-Bound Tyrosyl Adenylate[a]

enzyme	pH	k_3 (s^{-1})	K'_a (mM)	K_t (μM)	$k_3/K_tK'_a$ (s^{-1} M^{-2})
wild type[b]	7.8	38	4.7	12	6.74×10^8
Δwild type[b,c]	7.8	34	5.2	12	5.45×10^8
Δwild type[c,d]	6.0	29	6.2	9.6	4.87×10^8
Gln→Ala-173	7.8	8–40[e]	36–180[e,f]	700	3.2×10^5
ΔAsp→Ala-78[c]	6.0	~0.04	~54[f]	~900	8.2×10^2
ΔAsp→Ala-38[c]	7.8			620	1.0×10^5

[a] At 25 °C in presence of 10 mM $MgCl_2$ (free), 10 mM 2-mercaptoethanol, and 0.1 mM phenylmethanesulfonyl fluoride in either 144 mM Tris-HCl at pH 7.78 for 128 mM Bis-Tris-HCl at pH 6.0. k_3 is the first-order rate constant for the formation of E·Tyr-AMP·PP$_i$ from E·Tyr·ATP; K'_a is the dissociation constant of ATP from that complex, and K_t is the dissociation constant of tyrosine from E·Tyr. [b] From Wells and Fersht (1986). [c] Truncated enzyme (Waye et al., 1983). [d] J. Knill-Jones and A. R. Fersht, unpublished results. [e] Lower limit was determined from pre-steady-state kinetics in the presence of subsaturating concentrations of reagents. Upper limit is estimated from steady-state kinetics and is probably closer to correct value. [f] These were determined in the presence of 200 μM tyrosine, which is much less than the values of K_t for TyrTS(Ala-173) and TyrTS(Ala-78). The dissociation are, therefore, closer to K_a, the dissociation constant of ATP from the E·ATP complex. K_a for wild-type enzyme is 3.3 mM, close to the value of K'_a (unpublished data).

Table II: Pyrophosphorolysis of Enzyme-Bound Tyrosyl Adenylate[a]

enzyme	pH	k_{-3} (s^{-1})	K_{pp} (mM)	k_{-3}/K_{pp} (s^{-1} M^{-1})	K_{eq} (k_3/k_{-3})
wild type	7.8[b]	16.6	0.61	27 200	2.29
Δwild type	7.8[b]	15.3	0.68	22 500	2.22
Δwild type	6.0	15.5	0.9	17 200	1.87
Gln→Ala-173	7.8	40.0	1.7	23 500	0.2–1.0[c]
ΔAsp→Ala-78	6.0	≈0.12	≈10	12	~0.3
ΔAsp→Ala-38	7.8			640	

[a] Conditions as for Table I. k_{-3} is the first-order rate constant for the formation of E·Tyr·ATP from E·Tyr-AMP·PP$_i$; K_{pp} is the dissociation constant of PP$_i$ from that complex. [b] From Wells and Fersht (1986). [c] Upper estimate probably more correct (see Table I).

The changes in activity are illustrated clearly by the difference energy diagram (Figure 2). On mutation of Gln → Ala-173, the energy level of the E·T complex is raised by 2.4 kcal mol^{-1}, the transition state by 4.5 kcal mol^{-1}, the E·T-A·PP complex by 5.1 kcal mol^{-1}, and the final E·T-A complex by 4.5 kcal mol^{-1} [all calculated relative to the free mutant enzyme (Table III)].

ΔTyrTS(Asp→Ala-78). Mutation of Asp-78 to Ala-78 removes a possible hydrogen bond as well as a charge–charge electrostatic interaction between the side-chain carboxyl and the α-ammonium group of tyrosine. The resulting loss of binding energy is seen in an increase of about 94-fold in K_t and an increase of about 9-fold in K_a, combined with a large

Table III: Gibbs Free Energies of Complexes of Wild-Type and Mutant Enzymes[a]

enzyme	pH	free energy of enzyme-bound complexes (kcal mol^{-1})				
		G_{ET}	G_{ETA}	$G_{E[T-A]^*}$	$G_{ET-A-PP}$	G_{ET-A}
wild type	7.8	−6.7	−9.9	5.4	−10.4	−6.0
Δwild type	7.8	−6.7	−9.8	5.5	−10.3	−6.0
Δwild type	6.0	−6.8	−9.8	5.6	−10.2	−6.1
Gln→Ala-173	7.8	−4.3	−5 to −7	9.9	−5.3	−1.5
ΔAsp→Ala-78	6.0	∼−4.1	∼−6	13.4	−5.2	−2.5
ΔAsp→Ala-38	7.8	−4.4		10.7		−2.8

[a] Standard states of 1 M for tyrosine, ATP, and pyrophosphate. Free energy of enzyme is defined as zero. Energy levels of the enzyme-bound complexes (ET = E·Tyr; ETA = E·Tyr·ATP; [T-A]* = transition state for k_3 and k_{-3}; etc.).

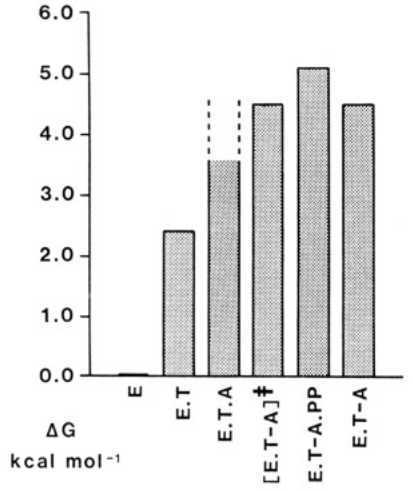

FIGURE 2: Difference energy diagram for TyrTS(Ala-173) vs. wild type.

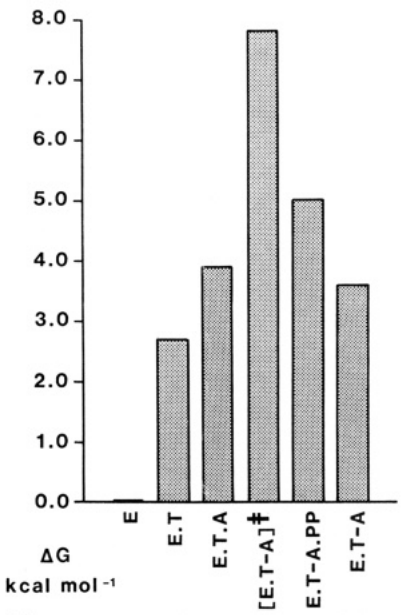

FIGURE 3: Difference energy diagram for TyrTS(Ala-78) vs. wild type.

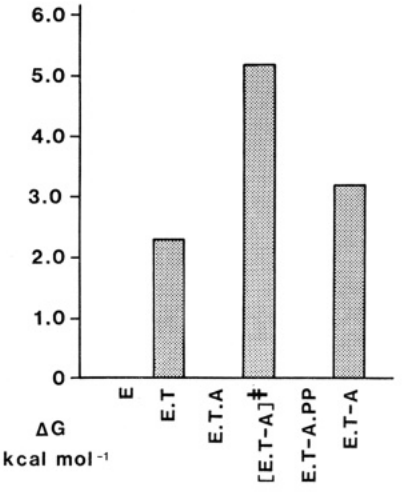

FIGURE 4: Difference energy diagram for TyrTS(Ala-38) vs. wild type.

decrease of about 700-fold in k_3 (Table I). The time course for a single turnover of the enzyme can be followed by nitrocellulose filter assays over a period of 30 min. This mutation results in a very unstable enzyme-bound tyrosyl adenylate complex being formed, so that experiments were performed at a lower pH (pH 6.0) at which it has a longer lifetime. This spontaneous breakdown of the enzyme-bound tyrosyl adenylate complex complicates measurements of the reverse reaction and entails a large correction factor. The value of k_{-3} is reduced by about 100-fold, while K_{pp} is raised by over 10-fold (Table II). (Measurements were limited by the solubility of magnesium pyrophosphate.) Thus, the specificity constant for the reverse reaction is reduced by about 1400-fold, and the equilibrium constant (K_{eq}) between E·Tyr-AMP·PP$_i$ and E·Tyr·ATP is decreased 5.7-fold (Table II). As is readily seen in the difference energy diagram (Figure 3), mutation of Asp-78 destabilizes the transition-state structure more than any of the other complexes.

ΔTyrTS(Asp-38→Ala-38). Truncation of the side chain of Asp-38 in ΔTyrTS(Asp-38→Ala-38) removes an electrostatic interaction as well as disrupting a network of hydrogen bonds around the α-ammonium binding pocket. This results in a large increase in K_t, but there is also an increase in the dissociation constant for ATP. The forward rate constant is significantly lowered to the extent that measurements in the stopped-flow fluorometer, at the high concentrations of ATP and tyrosine required, become unreliable. It is not sufficiently slow, however, for manual sampling methods to be applied, hence the difficulties in obtaining estimates of k_3 and K'_a. The value of $k_3/K_tK'_a$ was determined from steady-state measurements of the pyrophosphate exchange reaction at subsaturating concentrations of ATP (2 mM) and tyrosine (variable). Although this mutation does result in a very unstable enzyme-bound tyrosyl complex being formed, it is just possible to perform pyrophosphorolysis experiments on freshly prepared complex with the manual sampling technique at low pyrophosphate concentrations. This gives an estimate of k_{-3}/K_{pp}, which is reduced by 35-fold from the equivalent value for wild-type ΔTyrTS. A partial difference energy diagram may be constructed (Figure 4).

Linear Free Energy Plots (Figure 5). Although there are gaps in the data, sufficient rate contants were determined to add to some of the linear free energy plots of the accompanying study (Fersht et al., 1987). The differences in rate and equilibrium constants for wild-type (unpublished data) and truncated enzyme (Tables I and II) between pH 7.8 and pH

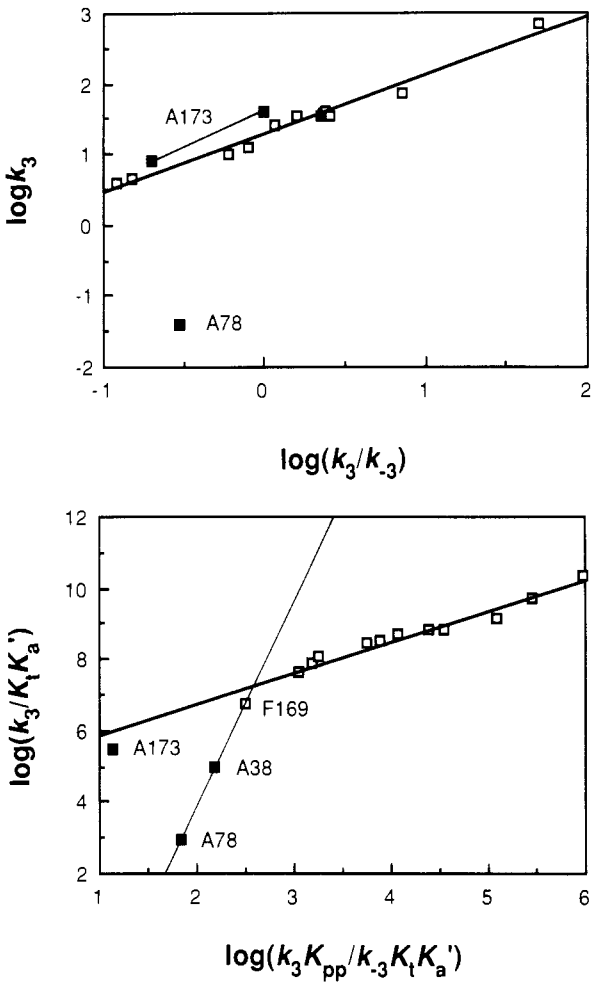

FIGURE 5: (Top) Plot of log k_3 vs. log k_3/k_{-3} for the nondisruptive mutations (□) described in Fersht et al. (1987) and for TyrTS-(Ala-173) and TyrTS(Ala-78). There is some uncertainty in the value of k_3 for TyrTS(Ala-173). There is little deviation from the regression line because of the slope is close to 1 (=0.8), and so the plot largely reflects the value of k_{-3}. (Bottom) Plot of log $(k_3/K_tK'_a)$ vs. log $(k_3K_{pp}/k_{-3}K_tK'_a)$. This plot compares transition-state binding with the binding of Tyr-AMP. The heavy line is the regression line through the nondisruptive mutants and the light line an artifact that could be misinterpreted (see text).

6.0 are sufficiently small compared with the overall differences for the new mutants that the data of Tables I and II were plotted without correction for some of the data being obtained at pH 6.0.

Discussion

The mutations that we have made all involve removal of either a highly polar or charged side chain on the enzyme that was interacting with a charged group on the substrate, the α-ammonium group of tyrosine. The resulting mutant proteins all have raised dissociation constants for tyrosine and ATP, even though the mutations are only in the region of the tyrosine binding pocket. The effects of the mutations are sufficiently large to suggest that, unlike most of our previous mutational studies (Fersht et al., 1985), thay cannot be assigned solely to the loss of a specific hydrogen-bond interaction but could result from longer range, subtle or gross, changes in enzyme conformation.

In the wild-type enzyme, these charged residues would be solvated so that on mutation the free energy of the unligated enzyme will have changed because of changes in hydration. There are several polar residues at the upper surface of the groove forming the tyrosine binding site, and it has been suggested that this polar character of the binding pocket might provide a low-energy route for water to be expelled on binding tyrosine (Blow & Brick, 1985). Deletion of the polar side chains of residues 38, 78, and 173 could possibly leave sufficient space for one or more water molecules to become trapped in the remaining hydrophobic region when the substrate binds. The binding of these water molecules to the α-ammonium group of tyrosine when bound to mutant enzymes would partly offset the loss of the interaction with the polar side chain present in wild-type enzyme and hence would attenuate the effect of mutation (Fersht et al., 1985).

Irrespective of the detailed structural interpretations, it is possible to analyze the effects of these mutations on catalysis by studying the pre-steady-state kinetics of the tyrosine activation reaction and calculating the free energy contributions to each state of the enzyme reaction (Table III). By comparing the free energy profiles for wild-type and the mutant proteins, a picture emerges of how these residues are contributing to binding the substrates, transition state, and products of the enzyme reaction (Figures 2–4). Although the nature of these mutants in the tyrosine binding site presented practical difficulties in obtaining accurate estimates of various rate and equilibrium constants, enough information has been obtained to give a picture of the distribution of binding energy over the different states of the reaction.

Deletion of any one of the three groups that are responsible for binding the α-ammonium group of the substrate tyrosine weakens binding by some 2.3–3 kcal mol^{-1}. Determination of the complete difference energy diagrams for the reaction reveals that mutation affects, in general, the whole reaction profile (Figures 2–4). Earlier work on the mutation of Tyr → Phe-169 showed that deletion of the –OH group weakens the binding of tyrosine by 2.8 kcal mol^{-1}, and this remains almost unchanged as ATP binds and reacts (Wells & Fersht, 1985, 1986). Mutation of Gln → Ala-173, however, weakens the binding of ATP by about 2 kcal mol^{-1} as does the mutation of Asp → Ala-78. This indicates that the mutations of Gln-173, Asp-38 and Asp-78 are disruptive, propagating structural changes into the ATP-binding site.

The larger effects of mutation on the binding of the transition state than on the binding of tyrosine may have a component from the more stringent requirements for correct binding in the transition state than in the initial complexes. For example, the dissociation constant of tyrosine measures dissociation from the lowest energy binding mode. In a seriously perturbed mutant, this could be a nonproductive mode, which is different from the binding of tyrosine to wild-type enzyme. But, the formation of the transition state requires that tyrosine and ATP be correctly positioned for reaction to take place, and this could require the reagents taking up higher energy binding modes that are closer to the geometries in the wild-type enzyme. Transition-state binding should thus reflect the truer effects of mutation on binding energy, as is generally so (Fersht, 1985). A similar argument may be made for the larger effects of mutation on the binding of tyrosyl adenylate than on binding of tyrosine; the position of tyrosine in Tyr-AMP is constrained so that there is less freedom for nonproductive binding.

Longer range electrostatic effects become important when a charged group is deleted and will modulate the effects on direct interactions. Removal of the carboxylate from Asp-38 or Asp-78 should reduce electrostatic repulsion with the negatively charged phosphates on ATP. Similarly, mutation of Asp-38 and Asp-78 reduces electrostatic repulsion with the carboxylate of the tyrosine substrate. Asp-38 and Asp-78 will

also interact favorably to some extent with the Mg^{2+}, which is coordinated to ATP or pyrophosphate, and the transition state. The large changes in energy on mutation of charged groups are consistent with the avoidance of unsolvated charges at enzyme–substrate interfaces being one of the major deteminants of specificity (Fersht et al., 1985).

Evidence from Linear Free Energy Plots (*Figure 5*). A plot of log k_3 vs. log (k_3/k_{-3}) compares the binding of the transition state with the E·Tyr·ATP and E·Tyr-AMP·PP_i complexes. TyrTS(Ala-173) only slightly deviates from the regression line of slope 0.79 through the nondisruptive mutants, despite there being an uncertainty of factor of 5 in the value of k_3. This is because of the phenomena described in the previous paper (Fersht et al., 1987): The values of log k_3 and log (k_3/k_{-3}) are linked such that, for a data point on a plot of slope close to unity and the value of k_{-3} being correct, the data point slides along the regression line with only a small increase in deviation as k_3 varies over the limited range. Nevertheless, in order for the fit to be so good, there must be the same value of the constant A in the Brønsted equation for the TyrTS(Ala-173) mutant as for the nondisruptive mutants, and the value of k_3 must be in the right range. The approximate conformity of TyrTS(Ala-173) to the linear free energy plot indicates that truncation of the side chain of Gln-173 does not have a major effect on the stages of the reaction from the E·Tyr·ATP complex to the aminoacyl adenylate complex. ΔTyrTS(Ala-78) clearly belongs to another class of mutations: it deviates by 2 orders of magnitude from the regression line.

A plot of log ($k_3/K_tK'_a$) vs. log ($k_3K_{pp}/k_{-3}K_tK'_a$) compares the enzyme-bound transition state with free enzyme and E·Tyr-AMP complexes. Again, TyrTS(Ala-173) is well behaved, falling on the regression line. But, ΔTyrTS(Ala-38) and ΔTyrTS(Ala-78) deviate significantly. It is clear that the latter two mutants belong to a different class from the simple nondisruptive mutants.

It should be noted in the latter linear free energy plot that a line may be drawn through the points for three mutants, ΔTyrTS(Phe-169), ΔTyrTS(Ala-78), and ΔTryTS(Ala-38), of slope 6.0! Thus, if the reasoning of the accompanying paper (Fersht et al., 1987) was applied blindly, without examining the three-dimensional structure of the enzyme and consulting difference energy diagrams, it would be falsely concluded that the three side chains were responsible for transition-state stabilization in the wild-type enzyme. Linear free energy relationships must be analyzed with caution.

The detailed structural reasons why these mutations lead to such dramatic effects on ATP binding when they are located in the tyrosine binding pocket will have to await the solution of the crystal structures. The linear free energy plots and difference energy diagrams do, however, pinpoint which mutants are liable to differ significantly from wild-type enzyme and warrant further structural analysis: those that have gross deviations from the well-behaved nondisruptive mutants.

ACKNOWLEDGMENTS

We thank Paul Dear for expert technical assistance in the construction of TyrTS(Asp→Ala-38) and TyrTS(Asp→Ala-78).

REFERENCES

Blow, D. M., & Brick, P. (1985) in *Biological Macromolecules and Assemblies* (Jurnak, F., & McPherson, A., Eds.) Vol. 2, pp 442–469, Wiley, New York.

Brick, P., & Blow, D. M. (1987) *J. Mol. Biol. 194*, 287–297.

Calendar, R., & Berg, P. (1966) *Biochemistry 5*, 1681–1690.

Fersht, A. R. (1985) *Enzyme Structure and Mechanism*, Chapter 12, Freeman, New York.

Fersht, A. R., Shi, J. P., Knill-Jones, J., Lowe, D. M., Wilkinson, A. J., Blow, D. M., Brick, P., Carter, P., Waye M. M. Y., & Winter, G. (1985) *Nature* (*London*) *314*, 235–238.

Fersht, A. R., Leatherbarrow, R. J., & Wells, T. N. C. (1987) *Biochemistry* (preceding paper in this issue).

Lowe, D. M., Fersht, A. R., Wilkinson, A. J., Carter, P., & Winter, G. (1985) *Biochemistry 24*, 5106–5109.

Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891–2899.

Waye, M. M. Y., Winter, G., Wilkinson, A. J., & Fersht, A. R. (1983) *EMBO J. 2*, 1827–1829.

Wells, T. N. C., & Fersht, A. R. (1985) *Nature* (*London*) *316*, 656–657.

Wells, T. N. C., & Fersht, A. R. (1986) *Biochemistry 25*, 1881–1886.

Wilkinson, A. J., Fersht, A. R., Blow, D. M., & Winter, G. (1983) *Biochemistry 22*, 3581–3586.

Winter, G., Fersht, A. R., Wilkinson, A. J., Zoller, M., & Smith, M. (1982) *Nature* (*London*) *299*, 756–758.