Reversible Dissociation of Dimeric Tyrosyl-tRNA Synthetase by Mutagenesis at the Subunit Interface†

D. Hugh Jones, Andrew J. McMillan, and Alan R. Fersht*
Department of Chemistry, Imperial College of Science and Technology, London SW7 2AY, U.K.
Greg Winter
MRC Laboratory of Molecular Biology, MRC Centre, Cambridge CB2 2QH, U.K.
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ABSTRACT: Dimeric tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* exhibits half-of-the-sites reactivity and negative cooperativity in binding of tyrosine. Protein engineering has been applied to the enzyme to determine whether it can be reversibly dissociated into monomers and if the monomers are active. The target for mutation is the residue Phe-164. The side chain of Phe-164 in one subunit interacts with its symmetry-related partner in the other. Mutation of Phe-164 → Asp-164 gives a mutant [TyrTS(Asp-164)] that undergoes dissociation at high pH when the aspartate residues are ionized. The monomer is inactive and does not bind tyrosine. Dissociation is enhanced at low concentrations of enzyme by a mass action effect. Kinetic and binding measurements on TyrTS(Asp-164) with tyrosine and tyrosyl adenylate show that the monomer has very weak affinity for these ligands. Accordingly, dimerization is favored by high concentrations of tyrosine and ATP since the dimeric form has a high affinity for the ligands. The presence of tRNA does not encourage dimer formation, and so it must bind to the monomer. TyrTS(Asp-164) is fully active at pH 6 where dimerization is favored but has low activity at pH 7.8 where dissociation is favored. It should now prove possible to engineer heterodimers that may be used to investigate the subunit interactions further.

Tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* is a well-established example of an enzyme that exhibits half-of-the-sites reactivity and negative cooperativity of substrate binding. The enzyme is a dimer composed of two identical polypeptide chains, each of M_r 47 500. The dimer has been shown by X-ray crystallography to be composed of symmetrically arranged equivalent subunits, each of which contains a complete active site that does not interact directly through space with the other (Blow & Brick, 1985). Yet, despite each active site being physically separate, solution studies have shown that the dimer binds only 1 mol of tyrosine and 1 mol of (tightly bound) tRNA (Fersht, 1975; Jakes & Fersht, 1975; Dessen et al., 1982) and forms (rapidly) only 1 mol of tyrosyl adenylate (Fersht et al., 1975a). We have subsequently found that every active mutant tyrosyl-tRNA synthetase we have produced by protein engineering also binds only 1 mol of tyrosyl adenylate per mole of dimer (unpublished data). The binding of ligands must cause the subunits to lose their symmetry and equivalence according to the induced-fit mechanism of Koshland et al. (1966).

Neither the molecular mechanism nor the mechanistic role of the half-of-the-sites reactivity is known. We have decided to apply protein engineering to these problems in an attempt to discover the reason why the tyrosyl-tRNA synthetase is a dimer. We wish first to establish whether the enzyme can be engineered to dissociate reversibly into monomers and whether the monomers are catalytically active. The initial target for mutation is the residue Phe-164, which is one of the residues involved in subunit interactions. It lies on the symmetry axis so that the side chain of one Phe-164 is in contact with its symmetrically related partner in the other subunit. In this study, we mutate Phe-164 → Asp-164 in the expectation that at higher values of pH the carboxyl groups will ionize and force the subunits to dissociate.

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Experimental Procedures

Materials

Reagents were obtained from Sigma (London), Cambridge Biotechnology Ltd., Amersham International, and New England Nuclear. Crude tRNA from *B. stearothermophilus* was purified by chromatography on benzoylated DEAE-cellulose (BD-cellulose) to a tyrosine acceptance of 100 pmol/A_{260} unit.

Construction of Gene for TyrTS(Asp-164). The Phe-164 → Asp-164 mutant of the *B. stearothermophilus* tyrosyl-tRNA synthetase gene cloned in the vector M13mp93 (Winter et al., 1982) was constructed by site-directed mutagenesis using the primer 5′AACTCGGTAT*C*TGAAATGC3′ (* = mismatched base) (Carter et al., 1984). The mutation was verified by dideoxy sequencing (Sanger et al., 1977; Biggin et al., 1983) of the entire TyrTS gene (Wilkinson et al., 1984).

Purification of the Enzyme. A late log-phase culture (200 mL) of *Escherichia coli* JM101 at 37 °C was infected with mutant phage (ca. 10^{12} plaque-forming units) and immediately used to inoculate 10 L of 2 × TY medium [1.6% (w/v) tryptone, 1.0% (w/v) yeast extract, 0.5% (w/v) NaCl] in an MBR Bioreactor fermenter (Wilkinson et al., 1983). The culture was grown for 16 h at 37 °C, pH 6.8, with vigorous stirring (600 rpm) and aeration (10 L min^{-1}) to an A_{550} = 10–15. The cell suspension was concentrated to about 1 L by using a Millipore Pellicon XX4200080 filtration apparatus, and the cells were harvested by centrifugation. Cell lysis, ammonium sulfate fractionation, and anion-exchange chromatography were carried out as previously detailed (Wilkinson et al., 1983). An additional fast protein liquid chromatography (FPLC) (Pharmacia) anion-exchange chromatography step was performed as described (Lowe et al., 1985), yielding an apparently homogeneous preparation on analysis by sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis. The enzyme preparation was then dialyzed against 144

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mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 7.78) containing 0.1 mM phenylmethanesulfonyl fluoride (PMSF) and 5 mM 2-mercaptoethanol. It was rapidly frozen in small aliquots in liquid nitrogen and stored at −70 °C or under liquid nitrogen.

Methods

Kinetic Procedures. Active site titration by nitrocellulose disk filtration was routinely used to determine the concentration of enzyme (Wilkinson et al., 1983). The pyrophosphate exchange procedure of Calendar & Berg (1966) was used to measure activation of tyrosine. Equilibrium dialysis and tRNA charging procedures were as previously described (Jakes & Fersht, 1975). The two buffers most commonly employed were 144 mM Tris-HCl (pH 7.78) and 128 mM [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane hydrochloride (Bistris-HCl) (pH 6.00), each containing 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 0.1 mM phenylmethanesulfonyl fluoride, at 25 °C.

FPLC Gel Filtration. The molecular mass distribution of mutant enzyme under different conditions was examined by gel filtration. An LKB Ultro Pac TSK-G3000 SW column (7.5 × 600 mm) and precolumn (7.5 × 75 mm) was connected to a Pharmacia FPLC chromatography system and run at 0.5–1.0 mL min^{-1} (1.5–2.9 MPa) at room temperature. Sample (50–200 μL) was loaded via a loop and valve assembly and the effluent continuously monitored at 280 nm before collection of 0.25–0.5-mL fractions. NaCl (0.2 M) was incorporated in later experiments to counteract possible ionic effects. Wild type enzyme was frequently used as an internal standard. The pyrophosphate exchange assay may be used to assay mutant in the presence of wild type enzyme since, as discussed below, the mutant has low activity at pH 7.78 and low concentration of tyrosine but is fully active at pH 6. Assays on mixtures of wild type and mutant enzymes were performed in parallel at pH 6.0 and at pH 7.78, and the proportion of the two enzymes was found from the relative activities.

Results

Parameters Affecting Active Site Titration of TyrTS-(Asp-164). (1) Active Site Titration of Mutant Enzyme Is Time Dependent, and the Time Course Varies with pH (Figure 1). The stoichiometry of tyrosyl adenylate formed with wild type enzyme reaches 1 mol per mole of dimer in a few seconds and remains stable for several minutes (Fersht et al., 1975b). [It was reported (Mulvey & Fersht, 1977) that the wild type enzyme slowly forms a second mole of enzyme-bound tyrosyl adenylate with a half-time of several minutes. This has now been found to be a reproducible artifact of enzyme preparations that have been stored for extended periods at −20 °C in 50% v/v glycerol. All recent work on fresh preparations of enzymes substantiates the original reports of Fersht et al. (1975a,b) and Fersht (1975) that indicate only 1 mol of aminoacyl adenylate accumulates. All enzyme preparations that are stable to freezing and thawing are now stored frozen at <−70 °C, under which conditions the properties of the enzymes do not change.] The active site titration of mutant enzyme takes several minutes, varying with precise conditions, to reach a plateau (Figure 1). There is an initial sharp rise in active site titer which is more pronounced at pH 6.0 than at pH 7.78. This is followed by a gradual rise (of doubling time approximately 3 min at pH 6.0 and >8 min at pH 7.78). By 15 min, a plateau is approached which reaches a higher titer at pH 6.0 than at pH 7.78. The active site titer of mutant enzyme at pH 6.0 is constant at high concentrations of tyrosine (>10 μM) over a wide range of enzyme concentrations (1–30 μM).

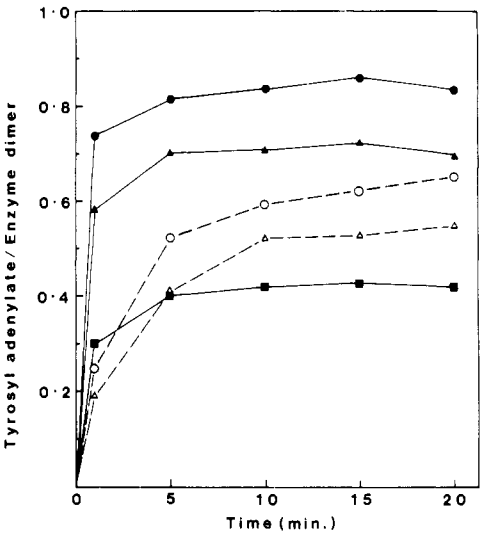

FIGURE 1: Time courses of active site titration for different concentrations of TyrTS(Asp-164) at pH 6.0 and 7.78. Titrations were performed in triplicate at 25 °C according to Wilkinson et al. (1983). Equal volumes (50 μL) of assay cocktail and enzyme were mixed, and 20-μL samples were withdrawn at the times indicated, immediately spotted onto presoaked nitrocellulose filters, and washed with 5 mL of assay buffer. The final concentrations of reagents in the assay were as follows: buffer, Tris-HCl, 144 mM (pH 7.78), or Bistris-HCl, 12.8 mM (pH 6.0); $MgCl_2$, 10 mM; PMSF, 0.1 mM; 2-mercaptoethanol, 10 mM; ATP, 2 mM; inorganic pyrophosphatase, 1 unit mL^{-1}; [^{14}C]tyrosine, 10 μM (522 Ci mol^{-1}). The data are plotted as moles of tyrosyl adenylate formed per mole of dimeric enzyme. The closed symbols and solid lines are at pH 6.00 and the open symbols and broken lines at pH 7.78; enzyme concentrations were 2.4 (● and ○), 1.2 (▲ and △), and 0.6 μM (■).

(2) Active Site Titer Decreases Reversibly with Increasing pH and Decreasing Enzyme Concentration. Whereas active site titration of the wild type enzyme is insensitive to pH in the range 6.0–8.5, the mutant Phe-164 → Asp-164 shows a rapid drop in activity above pH 7.5 (Figure 2). This drop in activity is more pronounced at lower enzyme concentration. Within this range, pH-dependent inactivation is reversible: when the pH was restored from 8.5 to 6.0, active site titer increased to the expected value. There is a marked decrease in active site titer when enzyme concentration (estimated from A_{280}) falls below approximately 5 μM (Figure 3). The maximum activity obtained corresponds to 1.04 active sites per dimer at pH 6.0 (on the basis of A_{280} = 1.05 for a 1 mg mL^{-1} solution of enzyme).

This concentration dependence of the active site titer was investigated further by preparing dilutions of enzyme of known A_{280}, measuring titer, and plotting the results as shown in Figure 4. The plot in Figure 4 is based on the assumption of a monomer–dimer equilibrium in which only the dimer binds tyrosyl adenylate (a model consistent with all the results in this study). Suppose the dimerization in the *absence* of ligands may be described by eq 1, where the dissociation constant K_d

$$2E \rightleftharpoons E_2 \quad (1)$$

$= [E]^2/[E_2]$. Let the total amount of enzyme present be $[E]_0$, as measured by A_{280} of the purified protein. Expressing $[E]_0$ as molarity of *dimer* equivalents gives

$$[E]_0 = [E_2] + \tfrac{1}{2}[E] \quad (2)$$

Active site titration gives $[E_2]$. Eliminating [E] gives

$$K_d[E_2] = 4\{[E]_0 - [E_2]\}^2 \quad (3)$$

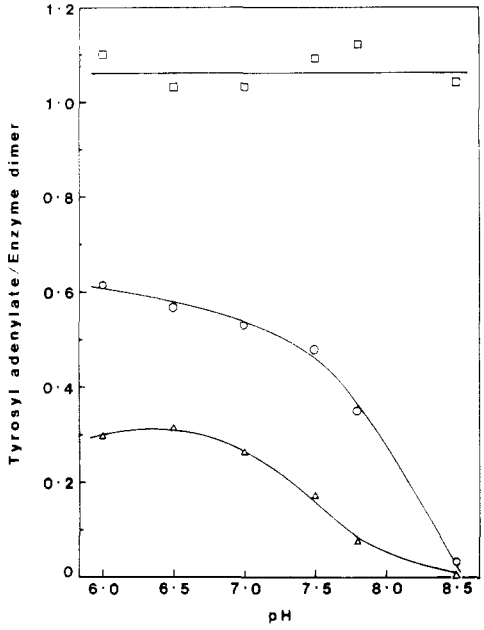

FIGURE 2: Effect of pH on active site titer of TyrTS(Asp-164) and TyrTS(Phe-164). The active site titration procedure was similar to that of Figure 1. Incubation time was 5 min at 25 °C. The buffers used and their final concentrations were as follows: pH 6.0, 12.8 mM Bistris-HCl; pH 6.5 and 7.0, 100 mM KH_2PO_4/K_2HPO_4; pH 7.5, 100 mM Tris-HCl; pH 7.78, 144 mM Tris-HCl; pH 8.5, 329 mM Tris-HCl. The enzyme concentrations were as follows: wild type enzyme, 0.25 μM (□); TyrTS(Asp-164), 1 μM (○); TyrTS(Asp-164), 0.1 μM (△).

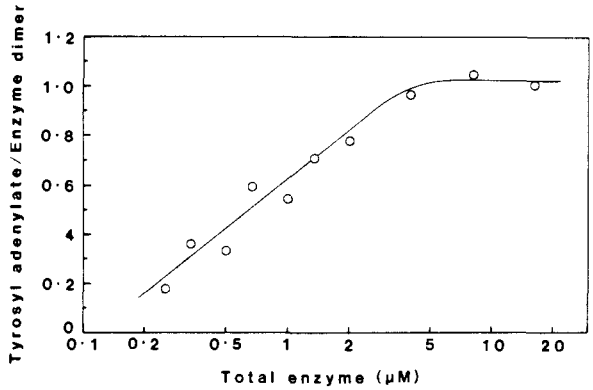

FIGURE 3: Effect of enzyme concentration $[E]_0$ on active site titer of TyrTS(Asp-164) at pH 6.0. The procedure of Figure 1 was used for active site titrations with 128 mM Bistris-HCl at pH 6.0, and 10 μM tyrosine. The dilution series was initially prepared in the absence of tyrosine. Enzyme concentration $[E]_0$ as molarity of dimer was estimated spectrophotometrically (on the basis of A_{280} = 1.05 for a 1 mg mL^{-1} solution of enzyme).

The addition of ATP and tyrosine and the formation of tyrosyl adenylate will perturb the system since additional intermediates will accumulate. However, if the only species that binds ligands tightly is the dimer, then eq 3 will still hold, but it may be shown that the K_d that is observed is a complex term composed of the true K_d of eq 1 multiplied by an attenuation factor comprised of various rate and binding constants. The plot in Figure 4 is thus consistent with a monomer–dimer equilibrium in which only the dimer is active.

Equilibrium Dialysis Experiments Show That the Affinity of the Mutant Enzyme for Tyrosine Decreases at Higher pH. At pH 7.78, wild type enzyme and tyrosine have a dissociation constant (K_s) of 11.6 μM and a stoichiometry (n) of 0.92 mol of tyrosine bound per mole of enzyme dimer [see Figure 3 of Fersht et al. (1975b)]. However, under the same conditions,

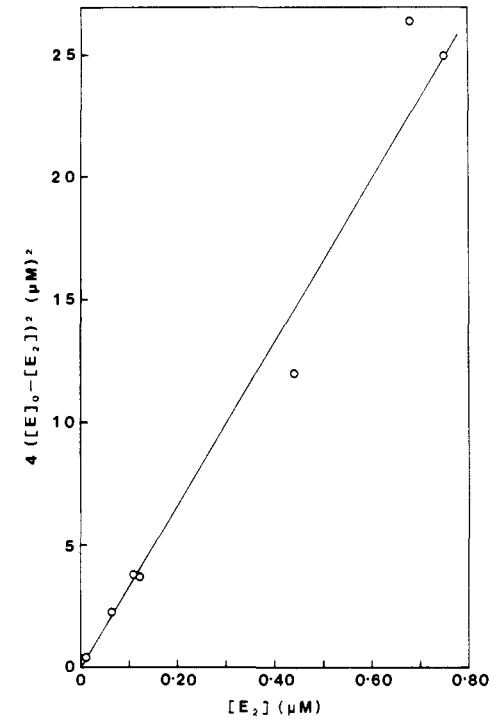

FIGURE 4: Estimate of apparent dissociation constant (K_d) of the subunits of TyrTS(Asp-164) at pH 6.0. This experiment was carried out exactly as in Figure 3. $[E]_0$ is the total concentration of enzyme present, expressed as molarity of dimer equivalents and estimated from A_{280} of the purified protein. $[E_2]$ is the concentration of dimer obtained from active site titration. The "apparent" K_d was estimated as 33 μM from the slope of the plot $K_d[E_2] = 4\{[E]_0 - [E_2]\}^2$ (for details and derivation, see text).

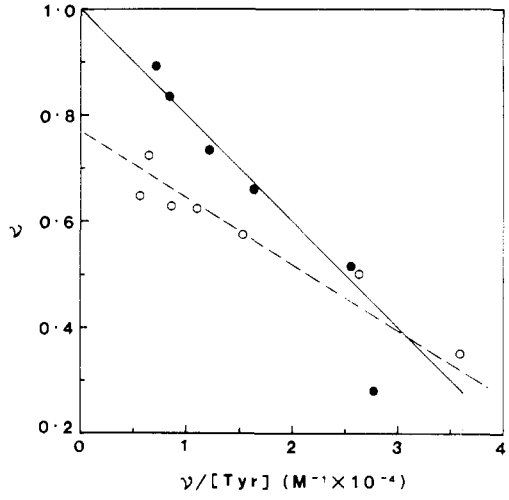

FIGURE 5: Scatchard plots of equilibrium dialysis data for tyrosine binding to TyrTS(Asp-164) and TyrTS(Phe-164) at pH 6.0. Equilibrium dialysis was carried out as described by Jakes & Fersht (1975) in 64 mM Bistris-HCl, pH 6.0 at 25 °C. [^{14}C]Tyrosine concentrations ranged from 10 to 250 μM (52.2 Ci mol^{-1}). ν is the stoichiometry in moles of tyrosine bound per mole of dimeric enzyme. TyrTS(Asp-164) (●) was present at 14 μM and gave K_s = 20 μM and n = 1.0. TyrTS(Phe-164) (○) was present at 9 μM and gave K_s = 12.5 μM and n = 0.8.

binding of tyrosine to the mutant enzyme (21 μM) was only just detectable. At pH 6.0, the two forms of the enzyme behave fairly similarly (Figure 5), giving K_s = 12.5 μM and n = 0.8 for wild type (consistent with the earlier data) and K_s = 20 μM and n = 1.0 for TyrTS(Asp-164). The concentration of mutant enzyme (14 μM) present in the dialysis cells at pH 6.0 was well in excess of that at which the active site titer begins to vary with enzyme dilution (Figure 3).

Table I: Effect of Subunit Interface Mutation on ATP Dependence of Pyrophosphate Exchange Kinetics[a]

pH		k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1} M^{-1})
6.0[b]	TyrTS(Phe-164)	9.9	0.92	1.1×10^4
6.0[b]	TyrTS(Asp-164)	6.3	0.62	1.0×10^4
7.78[c]	TyrTS(Phe-164)	9.5	1.10	8.6×10^3
7.78[c]	TyrTS(Asp-164)	7.1	2.20	3.2×10^3

[a] All kinetic experiments were performed at 25 °C in either 144 mM Tris-HCl, pH 7.78, or 12.8 mM Bistris-HCl, pH 6.0 (as indicated), each containing 10 mM $MgCl_2$ and 2 mM pyrophosphate. [b] [Tyrosine] = 50 μM. [c] [Tyrosine] = 250 μM.

Table II: Effect of Subunit Interface Mutation on Tyrosine Dependence of Pyrophosphate Exchange Kinetics[a]

pH		k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (s^{-1} M^{-1})
6.0	TyrTS(Phe-164)	11.9	1.9	6.3×10^6
6.0	TyrTS(Asp-164)	8.2	4.0	2.1×10^6
7.78	TyrTS(Phe-164)	7.0	2.9	2.4×10^6
7.78	TyrTS(Asp-164)	4.3	107.0	4.0×10^4

[a] Conditions as in Table I but with 2 mM MgATP present.

Pyrophosphate Exchange Kinetics. Analysis of the kinetic data was complicated because of deviations from Michaelis–Menten kinetics under certain conditions. The effects of the slow formation of active TyrTS(Asp-164) were minimized by using long time courses compared with those seen in Figure 1. At the concentrations of reagents used, linear Eadie–Hofstee plots were generally obtained, *except* for the mutant enzyme at pH 7.78 with low tyrosine concentrations (Figure 6). Below about 100 μM tyrosine ($\sim K_M$) the reaction rate falls very rapidly and Michaelis–Menten kinetics no longer hold. When the enzyme concentration is decreased further, the deviation from linearity is apparent at even higher tyrosine concentrations. Kinetic constants were calculated from the data at high substrate concentrations where the plots are linear. The results of these determinations for mutant compared with wild type enzyme at pH 6.0 and 7.78 are listed in Tables I and II. [The concentration of mutant enzyme at pH 6.0 giving $K_{M(Tyr)}$ = 4.0 μM was 1.0 μM, compared with 14 μM in the equilibrium dialysis experiment when $K_{s(Tyr)}$ was found to be 20 μM. At pH 7.78 a measurable $K_{M(Tyr)}$ (=107 μM) was obtained for the mutant with 2 mM ATP present in the pyrophosphate exchange assay; in equilibrium dialysis at pH 7.78, where binding was almost undetected, there was no ATP present.] The ATP dependence of the wild type enzyme is not very sensitive to the change in pH. For the mutant enzyme $k_{cat(ATP)}$ hardly changes, but $K_{M(ATP)}$ is reduced some 3.5-fold at pH 6.0, which accounts for the 3-fold improvements in k_{cat}/K_M for ATP at the lower pH. The net effect of the lower pH is to make the mutant resemble the wild type enzyme more closely in its ATP dependence with k_{cat}/K_M approximately 1×10^4 s^{-1} M^{-1} in each case.

The wild type enzyme has a specificity constant (k_{cat}/K_M) for tyrosine dependence that is 3 times higher at pH 6.0 than at pH 7.78. In contrast, there is a dramatic effect of lowering the pH on the behavior of the mutant toward tyrosine: $k_{cat}/K_{M(Tyr)}$ is more than 50-fold greater at pH 6.0. This is largely due to an increased affinity for tyrosine at the lower pH.

tRNA Charging Kinetics. Aminoacylation kinetics were performed in the standard buffers containing 14 μM $tRNA^{Tyr}$, 10 mM MgATP, 100 μM [^{14}C]Tyr, and 0.002 units mL^{-1} inorganic pyrophosphatase. The order of addition of reagents was varied: either the enzyme was preincubated with tRNA for 15 min, and the ATP and tyrosine were added subsequently, or the enzyme was preincubated with tyrosine and ATP to form the tyrosyl adenylate complex in situ, and then the tRNA was added. In either case at pH 7.78, the wild type enzyme charged tRNA at $v/[E]$ = 3 s^{-1}. TyrTS(Asp-164) (1.1 μM) preincubated with tRNA at pH 7.78 charged tRNA at $v/[E]$ = 0.02 s^{-1}. Note that under similar conditions (100 μM tyrosine and 2 mM ATP), pyrophosphate exchange takes place with $v/[E]$ = 2.1 s^{-1} (Figure 6). Preincubation of the enzyme with tyrosine and ATP, however, gave complex kinetics with an initial rate of charging that was far higher than that on preincubation with tRNA alone. The kinetics were qualitatively consistent in this case with the enzyme-bound tyrosyl adenylate complex being formed in situ and rapidly transferring the tyrosine to tRNA.

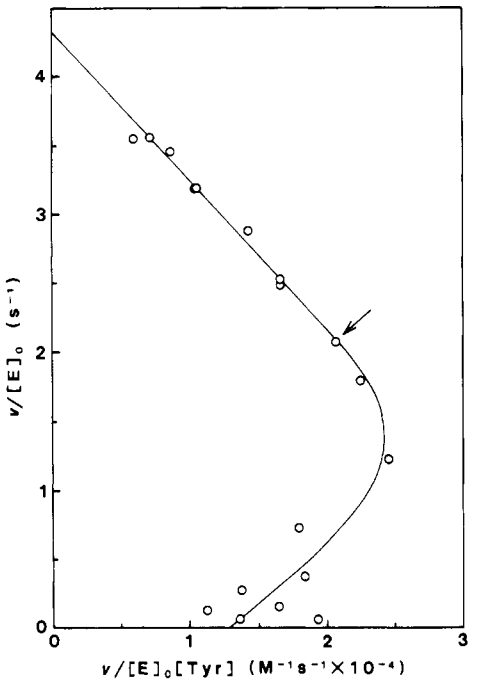

FIGURE 6: Eadie–Hofstee plot for tyrosine dependence of pyrophosphate exchange catalyzed by TyrTS(Asp-164) at pH 7.78. Assays were performed at 25 °C in 144 mM Tris-HCl, pH 7.78, containing 10 mM $MgCl_2$, 2 mM pyrophosphate, and 2 mM ATP. The data point corresponding to 100 μM tyrosine is indicated (→).

FPLC Gel Filtration Detects a Concentration-Dependent Molecular Weight for TyrTS(Asp-164) Which Also Varies with Ligands and pH. The FPLC system enabled gel filtration studies to be performed in a very short time (ca. 40-min elution period) compared with conventional column chromatography. Preliminary experiments at pH 7.78 (144 mM Tris-HCl) showed that TyrTS(Asp-164) eluted in a volume (residence time) corresponding to the monomer (M_r 47 500), whereas the wild type enzyme corresponded to M_r 95 000 (see Figure 7 at slightly different experimental conditions).

This behavior at pH 6.0 was investigated in more detail after improving the resolution of the technique by reducing the sample volume to the minimum practicable (50 μL) and increasing the ionic strength by inclusion of NaCl at 0.2 M to reduce possible binding effects on the column. It was found that the elution volume of the mutant was reduced either by increasing the enzyme concentrations or by the addition of tyrosine concentrations greater than 20 μM. The presence of 0.1 mM ATP, $MgCl_2$, and concentrations of tyrosine >10 μM also caused a reduction in the elution volume. Apparent values for the molecular weight were estimated from a calibration under identical conditions (Figure 7). The elution volumes at higher enzyme or ligand concentrations tended to that of

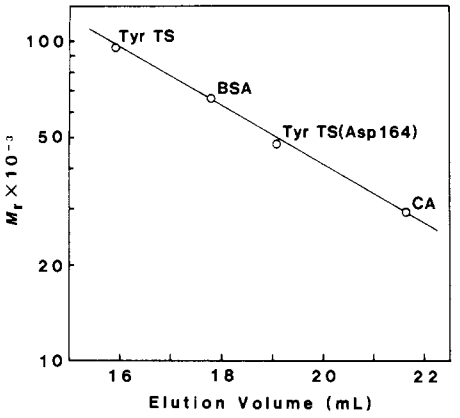

FIGURE 7: Calibration of FPLC gel filtration. The LKB TSK-G3000 SW column was run at 1 mL min^{-1} in 25 mM Bistris-HCl, pH 6.0, containing 0.2 M NaCl, 10 mM 2-mercaptoethanol, and 0.1 mM PMSF at 25 °C. Samples were loaded in a volume of 50 μL: TyrTS = 5 μM TyrTS(Phe-164) (M_r 95 000); BSA = 2 mg mL^{-1} bovine serum albumin (M_r 66 000); TyrTS(Asp-164) = <0.50 μM TyrTS-(Asp-164) (estimated dilution limit) (M_r 47 500); CA = 1.2 mg mL^{-1} carbonic anhydrase (M_r 29 000).

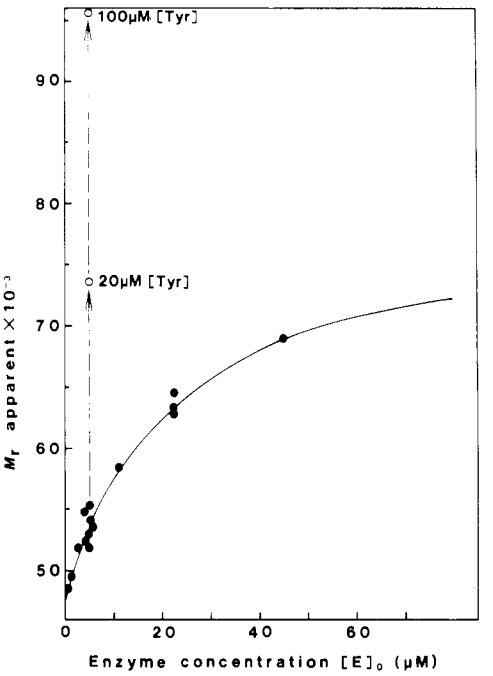

FIGURE 8: FPLC gel filtration of TyrTS(Asp-164): dependence of apparent M_r on enzyme and tyrosine concentrations. Gel filtration was performed as described in the legend to Figure 7. $[E]_0$ represents the concentration of enzyme initially applied to the column as a 50-μL sample, expressed as molarity of dimer equivalents based on A_{280} (as in Figures 3 and 4). The solid line (●) shows the concentration dependence in the absence of tyrosine. To examine the effects of tyrosine (○), the column was fully equilibrated by the passage of >2 column values of buffer containing tyrosine at the concentrations shown before applying samples of enzyme (5 μM).

Discussion

We have engineered a mutation (Phe-164 → Asp-164) at the subunit interfaces that by all established criteria induces the fully active dimeric enzyme to dissociate under the appropriate conditions to give an inactive monomer. Active site titration, gel filtration, and equilibrium dialysis at varying values of pH show that the dimeric form of TyrTS(Asp-164) is similar to wild type enzyme whereas in comparison the monomer does not bind tyrosine or form tyrosyl adenylate (or does so very weakly when compared with the dimer). The effects of the mutation, the juxtaposition of two symmetry-related aspartate residues at the subunit interface, are in accord with predictions. The monomer–dimer equilibrium position depends on three variables: pH, ligand concentration, and enzyme concentration. Association is favored by low pH, high concentrations of tyrosine, and high concentration of enzyme. Conversely, dissociation is favored by high pH, the absence of ligands, and low concentration of enzyme. These effects may be readily accounted for on qualitative grounds: (i) the enzyme presumably dissociates at high pH because the carboxyl groups of Asp-164 ionize and repel each other; (ii) high concentrations of tyrosine (and ATP) favor the formation of dimers since the dimeric form binds ligands while the monomeric form does not (eq 4); (iii) high concentration of enzyme favors association by a simple mass action effect.

$$2E \rightleftharpoons E_2 \underset{}{\overset{Tyr}{\rightleftharpoons}} E_2\cdot Tyr \underset{PP_i}{\overset{ATP}{\rightleftharpoons}} E_2\cdot Tyr\text{-}AMP \qquad (4)$$

There is also qualitative evidence from the experiments on aminoacylation of tRNA that tRNA binds to the monomer. We find that preincubating TyrTS(Asp-164) with tyrosine and ATP at pH 7.78 at concentrations where E_2·Tyr-AMP is formed in situ leads to an initial rapid aminoacylation of tRNA when it is added. But, preincubation of the enzyme with tRNA gives very slow charging on the addition of tyrosine and ATP. This occurs under conditions where, in the absence of tRNA, E_2·Tyr-AMP is formed very rapidly. Presumably, tRNA binds to the monomer and prevents the rapid formation of E_2·Tyr-AMP either directly or indirectly by inhibiting the reassociation of the subunits. If tRNA mimicked tyrosine and bound to the dimer and not to the monomer, as in eq 4, then tRNA would displace the monomer–dimer equilibrium in favor of dimer.

The quantitative analysis of the equilibria involved is not possible using the methods employed in this study. As explained under Results, analysis of the dissociation equilibrium by active site titration perturbs the system because of the secondary equilibria set up with enzyme–ligand complexes. Gel filtration of the unligated enzyme also gives data that are not readily analyzed quantitatively. As reviewed by Ackers (1970, p 427), gel filtration is a useful qualitative means of detecting association and dissociation, but there is no theoretical basis for the quantitative interpretation of such experiments—because of the continuous dilution of the sample by axial dispersion, no stable equilibrium composition is attained within the peak. The apparent molecular weight does not simply represent a weighted average of dimer and monomer molecular weights and cannot be related to K_d (Stevens & Schiffer, 1981). Although apparent dissociation constants may be fitted to such curves, they may be in error by as much as 5 orders of magnitude (Zimmerman & Ackers, 1971). At the limit of infinite dilution, the molecular weight of the monomer is obtained [Ackers (1970), p 427]—see Figures 7 and 8. We can conclude that association–dissociation of the mutant is fast compared with the residence time of the system on the column (Zimmerman, 1974).

The pH dependence of the monomer–dimer equilibrium will also be complicated because several sets of equilibria are involved that are superimposed upon the intrinsic pK_a of Asp-164 as follows. Presumably, either both Asp-164 residues or one of them is un-ionized in the dimeric form of the enzyme since there would be electrostatic repulsion when both are ionized. At very high enzyme concentrations, mass action will favor dimerization and force at least one of the Asp-164 residues to be un-ionized. The observed pK_a value for the pH dependence of the equilibrium will thus be forced to higher values at higher enzyme concentrations. The observed pK_a value of Asp-164 will be a function of the concentration of the enzyme, the dissociation constant (K_d), and the intrinsic pK_a value.

It is likely that the change of quaternary structure on dissociation of the dimer into monomers also leads to a reversible change in the tertiary structure of the subunit. The evidence for this is that the isolated monomer, unlike the dimer, does not bind tyrosine. It is expected that half-of-the-sites activity would lower the affinity of a subunit for its ligand compared with an isolated subunit in the same tertiary structure. This is because some of the binding energy of the ligand and the oligomer would be used in inducing the conformational change that is required on the Koshland–Nêmethy–Filmer model. Therefore, if the isolated subunit was in the same tertiary structure as it is in the dimer, it would be expected to bind tyrosine more tightly than the dimer does. In fact, we find the monomer binds tyrosine less tightly.

We have demonstrated in this study that we can produce stable monomers of the tyrosyl-tRNA synthetase by protein engineering. Our next aim is to use this technology to manufacture hybrid dimers to investigate the subunit interactions. For example, it may be possible to engineer another mutant with a positively charge residue (histidine, lysine, or arginine) at position 164 so that the hybrid dimer $TyrTS^+$–$TyrTS^-$ would be readily formed. This will enable specific mutations to be made in one subunit and coupled with specific mutations in the other.

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