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Asymmetry of Tyrosyl-tRNA Synthetase in Solution†

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ABSTRACT: The tyrosyl-tRNA synthetase from *Bacillus stearothermophilus* crystallizes as a symmetrical dimer with each subunit having a complete active site. The enzyme–substrate complexes, however, are known to be asymmetrical in solution because the enzyme exhibits half-of-the-sites activity by binding tightly only 1 mol of tyrosine or 1 mol of tyrosyl adenylate per mole of dimer. Evidence is now presented that the unligated enzyme is also asymmetrical in solution. Symmetry was investigated by construction of heterodimers containing one full-length subunit and one truncated subunit, allowing the introduction of different mutations into each monomer. Each dimer is active at only one site, but the site used is randomly distributed between the subunits. Each heterodimer thus consists of two equal populations, one activating tyrosine at a full-length subunit and the other at the truncated subunit. No detectable interconversion is found between active and inactive sites over several minutes either in the absence of substrates or when the enzyme is turning over in the steady state. Kinetic evidence implies that wild-type enzyme is inherently asymmetrical even in the absence of substrate.

Tyrosyl-tRNA synthetase catalyzes the aminoacylation of tRNA as a two-step reaction (eq 1 and 2). The enzyme from

$$E + Tyr + ATP \rightleftharpoons E\text{-}Tyr\text{-}AMP + PP_i \quad (1)$$

$$E\text{-}Tyr\text{-}AMP + tRNA^{Tyr} \rightarrow E + Tyr\text{-}tRNA^{Tyr} + AMP \quad (2)$$

Bacillus stearothermophilus is composed of two identical subunits (YTS/YTS).[1] Each subunit has a complete active site (Blow & Brick, 1985). However, the enzyme displays half-of-the-sites activity by binding tightly only 1 mol of tyrosine and forming only 1 mol of tyrosyl adenylate per two active sites (Fersht, 1975; Fersht et al., 1975a). The mech-

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[1] Abbreviations: (for subunits of tyrosyl-tRNA synthetase) YTS, wild type; ΔYTS, truncated wild type [see Waye et al. (1983)]; YTS(Asn-45), His → Asn mutation at position 45; ΔYTS(Asn-45), truncated with His → Asn mutation; (general) Bistris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; EDTA, ethylenediaminetetraacetic acid; FPLC, fast protein liquid chromatography; M-tRNA, modified *B. stearothermophilus* tRNA with the 3′-terminal adenosine chemically removed by the method of Fersht (1977); PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane.

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anism of this extreme form of negative cooperativity is not known (Fersht, 1975). Further, the results of these solution studies appear to contradict those of X-ray crystallography where the enzyme is a symmetrical dimer and has electron density for 2 mol of tyrosine bound/mol of dimer (Monteilhet & Blow, 1978; Blow & Brick, 1985).

Deletion of the carboxyl-terminal domains produces truncated wild-type enzyme (ΔYTS/ΔYTS), abolishing the binding of tRNA but leaving the kinetics of formation of tyrosyl adenylate and pyrophosphate exchange (eq 1) virtually unaffected (Waye et al., 1983; Wells & Fersht, 1986). Heterodimers of YTS/ΔYTS have been prepared by reversibly denaturing a mixture of parent homodimers (Carter et al., 1986). These enzymes are powerful tools for investigating half-of-the-sites activity because different mutations can be introduced into each subunit of the dimer. Heterodimers use tyrosyl adenylate formed at the truncated subunit to acylate tRNA that is bound to the full-length subunit (Carter et al., 1986). Bedouelle and Winter (1986) formed 13 different heterodimers by reversible denaturation of parent homodimers. Each heterodimer charges tRNA in the steady state at approximately half the rate of reversible renatured wild-type enzyme.

The steady-state charging of tRNA by heterodimers must overcome the following problem. The stability of enzyme-bound tyrosyl adenylate (Fersht et al., 1975b) combined with half-of-the-sites activity could lead to all of the enzyme accumulating as an inactive complex with tyrosyl adenylate bound at the full-length subunit only and not at the truncated subunit that supplies tyrosine for transfer to tRNA. This distribution of intermediate would rise from random activation at either of the two active sites followed by transfer from the truncated subunit. At each turnover, half of the remaining active enzyme would form a stable inactive complex. Since the observed rate of charging does not decrease with time, there must be a mechanism for preventing random activation or selectively removing tyrosyl adenylate from the full-length subunit. We show in this study that heterodimeric enzymes retain charging activity because they have long-lasting functional asymmetry in solution.

Experimental Procedures

Materials

Reagents were purchased from Sigma (London), Cambridge Biotechnology Ltd., BDH, and Amersham International.

Genes Coding for Mutant Enzymes. ΔYTS/ΔYTS was produced by deleting the region coding for the carboxyl-terminal domains of YTS/YTS (Waye et al., 1983). The His → Asn-45 mutation was introduced into the genes coding for YTS/YTS and ΔYTS/ΔYTS by Carter et al. (1986).

Expression and Purification of Homodimeric Enzymes. All homodimers were expressed and prepared as described by Lowe et al. (1985). The genotype of the recombinant M13 phage directing expression of tyrosyl-tRNA synthetases was confirmed by dideoxy DNA sequencing (Sanger et al., 1977) of the entire gene in each case. Any tyrosyl-tRNA synthetase activity from the *Escherichia coli* host was destroyed by incubation at 56 °C for 30 min. Each preparation was homogeneous on $NaDodSO_4$–polyacrylamide gel electrophoresis.

Methods

Preparation of Heterodimeric Enzymes. Full-length and truncated parent homodimers were denatured by mixing in 8 M urea. Urea (BDH, Aristar grade) was prepared as a 10 M stock solution that was deionized by passing down a column of Amberlite MB-1 immediately before use [see Stark et al (1960)]. Equal concentrations of full-length and truncated parent homodimers (6–20 nmol each) were incubated at 25 °C for 20 min in 0.5–3.5 mL of 8 M urea containing 0.1 mM EDTA, 10 mM 2-mercaptoethanol, and 0.1 mM PMSF. The mixture was then dialyzed against buffer A (144 mM Tris-HCl, pH 7.8, containing 0.1 mM EDTA, 10 mM 2-mercaptoethanol, and 0.1 mM PMSF) containing 6 M urea for 4 h at room temperature (approximately 20 °C). The preparation was then dialyzed for 4 h at 4 °C against buffer A containing 4 M urea. This was followed by dialysis for 14–16 h at 4 °C against buffer A containing 2 M urea, and finally against 20 mM Tris-HCl, pH 7.8, containing 0.1 mM EDTA, 10 mM 2-mercaptoethanol, and 0.1 mM PMSF (buffer B) for 3 × 3 h at 4 °C.

The dialyzed preparation contained renatured parent homodimers and heterodimer. These three enzymes were resolved from each other by FPLC ion exchange. Up to 15 nmol of total protein was loaded onto an 8-mL Mono-Q column that had been preequilibrated with buffer B. The column was operated at a flow rate of 2 mL/min. First, it was washed with buffer B for 10 min, and then the concentration of NaCl was stepped up to 80 mM. The enzymes was eluted by using a gradient of 80–240 mM NaCl over 80 min.

Measurement of Enzyme Concentration. Active-site titration by filtration through nitrocellulose disks was used to determine concentration of tyrosyl-tRNA synthetase (Wilkinson et al., 1983). The assay consists of measuring accumulation of the stable enzyme-bound tyrosyl adenylate complex in the presence of inorganic pyrophosphatase (eq 1). The enzyme is normally incubated for 2 min at 25 °C with 10 μM tyrosine and 2 mM ATP. However, under these conditions, the intermediate does not accumulate fully on enzymes carrying the His → Asn-45 mutation. These enzymes were therefore incubated for 20 min at 37 °C in 30 μM tyrosine and 10 mM ATP. Under these conditions the enzymes form 1 mol of tyrosyl adenylate/mol of dimer in 5–10 min, and there is no further accumulation over an hour.

Concentration of dimers was determined spectrophotometrically on the basis of A_{280} = 1.05 for a 1 mg mL^{-1} solution of full-length dimers, A_{280} = 1.27 for a 1 mg mL^{-1} solution of truncated dimers, and A_{280} = 1.14 for a 1 mg mL^{-1} solution of heterodimers. Extinction coefficients were calculated from the amino acid compositions of the different dimers (Mulvey et al., 1974; Winter et al., 1983; Waye et al., 1983).

Kinetic Procedures. All experiments were performed at 25 °C and in 144 mM Tris-HCl, pH 7.8, containing 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 0.1 mM PMSF. Rates of pyrophosphate exchange were measured as described by Calendar and Berg (1966). The kinetics of aminacylation of tRNA were determined by the method of Wilkinson et al. (1983). Kinetic data were analyzed by using the ENZFITTER program (Leatherbarrow, 1987).

Preparation and Characterization of Enzyme-Bound Tyrosyl Adenylate. Enzymes were incubated as described for active-site titration except the buffer was 12.8 mM Bistris-HCl, pH 6.0, and then unreacted substrates were removed by gel filtration through Sephadex G-50 (medium grade) using 12.8 mM Bistris-HCl, pH 6.0, containing 10 mM $MgCl_2$ (Fersht & Jakes, 1975). Under these conditions, tyrosyl adenylate is lost from wild-type enzyme with $t_{1/2}$ of 8 × 10^4 min (T. N. C. Wells and A. R. Fersht, unpublished data), and ≥99.9% of the radiolabeled tyrosine is bound to the enzyme after gel filtration.

Results

Preparation of Heterodimeric Tyrosyl-tRNA Synthetases.

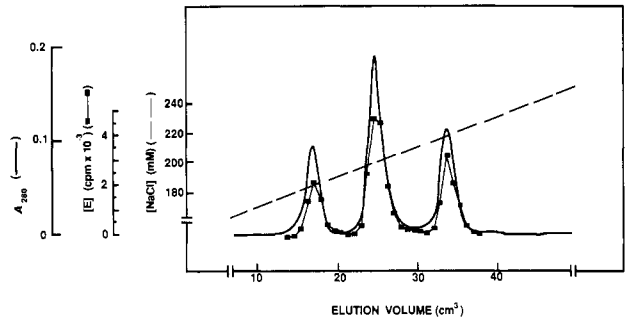

FIGURE 1: Elution profile for purification of YTS/ΔYTS by FPLC ion exchange. Details are given under Methods. A mixture of renatured YTS/YTS, YTS/ΔYTS, and ΔYTS/ΔYTS (approximately 10 nmol total of enzyme) was applied to the Mono-Q column. The heavy solid line gives the A_{280} of the eluant. Enzyme concentration was determined by active-site titration and is shown by the light solid line. [NaCl] in the elution buffer is given by the broken line.

Construction of heterodimeric enzymes allows introduction of different mutations into each subunit of the dimer. These enzymes are thus very useful for studying half-of-the-sites activity. Heterodimers were first prepared by reversibly denaturing a mixture of parent homodimers in urea (Carter et al., 1986). The urea was removed, and the enzymes were refolded and resolved from each other by polyacrylamide gel electrophoresis. Heterodimeric tyrosyl-tRNA synthetases have also been renatured from urea by dialysis in the presence of bovine serum albumin (Bedouelle & Winter, 1986). The carrier protein was required for regeneration of activity. This approach generates a rather crude preparation of heterodimeric enzyme that is not ideally suited to detailed kinetic analysis. Wild-type enzyme renatured after this dialysis procedure regains only 73% of its tRNA aminoacylation activity (Bedouelle & Winter, 1986). Yields of active enzyme from preparative polyacrylamide gel electrophoresis are generally low because of irreversible denaturation and difficulties in elution from gels. Heterodimeric tyrosyl-tRNA synthetases have also been produced by mutagenesis at the subunit interface (Ward et al., 1987). Dissociation into inactive monomers makes these enzymes difficult to use in study of half-of-the-sites activity (Ward et al., 1987).

We have improved the procedure for preparation of heterodimers as follows. Equimolar amounts of the full-length and truncated parent dimers were mixed in 8 M urea. The denaturant was then slowly removed by dialysis against progressively lower concentrations of urea, allowing the enzymes time to refold. Renatured parent homodimers and heterodimers were resolved from each other by FPLC ion exchange (Figure 1). Truncated dimer elutes at about 185 mM NaCl, heterodimer at 200 mM NaCl, and full-length dimer at 215 mM NaCl. The enzymes were shown to be pure by Na-DodSO_4–polyacrylamide gel electrophoresis for the homodimers and by FPLC gel filtration (Ward et al., 1986) for the heterodimers. The yield of protein was between 40 and 81%.

Fully active heterodimers were thus produced at high yield and high purity. The ratio of total renatured parent homodimers (full-length plus truncated) to heterodimers was 1.0 ± 0.1 for each of three preparations. This suggests that the presence or absence of carboxyl-terminal domains has no direct or indirect effect on association of subunits to form dimers. This conclusion extends the observation from X-ray crystallography that the carboxyl-terminal domains do not contribute to the structure of the subunit interface (Blow & Brick, 1985).

Detection of Half-of-the-Sites Activity in Tyrosyl-tRNA Synthetase. This requires precise knowledge of the concentration of binding sites in the enzyme solution. A preparation containing either contaminants or inactive enzyme could appear to have negative cooperativity. The concentration of protein in preparations of tyrosyl-tRNA synthetase is measured accurately by spectrophotometry since each enzyme is purified to electrophoretic homogeneity and the extinction coefficient is precisely known. Concentration of active enzyme is accurately determined by active-site titration (Fersht et al., 1975b) and shows that 1 mol of tyrosyl adenylate is formed per mole of enzyme dimer. This could be caused by half-of-the-sites activity or by exactly half of the preparation being inactive. Such contamination is very unlikely because a second mole of tyrosine is bound only after formation of tyrosyl adenylate (Fersht, 1975), indicating that the second half of the preparation has activity which is expressed only after the first half has reacted. Further, each of many preparations of wild-type enzyme has the same stoichiometry of formation of tyrosyl adenylate. The level of contamination by inactive enzyme would be expected to vary between preparations. Most characterized mutants of tyrosyl-tRNA synthetase (over 20 enzymes in all) appear to have half-of-the-sites activity in formation of tyrosyl adenylate, suggesting that this is a genuine characteristic of the enzyme and not artifactual. Further mutants tend toward, but never reach, 1 mol of tyrosyl adenylate/mol of dimer due to slow formation or rapid loss of the intermediate. However, each of the enzymes in the present work forms 0.92–1.07 mol of tyrosyl adenolate/mol of dimer both before and after treatment with urea. Evidence, therefore, strongly suggests that each enzyme has half-of-the-sites activity.

Table I: Pyrophosphate Exchange Kinetics[a]

enzyme	K_M for Tyr (μM)	K_M for ATP (mM)	k_{cat} (s^{-1})	rel k_{cat}[b]
YTS/YTS	2.1	1.0	8.2	1.0
renatured YTS/YTS	2.2	0.93	8.5	1.0
ΔYTS/ΔYTS	2.4	1.1	7.5	0.91
renatured ΔYTS/ΔYTS	2.8	1.2	7.2	0.88
YTS/ΔYTS	2.3	1.3	7.7	0.93
YTS(Asn-45)/ΔYTS	2.3	1.2	3.9	0.48
YTS/ΔYTS(Asn-45)	2.0	1.2	3.7	0.46

[a] Experimental conditions given under Methods. Pyrophosphate was at 2 mM, and the concentration of $MgCl_2$ was maintained at 10 mM greater than that of ATP. Enzyme concentration was 0.1–0.2 μM. Dependence upon tyrosine was measured at 2 mM ATP, and dependence upon ATP was determined at 50 μM tyrosine. Values of k_{cat} were obtained by extrapolation to infinite concentration of both tyrosine and ATP, assuming Michaelis–Menten kinetics. The following enzymes showed no detectable activity: YTS(Asn-45)/YTS(Asn-45), renatured YTS(Asn-45)/YTS(Asn-45), ΔYTS(Asn-45)/ΔYTS(Asn-45), and renatured ΔYTS(Asn-45)/ΔYTS(Asn-45). [b] Relative k_{cat} is the value for each enzyme over that for the native wild type.

Kinetic Properties of Reversibly Denatured Enzymes and Heterodimers. The enzymes in the current work each form 0.92–1.07 mol of tyrosyl adenylate/mol of dimer. Thus at least 92% of every preparation appears to be active enzyme. The kinetic properties of the native and renatured enzymes were characterized as follows.

(*a*) *Pyrophosphate Exchange Kinetics.* Deletion of the carboxyl-terminal domains of YTS/YTS to produce ΔYTS/ΔYTS has little effect on pyrophosphate exchange kinetics. The values of K_M for tyrosine, K_M for ATP, and k_{cat} are all very similar for the two enzymes (Table I). The lack of effect of the deletion on pyrophosphate exchange kinetics is well documented (Waye et al., 1983; Wells & Fersht, 1986).

In wild-type enzyme, His-45 stabilizes the transition state but interacts weakly with substrates in the ground state (Leatherbarrow et al., 1985). Mutation of His → Asn-45 removes this interaction with the transition state, drastically

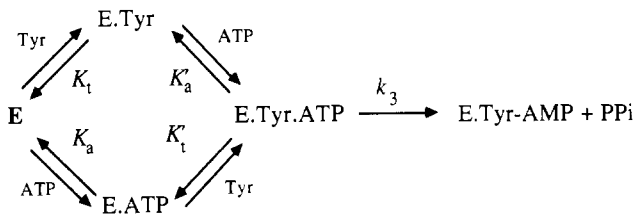

FIGURE 2: Kinetic scheme for formation of enzyme-bound tyrosyl adenylate.

Table II: Pre-Steady-State Kinetics for Formation of Enzyme-Bound Tyrosyl Adenylate[a]

enzyme	K_t' (μM)	K_a' (mM)	k_3 (s^{-1})
YTS/YTS	8.5	4.7	38
ΔYTS/ΔYTS	12[b]	5.2	34
YTS(Asn-45)/YTS(Asn-45)	7.0	1.5	0.0037
renatured YTS(Asn-45)/YTS(Asn-45)	6.8	1.8	0.0038
YTS(Asn-45)/ΔYTS[c]	7.6	1.6	0.0039
ΔYTS(Asn-45)/ΔYTS(Asn-45)	6.5	1.2	0.0030
renatured ΔYTS(Asn-45)/ΔYTS(Asn-45)	7.1	1.5	0.0032
YTS/ΔYTS(Asn-45)[c]	8.6	1.3	0.0034

[a] Experimental conditions given under Methods. The assay consisted of following the time course of active-site titration at 25 °C (see Figure 2) except for YTS/YTS and ΔYTS/ΔYTS, where values are from Wells and Fersht (1986). Concentration of enzyme was 0.3–0.6 μM, and $MgCl_2$ was maintained at 10 mM greater than ATP. Tyrosine dependence was measured at 30 mM ATP, and ATP dependence was determined at 30 μM tyrosine. Values of k_3 were obtained by extrapolation to infinite concentration of both tyrosine and ATP, assuming Michaelis–Menten kinetics. [b] Value of K_t. K_t' is probably very similar to that for YTS/YTS. [c] Values determined at high substrate concentrations, 1.5–30 μM tyrosine or 0.2–30 mM ATP, so that formation of tyrosyl adenylate by wild-type active sites reaches completion by the first time point (Figure 3), which allows subsequent monitoring of slow formation of the intermediate at the Ans-45 active site. Values given, therefore, apply to the mutant active site.

decreasing the catalytic rate but having little effect on affinity for free substrates (Leatherbarrow & Fersht, 1987). All homodimers carrying the His → Asn-45 mutation show no detectable pyrophosphate exchange activity (Table I), an observation that is expected on extrapolation from the pre-steady-state data.

Reversibly denaturing YTS/YTS and ΔYTS/ΔYTS does not cause a significant change in K_M for tyrosine, K_M for ATP, or k_{cat} (Table I), indicating that the procedure has no apparent effect on the functioning of the amino-terminal domains which are responsible for catalyzing pyrophosphate exchange.

The heterodimer YTS/ΔYTS displays kinetic properties that are very similar to those of native YTS dimers (Table I), showing that denaturation followed by association to form heterodimers causes no significant change in the functioning of the amino-terminal domains.

The two heterodimers carrying the His → Asn-45 mutation at one of the active sites have similar kinetics for pyrophosphate exchange (Table I), displaying unaltered values of K_M for tyrosine and K_M for ATP relative to that of native YTS/YTS. The value of k_{cat} is approximately halved because half of the active sites are those of Asn-45 and so have no detectable activity under the conditions used. The wild-type active sites are thus functioning normally in each of these heterodimers.

(*b*) *Pre-Steady-State Kinetics of Formation of Enzyme-Bound Tyrosyl Adenylate.* Pyrophosphate exchange catalyzed by active sites containing Asn-45 is too slow to measure in the steady state, but the formation of 1 mol of enzyme-bound tyrosyl adenylate is readily monitored in the presteady state (Figure 2). YTS(Asn-45) dimers and ΔYTS(Asn-45) dimers each display similar values of K_t', K_a', and k_3 (Table II),

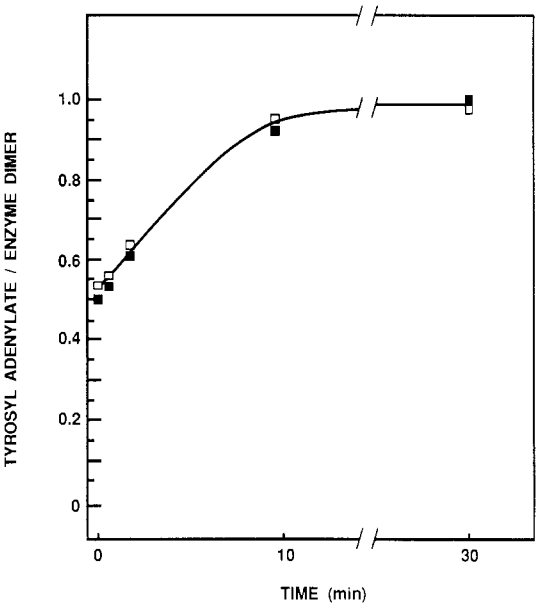

FIGURE 3: Time course for formation of enzyme-bound tyrosyl adenylate by heterodimers containing Asn-45 at one active site. Experimental details are given under Methods. Enzymes were at 0.5 μM, and the assays were at 37 °C. (Open symbols) YTS(Asn-45)/ΔYTS; (filled symbols) YTS/ΔYTS(Asn-45).

Table III: Activity in Charging of tRNA[a]

enzyme	$\nu/[E]$ (s^{-1})
YTS/YTS	3.4
renatured YTS/YTS	3.3
YTS/ΔYTS	1.8
YTS(Asn-45)/ΔYTS	2.0

[a] Experimental conditions given under Methods. Enzymes were at 20–50 nM; tyrosine, 100 μM; ATP, 10 mM; and $MgCl_2$, 20 mM. The following enzymes showed no detectable activity: ΔYTS/ΔYTS, renatured ΔYTS/ΔYTS, YTS(Asn-45)/YTS(Asn-45), renatured YTS(Asn-45)/YTS(Asn-45), ΔYTS(Asn-45)/ΔYTS(Asn-45), renatured ΔYTS(Asn-45)/ΔYTS(Asn-45), and YTS/ΔYTS(Asn-45).

indicating that deletion of the carboxyl-terminal domains has no significant effect on the formation of tyrosyl adenylate. This observation was expected because this deletion does not produce a large change in the pre-steady-state kinetics of ΔYTS/ΔYTS (Wells & Fersht, 1986). Mutation of His → Asn-45 produces a 10^4-fold decrease in k_3 and small changes in the values of K_t' and K_a' (Table II). Reversible denaturation of YTS(Asn-45) dimers or ΔYTS(Asn-45) dimers does not affect the kinetics of formation of tyrosyl adenylate (Table II).

Each of the two heterodimers containing one mutant active site displays biphasic kinetics for formation of tyrosyl adenylate (Figure 3). The kinetics of the second phase are very similar for each of the two enzymes. The values of K_t', K_a', and k_3 are almost identical with those for Asn-45 homodimers (Table II).

(*c*) *Kinetics of tRNA Charging.* As the carboxyl-terminal domains of YTS are required for binding of tRNA (Waye et al., 1983), all truncated homodimers display no ability to charge tRNA (Table III). The rate of tRNA charging by wild-type YTS dimers is virtually unaffected by reversible denaturation (Table III), indicating that the procedure has no apparent effect on the functioning of either the carboxyl- or amino-terminal domains since both are required. Mutation of His → Asn-45 in the full-length enzyme decreases the rate of turnover so that aminoacylation of tRNA by YTS(Asn-45)/YTS(Asn-45) is not detectable either before or after

Table IV: Efficiency of Transfer of Tyrosine to tRNA from Enzyme-Bound Tyrosyl Adenylate[a]

enzyme	fate of radiolabel after addition of tRNA to E·[^{14}C]Tyr-AMP: % remaining as E·Tyr-AMP[b]	% transferred to Tyr-tRNA[c]	% released into solution[d]
YTS/YTS	1.1	74	25
r YTS/YTS[e]	3.4	70	27
ΔYTS/ΔYTS	93	0.0	7.2
r ΔYTS/ΔYTS[e]	84	0.0	16
YTS/ΔYTS	48	37	15
YTS(Asn-45)/ YTS(Asn-45)	3.7	77	19
rYTS(Asn-45)/ YTS(Asn-45)[e]	3.3	72	25
YTS(Asn-45)/ΔYTS	44	42	14
ΔYTS(Asn-45)/ ΔYTS(Asn-45)	92	0.0	8.3
rΔYTS(Asn-45)/ ΔYTS(Asn-45)[e]	81	0.0	19
YTS/ΔYTS(Asn-45)	47	37	16

[a] Experimental details given under Methods and in Fersht and Jakes (1975). Each enzyme formed 0.92–1.07 mol of tyrosyl adenylate/mol of dimer. [b] Radioactivity remaining associated with enzyme (measured by filtration through nitrocellulose discs). [c] Radiolabel transferred to tRNA (determined by precipitation using trichloroacetic acid). [d] Radioactivity not accounted for in footnotes *b* and *c*. [e] r indicates renatured enzyme. Approximately 0.5 nmol of enzyme was used for each experiment.

denaturation. The YTS/ΔYTS(Asn-45) heterodimer has no detectable aminoacylation activity because tyrosine must be activated at the truncated subunit before it can be used to charge tRNA [this confirms an observation made by Carter et al. (1986)]. This absence of aminoacylation activity also indicates that the heterodimers do not dissociate reversibly either on storage or under assay conditions. Such dissociation would be easily detectable since it would re-form wild-type dimers that charge tRNA. YTS/ΔYTS and YTS(Asn-45)/ΔYTS heterodimers each aminoacylate tRNA at similar rates, and this rate is almost exactly half that of wild-type dimers (Table III).

(*d*) *Efficiency of Transfer of Tyrosine to tRNA from Enzyme-Bound Tyrosyl Adenylate.* Tyrosine activated in the first step of the reaction catalyzed by tyrosyl-tRNA synthetase has three possible fates on addition of tRNA. It may remain bound to the enzyme, be transferred to tRNA, or be liberated into solution. Dimers to YTS or YTS(Asn-45) each transfer 74–77% of the activated tyrosine to tRNA, an efficiency which is similar to that of wild-type enzyme from *E. coli* (Fersht & Jakes, 1975). Mutation of His → Asn-45, therefore, has no effect on the efficiency of transfer.

Truncated YTS dimers cannot bind or charge tRNA (Waye et al., 1983). Any tyrosine liberated from ΔYTS/ΔYTS or ΔYTS(Asn-45)/ΔYTS(Asn-45) on addition of tRNA represents dissociation or hydrolysis. These dimers fail to charge tRNA using enzyme-bound tyrosyl adenylate, and 7.2–8.3% of the intermediate is lost into solution (Table IV). Mutation of His → Asn-45 again has no significant effect, ΔYTS-(Asn-45) dimers having similar properties to ΔYTS dimers (Table IV). Reversibly denaturing each of the four homodimers YTS, ΔYTS, YTS(Asn-45), and ΔYTS(Asn-45) produced enzymes that release slightly more radiolabel into solution (Table IV). The reason for this observation is not known, but the effect is small and so does not interfere with interpretation of the data.

Each of the three heterodimers YTS/ΔYTS, YTS/ΔYTS(Asn-45), and YTS(Asn-45)/ΔYTS transfer tyrosine from enzyme-bound tyrosyl adenylate to tRNA with similar efficiency, which is almost exactly half that of native YTS dimers (Table IV). This indicates that each heterodimer forms the intermediate on the truncated subunit in about 50% of the population and on the full-length subunit in the remainder. Only tyrosine activated at the truncated subunit can be used to charge tRNA (Carter et al., 1986), and each of the three heterodimers transfers tyrosine at an efficiency similar to that found for full-length dimers. At least 88% of the tyrosine activated at the full-length subunit of the heterodimers remains bound to the enzyme.

Rigorous characterization of each of the enzymes used in this study thus shows that catalytic sites and binding sites in reversibly denatured and heterodimeric enzymes all function with essentially identical rate and binding constants to those found for the same sites in parental homodimers.

What Dictates Which of the Two Active Sites in Each Dimer Is Functional? Consider the complex of tyrosyl adenylate bound to YTS/ΔYTS heterodimer. Tyrosine is liberated from the truncated subunit when tRNA is bound, allowing the enzyme to recycle. Tyrosine activated on the full-length subunit cannot be transferred and could remain bound to the enzyme. This could lead to inhibition of the second active site because of half-of-the-sites activity. Tyrosyl adenylate is equally distributed between the two subunits of the dimer during the first turnover and so half of the enzyme could become inactivated. A similar loss in activity would occur on each subsequent turnover if the two active sites function randomly. This would lead to the enzyme displaying no activity in steady-state assays since the active site on the truncated subunit turns over rapidly. However, the YTS/ΔYTS heterodimer aminoacylates tRNA at half the rate of native enzyme (Table III). Four possible explanations for the charging activity of the heterodimer were tested.

(a) Binding of tRNA to full-length subunit induces the catalytic site on the truncated subunit to become active even if there is tyrosyl adenylate bound on the full-length subunit. Therefore, the enzyme no longer displays half-of-the-sites activity when tRNA is bound.

(b) Binding of tRNA to full-length subunit induces dissociation or hydrolysis of any tyrosyl adenylate bound to that subunit.

(c) tRNA first binds to one subunit and then directs the opposite subunit to form tyrosyl adenylate.

(d) Half the enzyme population turns over, repeatedly using only the truncated subunit. The remainder forms tyrosyl adenylate at the full-length subunit and so cannot turnover.

(*a*) *Half-of-the-Sites Activity Is Displayed Even When tRNA Is Bound.* The effect of tRNA binding on half-of-the-sites activity in the formation of enzyme-bound tyrosyl adenylate was determined by using tRNA that had been modified (referred to as M-tRNA) to remove the 3′-terminal adenosine which accepts the amino acid. The analogue was produced [as described by Fersht (1977)] by periodate oxidation followed by an addition–elimination reaction with lysine. M-tRNA has no detectable capacity to accept tyrosine, acceptance being at least 500-fold lower than that of native tRNA. M-tRNA is a competitive inhibitor of charging by YTS/YTS with $K_I = 0.97K_M$, indicating that M-tRNA has similar binding properties to those of native tRNA. The number of moles of tyrosyl adenylate formed per mole of wild-type dimer is 1.00 ± 0.02 in the presence or absence of M-tRNA. Thus, only one active site functions per dimer even in the presence of M-tRNA. Similar results were obtained for each of the enzymes used in this study. The 3′-terminal

Table V: Effect of tRNA on Rate of Pyrophosphate Exchange[a]

enzyme	addition	$\nu/[E]$ (s^{-1})
YTS/ΔYTS(Asn-45)	none	0.85
YTS/ΔYTS(Asn-45)	M-tRNA[b]	0.84
YTS/ΔYTS(Asn-45)	native tRNA	0.81
YTS(Asn-45)/ΔYTS	none	0.82
YTS(Asn-45)/ΔYTS	M-tRNA[b]	0.83

[a] Rate was measured as described in legend to Table I. Enzyme was at 0.2 μM; tyrosine, 2 μM; and ATP, 1 mM. Where indicated, tRNA (final concentration, 12 μM) was added to the incubation before tyrosine and ATP. [b] M-tRNA is *B. stearothermophilus* tRNA with the 3′-terminal adenosine removed.

adenosine residue of native tRNA could possibly cause alleviation of half-of-the-sites activity. However, this seems most unlikely as its removal has no effect on binding affinity.

(*b*) *tRNA Does Not Induce Loss of Tyrosyl Adenylate from the Full-Length Subunit in Heterodimers.* The complex of radiolabeled tyrosyl adenylate bound to YTS/ΔYTS was separated from unreacted substrates by gel filtration, and then its stability was measured under various conditions. The complex decays exponentially with a rate constant of 4.3×10^{-4} s^{-1} in the presence of 100 μM unlabeled tyrosine and 10 mM ATP. This value represents the sum of the losses from each subunit of the dimer. Addition of saturating tRNA (10 μM) in the presence of 100 μM unlabeled tyrosine and 10 mM ATP leads to rapid loss of 52% of the radioactive tyrosine bound to the enzyme because tRNA is charged using tyrosyl adenylate formed at the truncated subunit. The remaining 48% of the intermediate (bound at the full-length subunit) is lost with a rate constant of 4.2×10^{-4} s^{-1}, indicating that tRNA has no significant effect on the stability of tyrosyl adenylate bound at the same subunit. Further, this rate constant is far too low to account for the charging activity of the enzyme under these conditions where the rate is 1.8 s^{-1}.

(*c*) *tRNA Does Not Direct Which Active Site Is Functional.* There are two possible mechanisms by which binding of tRNA to the carboxyl terminal domain of one subunit of the dimer could favor the functioning of the active site on the other subunit. First, tRNA could inhibit the activity of the full-length subunit, or, second, it could stimulate the activity of the truncated subunit. These possibilities were investigated.

(*1*) *tRNA Does Not Inhibit Catalysis by the Full-Length Subunit to Which It Binds.* This model predicts that addition of tRNA to YTS/ΔYTS(Asn-45) will decrease the rate of pyrophosphate exchange because the active site on the full-length subunit has a much higher turnover number than that on the truncated subunit. This enzyme is unable to charge tRNA at a significant rate; therefore, the effect of native tRNA on catalysis of pyrophosphate exchange can be observed. The effect of M-tRNA was also measured in order to test whether removal of the 3′-terminal adenosine residue resulted in any change in the properties of the tRNA. Neither of the two tRNA species significantly affects the rate of pyrophosphate exchange by YTS/ΔYTS(Asn-45) heterodimer (Table V).

Any inhibition of catalysis by binding of tRNA to the full-length subunit of heterodimers was also measured in the pre steady state. Very low ATP concentration was used in order to measure the rate of formation of enzyme-bound tyrosyl adenylate by the wild-type active site of the YTS/ΔYTS(Asn-45) heterodimer. Under these conditions, only the wild-type active site forms tyrosyl adenylate at a measurable rate. The heterodimer activates tyrosine at a rate similar to that of wild-type enzyme, both in the presence and absence of tRNA (Table VI), confirming that tRNA does not inhibit

Table VI: Effect of tRNA on Rate Constant for Formation of Enzyme-Bound Tyrosyl Adenylate[a]

enzyme	addition	obsd rate constant (s^{-1})
YTS/YTS	none	7.8×10^{-3}
YTS/ΔYTS(Asn-45)[b]	none	7.3×10^{-3}
YTS/ΔYTS(Asn-45)[b]	native tRNA	7.2×10^{-3}
YTS/ΔYTS(Asn-45)[b]	M-tRNA[c]	7.3×10^{-3}
YTS(Asn-45)/ΔYTS[b]	none	7.2×10^{-3}
YTS(Asn-45)/ΔYTS[b]	M-tRNA[c]	7.2×10^{-3}

[a] Experimental details given under Methods. The first-order rate of formation of E·[^{14}C]Tyr-AMP (see Figure 2) was followed at 25 °C. Where indicated, tRNA (final concentration, 10 μM) was added to the incubation before tyrosine and ATP. Concentration of enzyme was 0.5 μM; tyrosine, 5 μM; ATP, 4 μM; and $MgCl_2$, 10 mM. [b] At such low concentrations of ATP, formation of tyrosyl adenylate by the mutant active sites is negligible during the course of the experiment. Measured rate constants, therefore, follow formation of the intermediate at the wild-type active site. [c] M-tRNA is *B. stearothermophilus* tRNA with the 3′-terminal adenosine removed.

Table VII: Effect of Preincubation with Tyrosine and ATP on Rate of tRNA Charging[a]

enzyme	preincubation	$\nu/[E]$[b] (s^{-1})
YTS/YTS	tRNA	3.6
YTS/YTS	tyrosine + ATP	3.4
YTS/ΔYTS	tRNA	1.8
YTS/ΔYTS	tyrosine + ATP	1.6
YTS(Asn-45)/ΔYTS	tRNA	1.8
YTS(Asn-45)/ΔYTS	tyrosine + ATP	1.7

[a] Experimental conditions given under Methods. Enzymes were at 20–50 nM; tyrosine, 100 μM; ATP, 10 mM; and $MgCl_2$, 20 mM. Assays were preincubated in the presence of either tRNA or tyrosine and ATP for 2 min. This preincubation contained substrates at 125% of their final concentrations. [b] Mol of tRNA charged (mol of enzyme)$^{-1}$ s^{-1}.

the activity of the full-length subunit to which it binds.

(*2*) *tRNA Fails To Stimulate the Activity of the Truncated Subunit to Which It Does Not Bind.* This model predicts that addition of tRNA should increase the activity of the YTS(Asn-45)/ΔYTS heterodimer because the active site on the truncated subunit has a much higher turnover number than that on the full-length subunit. Effects of tRNA were studied by using M-tRNA since native tRNA would rapidly be aminoacylated. M-tRNA has no significant effect on the rate of pyrophosphate exchange (Table V) or formation of enzyme-bound tyrosyl adenylate (Table VI), suggesting that the model does not hold.

(*3*) *Analysis of Charging Confirms That tRNA Does Not Direct Which Active Site Is Functional.* Preincubation of heterodimers with tyrosine and ATP in the absence of tRNA allows enzyme-bound tyrosyl adenylate to accumulate without any possible direction by tRNA. If tRNA directs which subunit is active, then such preincubation would decrease the rate of subsequent aminoacylation by 50% relative to that of enzyme preincubated with tRNA. This would be because half of the population would form tyrosyl adenylate on the full-length subunit in the absence of tRNA (see Table IV) and so be unable to cycle. The effect of preincubation with tyrosine and ATP upon the rate of charging was measured for YTS/YTS, YTS/ΔYTS, and YTS(Asn-45)/ΔYTS (Table VII). Such preincubation does not significantly decrease the rate of charging, confirming that tRNA does not direct which subunit functions (Table VII).

(*d*) *The Same Active Site Is Used Each Time a Dimer Turns Over.* The following experiments show that the heterodimers we have studied each divide into two populations

of equal size. The full-length subunit catalyzes the first turnover in one population, and the truncated subunit is active in the other. Interchange between the two populations is very rare in the steady state, indicating that the enzyme preferentially utilizes the same active site for each turnover.

Random selection of the functional active site in heterodimers containing Asn-45 would lead to formation of tyrosyl adenylate on almost all of the wild-type active sites before the mutant active sites have time to form the intermediate. However, both YTS(Asn-45)/ΔYTS and YTS/ΔYTS(Asn-45) display biphasic time courses in the formation of tyrosyl adenylate at high substrate concentration (Figure 3). Under these conditions, half of the population forms the intermediate rapidly at the wild-type active site, and then the remaining enzyme forms the intermediate slowly at the mutant subunit. The two enzymes show almost identical behavior, indicating that the location of the mutation, on the full-length or truncated subunit, has no effect. The equal distribution of tyrosyl adenylate between the two subunits was confirmed by allowing formation of enzyme-bound tyrosyl adenylate to reach completion and then measuring the proportion of radiolabeled tyrosine transferred to tRNA (Table IV). These data demonstrate that, during the first turnover, the full-length subunit functions in half of the population and the truncated subunit is active in the remainder.

YTS/ΔYTS and YTS(Asn-45)/ΔYTS both charge tRNA at the same rate, which is half that of the wild-type enzyme (Table III). This is because, in the first turnover, half of each enzyme forms tyrosyl adenylate on the full-length subunit and remains as a dead-end complex that cannot charge tRNA. The remainder forms the intermediate on the truncated subunit and so can aminoacylate tRNA. The functional asymmetry is maintained for subsequent cycles so that half of the enzyme turns over at wild-type rate, and the rest remains as an inactive dead-end complex. The observed rate of charging for each of these enzymes is, therefore, half that of the wild-type.

Quantitative Analysis of Functional Asymmetry. Consider catalysis of aminoacylation of tRNA by a heterodimeric enzyme. During the first turnover, some of the enzyme (say, N_0 mol) forms tyrosyl adenylate at the truncated subunit. This enzyme can charge tRNA and then recycle. A proportion, P, does not maintain asymmetry but forms tyrosyl adenylate at the full-length subunit, producing a dead-end complex that is very stable (see above). The remaining fraction, $1 - P$, maintains asymmetry and forms the intermediate on the truncated subunit. This enzyme can charge tRNA and then recycle. The number of moles of enzyme that are still using the truncated subunit will be given by $N_t = N_0(1 - P)^n$, where n is the number of cycles completed.

(*a*) *Analysis of the First Turnover: Measurement of the Preference for Using the Full-Length Subunit in Half of the Population and the Truncated Subunit in the Remainder.* The YTS/ΔYTS heterodimer forms tyrosyl adenylate with an equimolar distribution between the two subunits. This distribution may arise simply by chance because the enzyme has two identical active sites. However, the theory described above can be applied to measure the strength of the tendency for equimolar distribution between the two subunits when the active sites are not identical. The ratio of values of k_3 for wild-type and Asn-45 active sites is 1.0×10^4 (Table II). Random selection of functional active site would cause heterodimers containing Asn-45 to form tyrosyl adenylate on almost all of the wild-type active sites before the mutant active sites have time to form the intermediate. However, at least 44% of the tyrosyl adenylate is formed at Asn-45 active sites in heterodimers (Table IV). Taking $N_t/N_0 = 0.88$ and $n = 10^4$, this gives $P = 1.3 \times 10^{-5}$. Note that these calculations do not allow for the small differences in affinity for substrates between the two active sites of the heterodimers (see Table II). Increased affinity for substrate could favor utilization of the mutant active site. This value of P indicates a very strong tendency to form tyrosyl adenylate with an equimolar distribution between the two subunits, an observation which strongly suggests that the asymmetrical structure of the heterodimers does not perturb a tendency for each of the enzymes to divide into two populations of equal size.

(*b*) *Direct Measurement of Maintenance of Asymmetry after the First Turnover.* The procedure was as follows. (1) YTS/ΔYTS was allowed to form tyrosyl adenylate using unlabeled tyrosine. Free substrates were removed by gel filtration (see Experimental Procedures), and then the pH was adjusted to 7.8. (2) Substrates were added (about 9-fold excess of radiolabeled tyrosine and at least 2000-fold excess of ATP). (3) An approximate 3-fold excess (over enzyme) of tRNA was added to remove unlabeled tyrosine from the truncated subunit, allowing the enzyme to cycle until all of the tRNA was used. (4) Transfer RNA was added to 27-fold excess so that the enzyme cycles until all of the tyrosine is utilized and finally discharged from the truncated subunit. (5) The amount of radiolabel bound to the enzyme was measured. This must all be at the full-length subunit and must have been bound there following the first cycle. Dissociation and hydrolysis of unlabeled tyrosine from the full-length subunit is minimal (see above); large amounts of radioactive tyrosine could only gain access by interchange of enzyme from cycling at the truncated subunit to using the full-length subunit.

The value of N_0 can be measured at step 3, and n is taken as the concentration of tRNA at step 4 divided by N_0. This gives the minimum value of n since it does not allow for accumulation of the inactive complex of enzyme with tyrosyl adenylate bound at the full-length subunit. The estimate of P obtained is, therefore, the maximum value. The value of N_t can be calculated from the data given by step 5. N_t is given by N_0 minus the amount of enzyme with radiolabeled tyrosine bound at the full-length subunit.

There is no detectable accumulation of tyrosyl adenylate on the full-length subunit after only a few turnovers, but a range of number of turnovers between 50 and 200 gives $P \leq 3.3\text{–}31 \times 10^{-4}$. The sequence of steps 2 and 3 was reversed, allowing incubation of enzyme in the absence of substrates for 5 min at 25 °C between these two steps. This treatment did not produce any significant change in the maintenance of asymmetry ($P \leq 28 \times 10^{-4}$), indicating that the unligated enzyme has long-lasting asymmetry in solution.

Direct measurement of the preference for using only one active site is difficult because tyrosyl adenylate is spontaneously lost from both subunits of the dimer during the course of the experiment. Any intermediate leaving the full-length subunit will lead to re-formation of radiolabeled tyrosyl adenylate preferentially at that subunit. Measurement of aminoacylation using enzyme-bound tyrosyl adenylate (Table IV) indicates that up to 2.5% of the intermediate is lost from the full-length subunit of YTS/ΔYTS during the experiment (Table IV). This would account for a value of $P = 250 \times 10^{-4}$ if it occurs on each turnover. This value is almost an order of magnitude greater than any of those measured above. Interchange from cycling at the truncated subunit ot using the full-length subunit cannot thus be detected because the rate is much lower than that for spontaneous loss of tyrosyl adenylate from the full-length subunit.

(*c*) *Measurement of Maintenance of Asymmetry by Analysis of tRNA Charging Kinetics.* Even when cycling 1.8 times/s, YTS/ΔYTS does not show any deviation from production of charged tRNA as linear function of time, indicating that there is no detectable increase in the proportion of inactive enzyme having tyrosyl adenylate bound at the full-length subunit. A decrease of 10% in the cycling population would be readily detectable but is not seen after approximately 320 turnovers. Taking $N_t/N_0 \geq 0.90$, $P \leq 3.3 \times 10^{-4}$. This indirectly determined value is in agreement with the directly determined value of $P \leq 31 \times 10^{-4}$.

Discussion

Mutant Tyrosyl-tRNA Synthetases Are Asymmetrical Dimers in Solution. Each heterodimer forms tyrosyl adenylate at only one of its two subunits. There is no preference for truncated or full-length subunit and so the intermediate is equally distributed between the two possible sites. The enzyme thus consists of two equal populations, one activating tyrosine at the full-length subunit and the other at the truncated subunit. There is no detectable interconversion between active and inactive subunits over several minutes either in the absence of substrates or when the enzyme is turning over in the steady state.

The most compelling and direct evidence for asymmetry comes from two sets of studies. First, random choice of active site in heterodimers containing Asn-45 in one subunit would lead to activation of tyrosine on almost all of the wild-type sites before mutant sites have time to react. However, both YTS/ΔYTS(Asn-45) and YTS(Asn-45)/ΔYTS display biphasic time courses in formation of tyrosyl adenylate (Figure 3): 0.5 mol of tyrosyl adenylate/mol of dimer is formed rapidly at the wild-type active site, and then a further 0.5 mol of intermediate/mol of dimer accumulates slowly at the mutant subunit. Second, addition of tRNA to preformed complexes of each heterodimer with bound tyrosyl adenylate leads to transfer, at wild-type efficiency, of that half of the tyrosyl adenylate bound at the truncated subunit. In the other half of the population, most of the tyrosyl adenylate bound to the full-length subunit remains bound (Table IV). This confirms equal distribution of tyrosyl adenylate between the two active sites.

Functional asymmetry is maintained for subsequent cycles of tRNA charging, so that half of each heterodimer cycles at the truncated subunit, leading to aminoacylation of tRNA. The remainder has tyrosyl adenylate bound as a stable complex at the full-length subunit. This dead-end complex cannot charge tRNA. YTS/ΔYTS and YTS(Asn-45)/ΔYTS thus both charge tRNA at the same rate, which is half that of the wild-type enzyme (Table III). Heterodimers have a very strong tendency to behave as two populations of equal size despite asymmetry in structure and the presence of a debilitated active site in Asn-45 heterodimers. The rate of interchange between the two populations is very low. The maximum frequency observed is once every 320 turnovers, and it may be less than once every 3000 turnovers. Unligated tyrosyl-tRNA synthetase has long-lasting asymmetry as there is no detectable interchange between sites even after incubation for 5 min in the absence of substrates.

Wild-Type Enzyme Is Probably Asymmetric in Solution. Wild-type enzyme comprises two subunits of identical composition, making direct demonstration of long-lasting asymmetry very difficult. Kinetic evidence, however, implies that the observation of asymmetric behavior in heterodimers may be extended to native enzyme. First, wild-type enzyme exhibits half-of-the-sites activity in binding substrate and activating tyrosine and so enzyme–substrate complexes are asymmetric. Second, truncation of one subunit does not impose any asymmetry on the choice of active site in heterodimers: the functional site in heterodimers is equally distributed between the two subunits and is independent of subunit composition. Since, in addition, denaturation and renaturation have no significant effect on the kinetics of any of the enzymes used in this study, the conclusions from studying heterodimers would appear to be valid for the parent homodimers, including wild-type enzyme.

Mechanisms of Half-of-the-Sites Activity. Many enzymes appear to show half-of-the-sites activity [for examples, see Fersht (1985) and Schimmel and Soll (1979)]. The mechanism of choice functional active site is known for only a few of these enzymes. The functional active site may be selected randomly, as in tryptophanyl-tRNA synthetase from beef pancreas (Trezeguet et al., 1986). Other enzymes are suggested to alternate between the two active sites. In this mechanism, reaction occurs at one subunit of the dimer, but the product is not released until substrate binds to the second active site (Lazdunski et al., 1971). This "flip-flop" mechanism may be used by succinyl CoA synthetase from *E. coli* (Wolodko et al., 1983). A further possibility is repeated utilization of the same active site. Here the enzyme remembers which active site was used for the first turnover and then uses this site for all subsequent turnovers. This mnemonic mechanism has been proposed to occur as follows (Ricard et al., 1974). Binding of substrate or catalysis stabilizes a rare, or new, conformation. This conformation has different properties to the ground state and is slow to relax to the native structure. The enzyme remembers previous events, and this leads to a change in its properties. Several enzymes display mnemonical behavior: proline racemase from *Clostridium stricklandii* (Rudnick & Abeles, 1975); glucokinase from rat liver (Storer & Cornish-Bowden, 1976, 1977); and inorganic pyrophosphatase from yeast (Svyato et al., 1984).

Neither random selection nor flip-flop accounts for the tRNA charging activity of YTS/ΔYTS heterodimers. Our kinetic analysis has demonstrated that tyrosyl-tRNA synthetase preferentially uses the same active site for each cycle in the steady state.

Tyrosyl-tRNA Synthetase Appears To Be Inherently Asymmetric and Not Mnemonic. Asymmetry in mnemonical enzymes is induced when the enzyme is altered by substrate binding or catalysis (Ricard et al., 1974).

(*a*) *Catalysis Is Not Required To Induce Asymmetry.* If this were the case, then Asn-45 heterodimers would not have biphasic time courses for formation of tyrosyl adenylate. Catalysis is so slow at the mutant active site that the intermediate would almost always be formed at the wild-type active site.

(*b*) *Binding of Substrate Is Not Required To Induce Asymmetry.* In a mnemonical mechanism, the binding of the first molecule of substrate to the enzyme is different from subsequent binding events because energy is required to induce the remembered conformation. However, this is not seen for wild-type tyrosyl-tRNA synthetase. The dissociation constant for the first molecule of tyrosine has been measured by determining the association and dissociation rate constants using stopped-flow fluorescence, giving $K_t = 10 \pm 1$ μM (Fersht et al., 1975a). Equilibrium dialysis measures average binding over several hours and gives a value of $K_t = 11.6 \pm 1$ μM (Fersht et al., 1975a). There is no significant change in affinity for tyrosine between the first and subsequent binding events. These data thus imply that the form of tyrosyl-tRNA

synthetase isolated in vitro is inherently asymmetrical.

(*c*) *Comparison of the Structure in Solution with That in Crystals.* Crystalline unligated wild-type tyrosyl-tRNA synthetase and enzyme–tyrosine complex are each symmetrical dimers (Monteilhet & Blow, 1978; Brick & Blow, 1985). The enzyme–substrate complex contains electron density for 2 mol of tyrosine/mol of dimer. The crystal structure of the ΔYTS/ΔYTS dimer is not fully symmetrical, but each dimer again has electron density for 2 molecules of tyrosine (Blow & Brick, 1987). Crystallization possibly selects a symmetrical conformation of tyrosyl-tRNA synthetase (Monteilhet & Blow, 1978). The structure of wild-type enzyme in solution appears to be different from that in crystals in terms of symmetry.

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