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A model of synthetase/transfer RNA interaction as deduced by protein engineering

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The recognition of transfer-RNA by their cognate aminoacyl-tRNA synthetases is the crucial step in the translation of the genetic code. In order to construct a structural model of the complex between the tyrosyl-tRNA synthetase (TyrTS) from *Bacillus stearothermophilus* and $tRNA^{Tyr}$, 40 basic residues at the surface of the TyrTS dimer have been mutated by site-directed mutagenesis and heterodimers created *in vitro* by recombining subunits derived from different mutants. As reported here a cluster of basic residues (Arg 207-Lys 208) in the N-terminal domain of one TyrTS subunit interacts with the acceptor stem of $tRNA^{Tyr}$ and two separated clusters of basic residues (Arg 368-Arg 371; Arg 407-Arg 408-Lys 410-Lys 411) in the C-terminal domain of the other subunit interact with the anticodon arm. The TyrTS would thus clamp the tRNA in a fixed orientation. The precise alignment of the flexible ... ACCA 3′ end of the tRNA for attack on the tyrosyl adenylate is made by contacts closer to the catalytic groups of the enzyme, such as with Lys 151.

TyrTS catalyses the aminoacylation of $tRNA^{Tyr}$ in a two-stage reaction. The tyrosine is first activated with ATP to form tyrosyl adenylate and pyrophosphate, then the adenylate is attacked by the 3′-terminal ribose of the tRNA to form Tyr-$tRNA^{Tyr}$ and AMP. TyrTS is a dimer which shows 'half-of-the-sites' reactivity, forming one tyrosyl adenylate and binding tightly one tyrosine and one $tRNA^{Tyr}$ per dimer in solution. The two subunits are related by symmetry through a 2-fold axis. Each subunit has an N-terminal domain (residues 1-319), which makes all the interactions with the tyrosyl adenylate and forms the subunit interface, and a C-terminal domain (residues 320-419), which is disordered in the crystal (reviewed in ref. 1). By creating a truncated TyrTS at the level of the gene, Waye *et al.*[2] have shown that the N-terminal domain of TyrTS catalyses the formation of tyrosyl adenylate with unchanged k_{cat} and K_M but does not charge and does not bind $tRNA^{Tyr}$; this shows that the C-terminal domain of TyrTS is essential for tRNA binding.

To identify residues of TyrTS that interact with $tRNA^{Tyr}$, we chose the following strategy: (1) We assumed that basic residues of the synthetase could form salt bridges with the phosphates of the tRNA backbone or hydrogen bonds with the nucleotide bases. (2) Because the *B. stearothermophilus* and *Escherichia coli* TyrTS have homologous sequences and similar properties[3,4], we considered mainly the conserved basic residues. (3) We changed the arginine and histidine residues to glutamine, and lysine to asparagine; such changes remove the charge but not the hydrophilic character of the residue. We therefore mutated 40 basic residues of the *B. stearothermophilus* TyrTS by oligonucleotide-directed mutagenesis of the encoding gene (*tyrS*) (see Fig. 1 legend).

To test the overall activity of the mutant synthetases, we devised an *in vivo* genetic complementation assay. In this assay, the *B. stearothermophilus tyrS* gene is carried by and expressed from a recombinant M13 phage[5]. The host is HB2111, an *E. coli* strain which harbours a thermosensitive mutation in its own *tyrS* gene, which is an essential gene. The HB2111 cells can grow at 42 °C, the non-permissive temperature, only if they are infected by a phage which directs the production of an active *B. stearothermophilus* TyrTS. Most of the 40 mutant phages could complement HB2111 and were therefore eliminated. However, 13 mutants were either unable to complement—KN82, RQ86, KN151, KN208, KN230, KN233, RQ368, RQ407, KN410 and KN411—or did so weakly—RQ207, RQ371 and RQ408 (Fig. 1).

The 13 TyrTS mutants identified from the complementation assay and the wild-type TyrTS were purified from phage-infected cells. All the mutant enzymes were able to form enzyme-bound tyrosyl adenylate, albeit slowly for mutants RQ86 and KN233 (half life, $t_{1/2} = 8$ and 27 min respectively, compared with 2 s for the wild-type enzyme)[6]. The pyrophosphate exchange assay showed that the mutant and wild-type enzymes had similar activities (4.6 s^{-1} at 2 mM ATP, 50 μM Tyr and 2 mM pyrophosphate) except for mutants KN82, RQ86, KN230 and KN233 ($<0.23\ s^{-1}$) (see Table 1). Thus, although these four mutants are able to form tyrosyl adenylate and are presumably correctly folded, there is nevertheless a lesion in the activation step. Additional experiments are needed to determine whether the wild-type residues at these four positions also interact with the tRNA; in the crystallographic structure, they lie on the rim of the tyrosyl adenylate binding site (Fig. 1).

Table 1 shows the kinetic results for the charging of tRNA by the nine mutants that exchange pyrophosphate at a normal rate. Eight of the nine mutant enzymes have altered K_M values for tRNA in the aminoacylation reaction, which indicates that most of the contacts between the synthetase and the tRNA are involved in the initial complex formation. The KN151 mutant has a K_M for tRNA which is identical to that of the wild-type enzyme and a k_{cat} which is reduced by 150-fold; this indicates that the wild-type residue, Lys 151, is not involved in the initial binding between the synthetase and the tRNA and that TyrTS and $tRNA^{Tyr}$ may make an additional contact, involving Lys 151,

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Table 1 Kinetic parameters for $tRNA^{Tyr}$ charging

Mutant	k_{cat} ($s^{-1} \times 10^3$)	K_M (μM)	k_{cat}/K_M ($s^{-1}\ M^{-1} \times 10^{-3}$)	ΔG_{app} (kcal mol^{-1})	Complementation
Wild type	450	1.4	315	0	+
KN151	3	1.2	2.3	2.9	−
RQ207	ND	>100	19.4	1.7	+/−
KN208	ND	>28	11.3	2.0	−
RQ368	ND	>100	2.4	2.9	−
RQ371	ND	>100	17.1	1.7	+/−
RQ407	ND	>100	11.9	1.9	−
RQ408	ND	>100	16.5	1.8	+/−
KN410	ND	>100	9.3	2.1	−
KN411	ND	>100	12.8	1.9	−

$\Delta G_{app} = -RT\ln(k_{cat}/K_M)_{mut}/(k_{cat}/K_M)_{wt}$ (mut, mutant; wt, wild type)[11]. For genetic complementation assay, see Fig. 1 legend. ND, not determined. Purification of the wild-type and mutant enzymes from phage-infected cells[7], active-site titration, pyrophosphate exchange and tRNA charging assays[11] were done as described elsewhere. We obtained different values for the wild-type TyrTS with pure *E. coli* $tRNA^{Tyr}$ (Boehringer: 1,000 pmol of tyrosine incorporation per A_{260} unit) and crude *E. coli* tRNAs ($K_M = 1.43$ versus 2.57 μM and $k_{cat} = 0.45$ versus 1.17 s^{-1}). The rate of charging by the wild-type enzyme gave a straight line in the Eadie-Hofstee plot (see ref. 12) only for pure $tRNA^{Tyr}$ concentrations less than 6 μM and was inhibited for higher values. We therefore used pure *E. coli* $tRNA^{Tyr}$ for the mutant enzymes, in the range 0.375-6 μM.

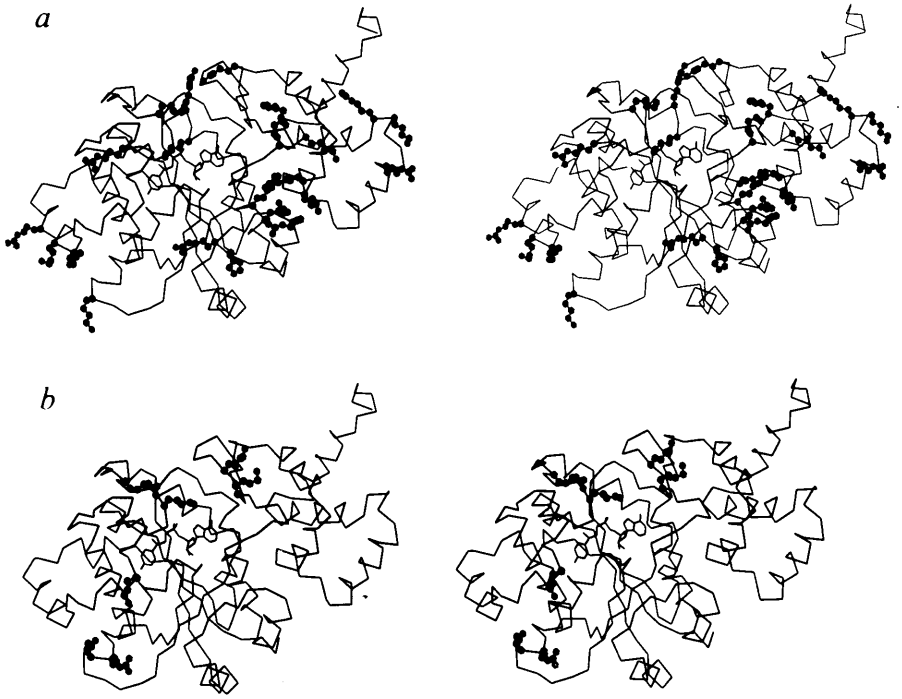

Fig. 1 Stereo view of the TyrTS backbone with mutated side chains. Forty basic residues—lysine (K), arginine (R) or histidine (H)—were mutated to asparagine (N) or glutamine (Q). Twenty-seven mutants gave full growth (+) in the genetic complementation assay while 13 mutants gave residual (+/−) or no growth (−): RQ10+, RQ56+, RQ57+, HQ63+, RQ64+, KN82−, KN83+, RQ86−, RQ100+, KN102+, RQ137+, KN141+, HQ142+, KN151−, RQ157+, RQ207+/−, KN208−, KN210+, KA225+, KN230−, KN233−, KN245+, RQ265+, KN268+, RQ286+, KN291+, RQ292+, KN304+, HQ307+, KN367+, RQ368−, RQ371+/−, RQ385+, RQ398+, RQ402+, RQ407−, RQ408+/−, KN410−, KN411−, RQ417+, wild type+. The N-terminal domain (residues 1-319) is represented with bound tyrosyl adenylate. *a*, 22 residues that, when mutated, score as +; *b*, 7 residues that score as +/− or −. From bottom to top of the figure: R207, K208, K151, K82 and R86 on the left, K230 and 233 on the right.

Methods. Primer design: In general, we used codons AAC for Asn and CAG for Gln. CAA was used in the mutants RQ64, RQ292 and RQ402 to improve discrimination during the screening of the mutants. In general, the oligonucleotides contained seven pairing residues on each side of the mismatched base(s) and were 15- or 16-mers. The oligonucleotides for mutants RQ137 and KN268 contained an additional pairing residue on the 3′ or 5′ side of the mismatch, respectively, to provide a G·C pair at this end of the primer. The oligonucleotide for mutant KA225 had the sequence 5′ TC CGC TGC CGT CAC AAG 3′; KA225 was constructed by P. Carter (unpublished). *Oligonucleotide synthesis*: The oligonucleotides were synthesized by Biosearch SAM1 DNA synthesizer using phosphotriester chemistry on a controlled-pore glass support, and were purified by either HPLC on an ion-exchange resin (Partisil 10SAX) or gel electrophoresis using ethidium bromide staining[14]. *Mutagenesis technique*: The mutagenesis was done on derivatives of the original M13mp93tyrS vector[5] that carry an 'up' mutation in the *tyrS* promoter (M. M. Y. Waye and G.W., in preparation) and, in some cases, an amber mutation in M13 gene IV[15]. The technique used was "priming all the way round/sucrose gradient" for 9 mutants and "double priming with amber selection" for 31 mutants[14]. The mutant phages were screened by hybridization with with the mutagenic oligonucleotides[14] followed by dideoxy sequencing using a family of synthetic primers[16]. Mutagenic oligonucleotides were also used as sequencing primers. *Complementation assay*: We constructed HB2111 (*tyrS*(Ts) *recA srl*::tn*10 argG thi lac*/F′*lac*$^+$) from the *E. coli* K-12 strain 565cN (ref. 17) and used it as host. About 10^7 cells of an exponential culture of HB2111, at 30 °C in complete medium, were spread on a pre-warmed minimal glucose plate containing tetracycline (15 μg ml^{-1}) and arginine. Drops (10 μl) of the phage suspensions (~5×10^{11} plaque-forming units (PFU) ml^{-1}) were spotted onto the lawn and the plate was incubated at 42.5-43 °C. Colonies were scored after 48 h. The mutant HN45, which does not activate tyrosine[1], and the truncated TyrTS[2] were both negative in the assay.

in the transition state.

Previously, we have shown that it is possible to form and purify heterodimers between truncated (lacking residues 319-417) and full-length TyrTS enzymes by mixing the two parental homodimers in equimolar amounts, denaturing the mixture with urea, then renaturing it by electrophoresis through a non-denaturing polyacrylamide gel. After renaturation, the two-refolded homodimers and the heterodimer are roughly in the proportion 1:1:2, as theoretically expected. Detailed kinetic studies of purified heterodimers carrying the Asn 45 mutation (which prevents tyrosine activation) in either the full-length or the truncated subunit, have shown that one tRNA molecule interacts with both TyrTS subunits[7]. These studies also show that the 3′-terminal adenosine of the tRNA attacks the tyrosyl adenylate in the truncated subunit of the heterodimer.

Here, we have formed heterodimers between the truncated TyrTS and each of the 13 mutants described above by stepwise dilution of the urea. As controls, we also mixed the two parental homodimers after the denaturation/renaturation process. We assayed the final mixtures for charging of tRNA with ^{14}C-tyrosine (Table 2). The seven mutants carrying a lesion in the N-terminal domain formed active heterodimers with the truncated TyrTS. Similarly, an active heterodimer could be made between the mutant enzymes KN151 and RQ368. Thus, a functional tRNA interaction site can be reconstituted by assembling two inactive monomers, one of which has a wild-type N-terminal domain but lacks the C-terminal domain (or has a lesion therein) and the other having a mutated N-terminal domain but a wild-

Fig. 2 Docking of tRNA and synthetase. The figure shows the backbone of the TyrTS dimer with tyrosyl adenylate bound to the right subunit and with the side chains of residues R207, K208, K151, K82, R86, K230 and K233 (for the identification of these residues, see Fig. 1 legend). Only the N-terminal domain (residues 1-319) of each subunit is represented. The structure of tRNA^{Phe} is indicated by the van der Waals' spheres of its phosphorous atoms. The flexible 3′-terminus of the tRNA (...ACCA 3′) was docked up to the tyrosyl adenylate binding site and the 3′-terminal adenosine could be fitted into the active site so as to attack the tyrosyl adenylate at the scissile bond; however, several rotations of the phosphodiester backbone were required to avoid bad contacts.

Table 2 Charging activity of TyrTS heterodimers

Mixture	Aminoacylation rate ($s^{-1} \times 10^3$) *a*	*b*
tr+KN82	270	59
tr+RQ86	277	4
tr+KN151	196	2
tr+RQ207	304	29
tr+KN208	328	25
tr+KN230	297	6
tr+KN233	250	9
tr+RQ368	3	2
tr+RQ371	32	22
tr+RQ407	16	11
tr+RQ408	31	23
tr+KN410	14	11
tr+KN411	20	13
KN151+RQ368	162	4

The truncated TyrTS (tr)[2] and each of the 13 mutants were mixed in equal molarities (according to active-site titration) before (*a*) or after (*b*) the denaturation/renaturation process. A similar experiment was performed with the KN151 and RQ368 mutants. The rates are calculated per input mole of one of the two parental homodimers; calculated in this way, they can reach 100% of the rate of the re-folded wild-type enzyme if there is random reassociation of the monomers. In case *a*, half of the monomers are theoretically engaged in heterodimer formation and therefore the contribution of the re-folded homodimers should be half the value in *b*. The rates for the re-folded truncated and wild-type enzymes were 0 and 421×10^{-3} s^{-1} respectively. The rate of charging by the active TyrTS heterodimers (46–75% that of reconstituted wild-type enzyme) implies that the tRNA affects the formation or the hydrolysis of tyrosyl adenylate. Each heterodimer has two potential sites for tyrosine activation and a single site for tRNA binding. However, due to half-of-the-sites reactivity, only one molecule of tyrosyl adenylate is formed per heterodimer. If tyrosyl adenylate is formed on the truncated subunit, it can be attacked by the tRNA (rate 0.45 s^{-1}; see Table 1) and the enzyme can recycle, whereas if adenylate is formed on the other subunit, it cannot be transferred to the tRNA and also prevents further tyrosine activation at the truncated subunit. In the latter case, the enzyme is blocked until the tyrosyl adenylate hydrolyses (rate 0.45×10^{-3} s^{-1}; ref. 13). If tyrosyl adenylate could form at random at either subunit, the heterodimers would quickly be blocked (50% of the remaining active heterodimers each cycle). Thus, we suspect that either the tyrosyl adenylate forms preferentially on the truncated subunit of the heterodimers (directed by the lie of the tRNA), or the binding of tRNA greatly stimulates hydrolysis of blocking tyrosyl adenylate. The experiments were done in 100 mM Tris-HCl, 44 mM Tris *p*H 7.78, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol and 0.1 mM phenyl methyl sulphonyl fluoride. For denaturation, the starting solutions (20 μl) were 10.4 μM in each enzyme; 1 μl bovine serum albumin (BSA, 20 mg ml^{-1}) was added, then urea until saturation (~25 mg; volume 40 μl, urea 10 M, BSA 0.5 mg ml^{-1} final concentration). For renaturation, the urea solutions were diluted using nine steps of 1 M each (15 min at room temperature for each step), keeping BSA at 0.5 mg ml^{-1}. Aliquots (2.25 μl) of the mixtures were assayed in 100 μl of 10 mM Mg-ATP, 20 μM ^{14}C-Tyr (514 mCi $mmol^{-1}$), 5 mg ml^{-1} crude *E. coli* tRNA (380 pmol of tyrosine incorporation per mg). The presence of BSA during the denaturation/renaturation process was necessary to recover activity. 73% of the activity of the wild-type TyrTS could be recovered using stepwise dilution of urea whereas only 7% was recovered after a one-step dilution. The addition of urea to the tRNA charging assay resulted in a progressive increase of the rate (2.5-fold maximum at 0.16 M urea).

type C-terminal domain[7]. The results show that $tRNA^{Tyr}$ interacts with the residues Lys 151, Arg 207 and Lys 208 (which correspond to mutated residues) in the truncated subunit of the heterodimer and therefore, taking into account our previous results[7], that it interacts with both tyrosyl adenylate and residues Lys 151, Arg 207 and Lys 208 in the same subunit of the dimer. The six mutants with a lesion in the C-terminal domain did not form active heterodimers with the truncated TyrTS; this result was expected because the C-terminal domain of TyrTS is necessary for tRNA charging[2].

In the active heterodimers, one of the constitutive monomers lacks the C-terminal domain. Therefore, in the wild-type TyrTS, only one of the two symmetrical copies of the C-terminal domain is essential for tRNA interaction. Similarly, only one of the two monomers has a wild-type N-terminal domain. Therefore, only one of the two symmetrical copies of residues Lys 151, Arg 207 or Lys 208 is essential for interaction with tRNA. These results show that TyrTS can function with only one productive tRNA binding site per dimer.

Model-building studies[4] indicate that $tRNA^{Tyr}$ may fold into a structure which is similar to the known structure of yeast $tRNA^{Phe}$ (ref. 8). We have attempted a docking of TyrTS[9] with $tRNA^{Phe}$ using the computer graphics program FRODO[10]. The 3′-hydroxyl of $tRNA^{Phe}$ was held near the tyrosyl adenylate binding site and the tRNA rotated around this fixed point so that the acceptor arm interacted with the tyrosyl adenylate and with residues Lys 151, Arg 207 and Lys 208 on the same subunit. With the path of the tRNA fixed as above, we examined the interaction of the anticodon arm with the second (nonproductive) subunit; this subunit interacts with the tRNA through its disordered C-terminal domain. In the crystallographic map, this domain may correspond to an island of electron density which is located in part on top of the α-helical domain and of the tyrosyl adenylate binding pocket. It is unlikely that the N-terminal domain of the nonproductive subunit makes important contacts to the tRNA through basic residues, as the heterodimers constructed between the truncated TyrTS and the non-complementing mutants at positions 82, 86, 151, 207, 208, 230 and 233 were all active in tRNA charging (Table 2). The model in Fig. 2 was generated by a more careful docking of the acceptor stem with the productive subunit and suggests that the anticodon arm lies in the plane of the subunits, where it would be in close proximity to the crystallographic island of density mentioned above (Fig. 2).

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