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Construction of heterodimer tyrosyl-tRNA synthetase shows $tRNA^{Tyr}$ interacts with both subunits

(site-directed mutagenesis/urea-induced protein unfolding)

PAUL CARTER*, HUGUES BEDOUELLE†, AND GREG WINTER

Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge, CB2 2QH, England

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ABSTRACT **The tyrosyl-tRNA synthetase (EC 6.1.1.1) from *Bacillus stearothermophilus* is a dimer of two identical subunits. The dimer shows "half-of-the-sites" reactivity in that only one molecule of tyrosyladenylate is formed and one molecule of $tRNA^{Tyr}$ binds per dimer. To identify whether the $tRNA^{Tyr}$ binds to a single subunit in the dimer, or to both subunits, heterodimers were constructed by mixing two variant dimers together in 8 M urea. As the unfolded protein is electrophoresed into a native polyacrylamide gel, it refolds and reassociates, and heterodimers can be purified from the parental dimers. Kinetic analysis of heterodimers formed between variant enzymes with defective tyrosine activation or tRNA aminoacylation shows that a molecule of $tRNA^{Tyr}$ interacts with the N-terminal region of one subunit and the C-terminal region of the other subunit in the dimer.**

The tyrosyl-tRNA synthetase ($tRNA^{Tyr}$ synthetase, EC 6.1.1.1) from *Bacillus stearothermophilus* catalyzes the aminoacylation of $tRNA^{Tyr}$ with tyrosine in a two-step reaction (1) in which the tyrosine is first activated to give enzyme-bound tyrosyladenylate (reaction **1**) and the tyrosyl moiety is then transferred to $tRNA^{Tyr}$ (reaction **2**).

$$E + Tyr + ATP \rightleftharpoons E{\cdot}Tyr\text{-}AMP + PPi, \quad [1]$$

$$E{\cdot}Tyr\text{-}AMP + tRNA^{Tyr} \rightleftharpoons Tyr\text{-}tRNA^{Tyr} + AMP + E. \quad [2]$$

A number of features of the $tRNA^{Tyr}$ synthetase system have led to extensive analysis of this enzyme by site-directed mutagenesis (2–6). The gene coding for the $tRNA^{Tyr}$ synthetase has been cloned (7) and sequenced (8). The x-ray crystallographic structures have been determined for both the native $tRNA^{Tyr}$ synthetase dimer (9, 10) and for the enzyme-bound tyrosyladenylate complex (11). The crystal structures show an ordered N-terminal domain (residues 1–317) and a disordered C-terminal region (residues 318–419). The $tRNA^{Tyr}$ synthetase is expressed in high yield directly from the M13 clone (2) and is readily purified from *Escherichia coli* proteins after a heat denaturation step. The amount of active enzyme may be assayed by an active site titration reaction (12).

Kinetic studies have shown that in solution the two active sites in the $tRNA^{Tyr}$ synthetase dimer interact in an anticooperative way, in that only one molecule of tyrosine (13, 14) and one molecule of $tRNA^{Tyr}$ (15, 16) are bound tightly per dimer and one molecule of tyrosyladenylate is formed per dimer (12, 14). The terminal adenosine residue in $tRNA^{Tyr}$ that is aminoacylated with tyrosine during the activation reaction must interact with the N-terminal domain of the enzyme, as the tyrosyladenylate binding site is located in this domain (11). However, the $tRNA^{Tyr}$ must also interact with the disordered C-terminal domain since a truncated variant WTt, in which this region was deleted, could not aminoacylate tRNA although it could form tyrosyladenylate (17). In principle, one molecule of $tRNA^{Tyr}$ could interact with either the N-terminal domain of one subunit and the C-terminal domain of the other subunit in the dimer (Fig. 1, model 1), or it could interact with both the domains of one subunit (Fig. 1, model 2).

To distinguish between these two models heterodimers were constructed between two different $tRNA^{Tyr}$ synthetase variants with lesions in either tyrosine activation (reaction **1**) or in tRNA aminoacylation (reaction **2**). For example, complementation between the two subunits could indicate that the tRNA interacts with both. The point mutation His-45→Asn (18) and the truncated enzyme (17) represent lesions in the activation and charging reactions, respectively. Mutation of the catalytic residue His-45→Asn reduces the k_{cat} for the activation reaction by 2- to 500-fold but with almost unchanged K_M for ATP (18). Kinetic parameters for the transfer of tyrosine from tyrosyladenylate to tRNA by the Asn-45 enzyme are almost identical to those of the wild-type (WT) enzyme (A. R. Fersht, personal communication). The truncated enzyme activates tyrosine with similar kinetic parameters to the full-length enzyme but does not aminoacylate $tRNA^{Tyr}$ (17).

In this paper we report a method for the construction of $tRNA^{Tyr}$ synthetase heterodimers by mixing two different $tRNA^{Tyr}$ synthetase variants in the presence of 8 M urea and then reassociating and refolding the different subunits during electrophoresis on a polyacrylamide gel. Alternative models for the interaction of $tRNA^{Tyr}$ with $tRNA^{Tyr}$ synthetase (Fig. 1) were then tested by the construction of appropriate $tRNA^{Tyr}$ synthetase heterodimers.

METHODS

Oligonucleotide Site-Directed Mutagenesis. The His-45→Asn mutation in the $tRNA^{Tyr}$ synthetase gene cloned in the phage M13mp93 (2) was constructed by using the synthetic oligonucleotide HN45 [5′d(GCCGATATTCAAACT-G)] as described (5). The His-45→Asn mutation in the truncated version of the $tRNA^{Tyr}$ synthetase gene (17) was constructed by annealing the HN45 primer to the M13 template, extending the primer for 4 hr with DNA polymerase I (Klenow fragment) in the presence of deoxynucleoside triphosphates, and directly transfecting the heteroduplex DNA into an *Escherichia coli* host (19). The host strain BMH 71-18 mutL (20), which is deficient in mismatch repair, was used to obtain the Asn-45 mutant at high frequency (19, 21,

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Abbreviations: WT, wild type; t, truncated.

*Present address: Genentech Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080.
†Present address: UPMTG, Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France.

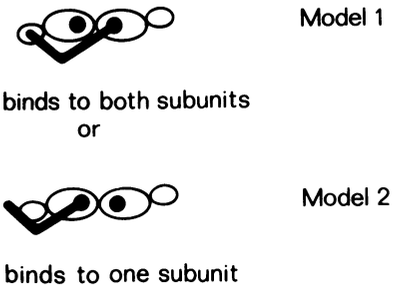

FIG. 1. Alternative models for interaction of $tRNA^{Tyr}$ with $tRNA^{Tyr}$ synthetase dimer. One molecule of $tRNA^{Tyr}$ (check mark) may interact with the active site (filled circle) in the N-terminal region (large ellipse) of one subunit in the $tRNA^{Tyr}$ synthetase dimer and the C-terminal region (small ellipse) of the other subunit (model 1) or alternatively with the N-terminal and C-terminal domains of one subunit in the dimer (model 2).

22). The complete nucleotide sequence of $tRNA^{Tyr}$ synthetase mutant genes was verified by dideoxy sequencing (23, 24) by using a family of five sequencing primers located at intervals throughout the $tRNA^{Tyr}$ synthetase gene (3).

Purification of $tRNA^{Tyr}$ Synthetase. An overnight culture of TG1 bacteria was diluted 1:100 with 500 ml of 2 × TY medium (25) and incubated at 37°C with shaking (300 rpm) until A_{600} = 0.5. The culture was then infected with 500 μl phage stock (about 10^{12} phage) and incubated at 37°C for 6 hr with shaking (300 rpm). The cells were harvested by centrifugation in a Sorvall GS-3 rotor at 7000 rpm, 4°C for 10 min. The cell pellet was resuspended in 25 ml of 50 mM Tris·HCl (pH 7.4), 1 mM EDTA, 10 mM 2-mercaptoethanol, and 0.1 mM phenylmethylsulfonyl fluoride (S buffer) and sonicated for 2 min on ice. The sonicated samples were heated at 58°C for 30 min, and solid debris was pelleted by centrifugation in a Sorvall SS-34 rotor at 18,000 rpm at 4°C for 30 min. The supernatant was dialyzed at 4°C overnight against 5 liters of S buffer and then loaded onto a 2 ml DE-52 (Whatman) column preequilibrated with the S buffer. The column was washed first with 10 ml of S buffer and then with 10 ml of S buffer containing 50 mM NaCl. The $tRNA^{Tyr}$ synthetase was eluted with 10 ml of S buffer containing 150 mM NaCl, and $tRNA^{Tyr}$ synthetase containing fractions [as shown by $NaDodSO_4$/PAGE (26)] were pooled, dialyzed at 4°C overnight against 5 liters of S buffer, and then frozen in aliquots in liquid nitrogen. The yield of $tRNA^{Tyr}$ synthetase was determined by active site titration (3). For the construction of heterodimers this material was used directly. For kinetic analysis of parent dimers the $tRNA^{Tyr}$ synthetase was further purified by fast protein liquid chromatography on a monoQ column (Pharmacia P-L Biochemicals) as described (17).

Urea Gradient Polyacrylamide Gel Electrophoresis. Urea gradient gels were cast and electrophoresed as described by Creighton (27) with the following modifications. The continuous buffer system used 50 mM Tris/50 mM *N,N*-bis[2-hydroxyethyl]glycine (Bicine, pH 8.4). The gels (10 cm × 10 cm × 0.05 cm) were cast with a linear gradient of 0–8 M urea and a compensating gradient of 10–7.5% acrylamide across the width of the gel. The gels were electrophoresed for 30–60 min at 10 mA in a fan-cooled apparatus (Raven Scientific) by using the microslab gel system of Matsudaira and Burgess (28).

Preparative Construction and Purification of Heterodimers. Two DE-52-purified $tRNA^{Tyr}$ synthetases (10 nmol each) (0.95 mg full-length, 0.73 mg truncated) were mixed in the presence of excess ultra-pure urea (Bethesda Research Laboratories) and electrophoresed on a 10% native polyacrylamide gel (buffer system as in ref. 26 but omitting the $NaDodSO_4$) (20 cm × 20 cm × 0.45 cm) at 30 mA for 12 hr. The protein was located within the gel by diffusion blotting onto nitrocellulose (Schleicher & Schüll) and staining the nitrocellulose with India ink (Pelikan AG) (29). $tRNA^{Tyr}$ synthetase heterodimers were electroeluted from gel slices placed in dialysis bags into 3 ml of electrophoresis running buffer and dialyzed at 4°C overnight against 5 liters of S buffer. The heterodimers were further purified on 200-μl DE-52 columns (as above), dialyzed at 4°C overnight against 5 liters 144 mM Tris·HCl (pH 7.78), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, and then frozen in aliquots in liquid nitrogen.

Kinetic Assays. The pyrophosphate exchange, active site titration (at 37°C instead of 25°C), and aminoacylation assays were as described (3). For the Asn-45 enzyme the time course for the active site titration was followed over 1 hr to ensure that the enzyme was saturated with tyrosyladenylate.

Amino Acid Analysis of Heterodimers. Aliquots of about 50 pmol of WT and heterodimer $tRNA^{Tyr}$ synthetase (as determined by active site titration) were hydrolyzed in 6 M HCl in the presence of 2 nmol of norleucine as an internal standard. Samples after 24, 48, and 72 hr of hydrolysis were subjected to amino acid analysis on a Durrum D500 amino acid analyzer (30). The amount of homodimer and heterodimer $tRNA^{Tyr}$ synthetase was then calculated from the amount of alanine determined from the amino acid analysis using the known amino acid composition of these enzymes (8).

RESULTS

Urea-Induced Unfolding of $tRNA^{Tyr}$ Synthetase. The urea-induced unfolding of the $tRNA^{Tyr}$ synthetase dimer was investigated by electrophoresis through a urea gradient gel. An unfolding transition at around 6 M urea was observed that presumably represents a transition from the native dimer to the unfolded momomer. Similar transitions were obtained for full-length $tRNA^{Tyr}$ synthetase dimers (WT and Asn-45 enzyme) and truncated $tRNA^{Tyr}$ synthetase dimers (WTt and Asn-45t enzymes). The unfolding profile for the Asn-45 variant is shown in Fig. 2*A*.

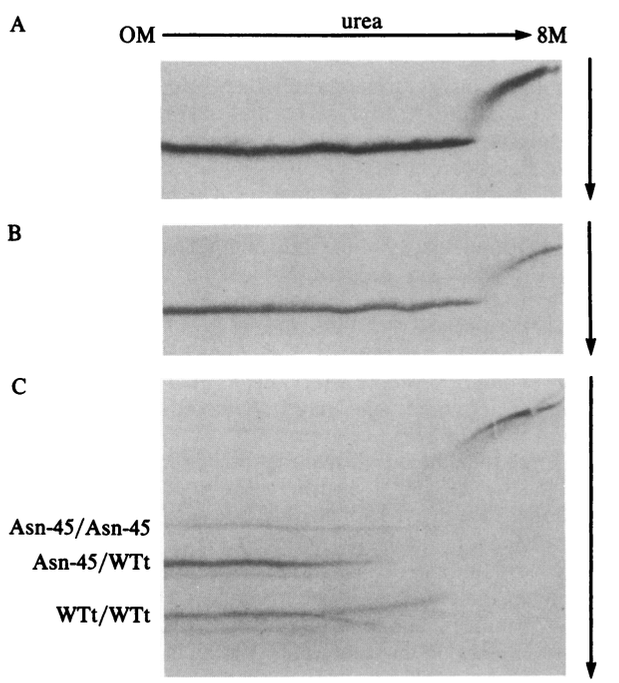

FIG. 2. Urea gradient PAGE of $tRNA^{Tyr}$ synthetase. Asn-45 (150 pmol) variant of $tRNA^{Tyr}$ synthetase was loaded on a urea gradient gel in the absence (*A*) or presence of 8 M urea (*B*) or in the presence of 150 pmol of WTt $tRNA^{Tyr}$ synthetase and 8 M urea (*C*).

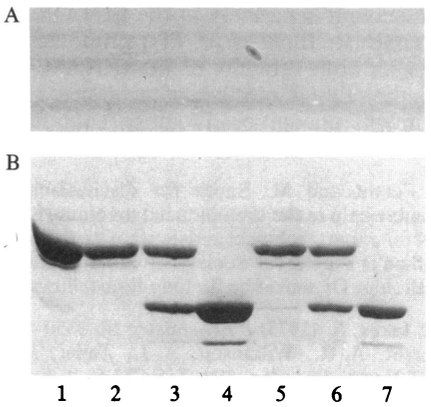

FIG. 3. Preparative construction of heterodimers. Two different $tRNA^{Tyr}$ synthetase variants (10 nmol each) were mixed in 8 M urea and electrophoresed on a native polyacrylamide gel. (*A*) The protein was located within the gel by diffusion blotting onto nitrocellulose and then staining the nitrocellulose with India ink. (*B*) Purified heterodimers were analyzed by $NaDodSO_4$/PAGE. Lanes: 1, WT parent dimer; 2, gel-eluted WT dimer; 3, WT/Asn-45t heterodimer; 4, Asn-45t parent dimer; 5, Asn-45 parent dimer; 6, Asn-45/WTt heterodimer; and 7, WTt parent dimer.

Refolding of $tRNA^{Tyr}$ Synthetase After Urea-Induced Unfolding. To investigate whether the unfolding and dissociation of $tRNA^{Tyr}$ synthetase dimers were reversible, the enzymes were first denatured in the presence of 8 M urea and then electrophoresed on a urea gradient gel. Refolding profiles similar to the unfolding profiles were obtained for full-length $tRNA^{Tyr}$ synthetase dimers (WT and Asn-45 enzymes) and truncated $tRNA^{Tyr}$ synthetase dimers (WTt and Asn-45t enzymes). The refolding profile for the Asn-45 variant is shown in Fig. 2*B*.

Construction of Heterodimer $tRNA^{Tyr}$ Synthetase. From the preliminary unfolding and refolding experiments described above, it appears that $tRNA^{Tyr}$ synthetase dimer may be unfolded and dissociated in the presence of urea and then refolded and reassociated into a native dimer during PAGE. In an attempt to construct a heterodimer, a full-length $tRNA^{Tyr}$ synthetase (Asn-45) was mixed with an equimolar amount of a truncated enzyme (WTt) in 8 M urea and then electrophoresed into a urea gradient gel as shown in Fig. 2*C*. Three main bands are observed at low urea concentrations, and around 6 M urea there is an unfolding transition. The three main bands at low urea concentrations presumably correspond to the refolded parent dimers and the intermediate band to the heterodimer (Asn-45/WTt).

Preparative Construction of Heterodimers. The experiment in Fig. 2*C* shows that heterodimer $tRNA^{Tyr}$ synthetase enzymes may be constructed and purified by PAGE after urea-induced unfolding of the parent homodimers. Heterodimer $tRNA^{Tyr}$ synthetase, Asn-45/WTt, (Fig. 3*A*) and WT/Asn-45t, were constructed to test alternative models (Fig. 1) for $tRNA^{Tyr}$ synthetase interaction with $tRNA^{Tyr}$.

To verify that the gel eluted protein corresponded to $tRNA^{Tyr}$ synthetase heterodimers, samples were analyzed by $NaDodSO_4$/PAGE (Fig. 3*B*). The putative heterodimers Asn-45/WTt and WT/Asn-45t gave rise to two bands of approximately equal intensity with mobilities corresponding to the full-length and truncated enzymes as expected.

Kinetic Analysis of $tRNA^{Tyr}$ Synthetase Heterodimers. The activity of the heterodimers in the activation reaction and in the aminoacylation reaction was then determined using the steady state assays (Table 1). The heterodimer Asn-45/WTt was found to be active for both the activation and aminoacylation reactions whereas the heterodimer WT/Asn-45t was found to activate tyrosine but not to aminoacylate $tRNA^{Tyr}$. The number of active sites per $tRNA^{Tyr}$ synthetase dimer was calculated from the active site titration and amino acid analysis data for the WT enzyme (1.2) and the heterodimers Asn-45/WTt (0.87) and WT/Asn-45t (1.1).

DISCUSSION

It is convenient to follow the urea-induced unfolding of a protein by electrophoresis through a polyacrylamide slab gel in which there is a gradient of urea concentration perpendicular to the direction of electrophoresis (27, 31). Furthermore, a number of proteins can be refolded during electrophoresis after urea-induced unfolding (32).

Preliminary experiments on the $tRNA^{Tyr}$ synthetase demonstrated that the $tRNA^{Tyr}$ synthetase dimer is dissociated and unfolded in the presence of 8 M urea but may be refolded and reassociated into a native-like structure during PAGE. The kinetic data obtained for gel-purified WT $tRNA^{Tyr}$ synthetase was identical to that obtained for WT enzyme purified by ion exchange fast protein liquid chromatography (unpublished data), which strongly suggests that the enzyme had refolded into the native structure. These observations led to the construction of heterodimer $tRNA^{Tyr}$ synthetase by urea-induced unfolding of different parent homodimers followed by PAGE. Entry of urea-unfolded $tRNA^{Tyr}$ synthetase into a polyacrylamide gel is associated with the very rapid removal of urea, concentration at the buffer/gel interface, and contact with the gel matrix. It has been argued that refolding of proteins after urea-induced unfolding is not affected by the electrophoretic process or by the gel matrix (32). However urea-unfolded $tRNA^{Tyr}$ synthetase (at 5 μM) can be readily refolded from urea by serial dilutions in the presence of bovine serum albumin (0.5 mg/ml), but not in its absence (unpublished data). We, therefore, suspect that the polyacrylamide gel matrix may help refolding, and since reassociation of $tRNA^{Tyr}$ synthetase monomers is required to form dimers, concentrating the sample at the gel interface may also be important.

Heterodimers have been constructed by preparative gel electrophoresis by using appropriate variant parent dimers to

Table 1. Kinetic activity of heterodimer $tRNA^{Tyr}$ synthetase enzymes

	Pyrophosphate exchange activity				Aminoacylation activity		
Enzyme	K_m(ATP), mM	k_{cat}, sec^{-1}	k_{cat}/k_{cat}(WT)	K_m(Tyr), μM	K_m(ATP), mM	k_{cat}, sec^{-1}	k_{cat}/k_{cat}(WT)
WT/WT	1.4	6.3	(1.0)	3.1	0.56	2.3	(1.00)
Asn-45/WTt	1.2	3.4	0.54	2.6	0.52	1.3	0.56
WT/Asn-45t	1.2	3.3	0.52	3.8	—	—	—

All kinetic assays were performed at 25 ± 0.2°C in a standard buffer containing 144 mM Tris·HCl (pH 7.78), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, and 0.1 mM phenylmethylsulfonyl fluoride as described (3). For the ATP dependence of pyrophosphate exchange and for aminoacylating 50 μM tyrosine and 100 μM tyrosine were used, respectively. For the tyrosine dependence of pyrophosphate exchange 2 mM ATP was used. Aminoacylation of tRNA by the WT/Asn-45t heterodimer could not be detected by this assay.

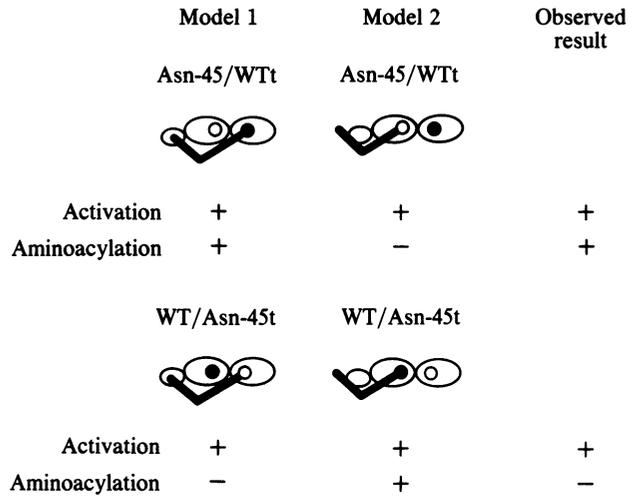

FIG. 4. Kinetic activity of heterodimer $tRNA^{Tyr}$ synthetase enzymes. Heterodimers Asn-45/WTt and WT/Asn-45t (symbols as in Fig. 1) were constructed. The kinetic activity of the heterodimers as predicted from models where the tRNA interacts with both subunits (Fig. 1, model 1) or one subunit (Fig. 1, model 2) of the dimer is compared with the observed activity (Table 1).

test models for interaction of $tRNA^{Tyr}$ with $tRNA^{Tyr}$ synthetase. From the two alternative models for tRNA interaction with $tRNA^{Tyr}$ synthetase shown in Fig. 1, we predict that both Asn-45/WTt and WT/Asn-45t $tRNA^{Tyr}$ synthetase heterodimers should catalyze the formation of tyrosyladenylate (reaction 1). If $tRNA^{Tyr}$ interacts with the N-terminal region of one subunit and the C-terminal region of the other subunit in the $tRNA^{Tyr}$ synthetase dimer (model 1), then we would expect the Asn-45/WTt heterodimer to aminoacylate tRNA. Thus we would create an enzyme active for aminoacylating from two inactive parent enzymes. We would also expect the WT/Asn-45t heterodimer to be inactive for aminoacylation. Conversely, if the tRNA interacts with just one subunit (model 2), then we would predict the Asn-45/WTt heterodimer to be inactive for aminoacylation and the WT/Asn-45t heterodimer to be active. The observed activities of the heterodimers (Fig. 4) show that both subunits in the dimer are involved in tRNA aminoacylation as in model 1. The possibility that tyrosyladenylate can migrate between the two active sites of the dimer is ruled out by the observation that the heterodimer WT/Asn-45t does not charge tRNA.

The heterodimers (Asn-45/WTt and WT/Asn-45t) have almost identical K_m values for ATP and tyrosine as the WT enzyme in the activation reaction (Table 1), and both the WT and heterodimer enzymes have one active site per dimer. However, the turnover number (k_{cat}) of the heterodimers Asn-45/WTt and WT/Asn-45t in the activation reaction and of the heterodimer Asn-45/WTt in the aminoacylation reaction is about half that of the WT enzyme (Table 1). This is probably a result of the anticooperativity between active sites in the dimer, in which substrate binding to one subunit "switches off" the other subunit in the dimer. Thus binding of tyrosine to a subunit with the His-45→Asn mutation switches off the other subunit in the heterodimer, while binding to a WT subunit (WT or WTt) yields tyrosyladenylate. Overall this results in half the turnover per active site.

Specific models have been suggested for the interaction of $tRNA^{Tyr}$ with the $tRNA^{Tyr}$ synthetase dimer by Reid (33) and by Blow *et al.* (34). Reid proposed that $tRNA^{Tyr}$ is in equilibrium between L shaped and U shaped conformations, and that one molecule of $tRNA^{Tyr}$ in the U conformation interacts with each subunit separately in the $tRNA^{Tyr}$ synthetase dimer. In contrast, Blow *et al.* (34) proposed that $tRNA^{Tyr}$ interacts with both subunits of the $tRNA^{Tyr}$ synthetase by spanning the subunit interface of the dimer. These heterodimer experiments unequivocally support the model proposed by Blow.

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