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Discrimination between transfer-RNAs by tyrosyl-tRNA synthetase

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Summary — We have constructed a model of the complex between tyrosyl-tRNA synthetase (TyrRS) from *Bacillus stearothermophilus* and $tRNA^{Tyr}$ by successive cycles of predictions, mutagenesis of TyrRS and molecular modeling. We confront this model with data obtained independently, compare it to the crystal structures of other complexes and review recent data on the discrimination between tRNAs by TyrRS. Comparison of the crystal structures of TyrRS and GlnRS, both of which are class I synthetases, and comparison of the identity elements of $tRNA^{Tyr}$ and $tRNA^{Gln}$ indicate that the two synthetases bind their cognate tRNAs differently. The mutagenesis data on $tRNA^{Tyr}$ confirm the model of the TyrRS:$tRNA^{Tyr}$ complex on the following points. TyrRS approaches $tRNA^{Tyr}$ on the side of the variable loop. The bases of the first three pairs of the acceptor stem are not recognized. The presence of the NH_2 group in position C6 and the absence of a bulky group in position C2 are important for the recognition of the discriminator base A73 by TyrRS, which is fully realized only in the transition state for the acyl transfer. The anticodon is the major identity element of $tRNA^{Tyr}$. We have set up an *in vivo* approach to study the effects of synthetase mutations on the discrimination between tRNAs. Using this approach, we have shown that residue Glu152 of TyrRS acts as a purely negative discriminant towards non-cognate tRNAs, by electrostatic and steric repulsions. The overproductions of the wild type TyrRSs from *E coli* and *B stearothermophilus* are toxic to *E coli*, due to the mischarging or the non-productive binding of tRNAs. The construction of a family of hybrids between the TyrRSs from *E coli* and *B stearothermophilus* has shown that their sequences and structures have remained locally compatible through evolution, for folding and function, in particular for the specific recognition and charging of $tRNA^{Tyr}$.

aminoacyl transfer-RNA synthetase / tyrosyl transfer-RNA synthetase / tRNA identity / tRNA discrimination / evolution

Introduction

Tyrosyl-tRNA synthetase (TyrRS) catalyses the aminoacylation of $tRNA^{Tyr}$ with tyrosine in two steps, first the activation of tyrosine with ATP to form tyrosyl-adenylate (Tyr-AMP), then the transfer of tyrosine from Tyr-AMP to the 3'-terminal ribose of $tRNA^{Tyr}$. Both steps of the reaction have been characterized by experiments of pre-steady state kinetics and the mechanism of the first step has been studied in great detail by mutagenesis of active site residues [1–3]. The crystal structure of TyrRS from *Bacillus stearothermophilus* (Bst-TyrRS) has been determined at 2.3 Å resolution [4, 5].

Abbreviations: aa, amino acid in the three letter code; aaRS, aminoacyl-tRNA synthetase; aa-AMP, aminoacyl-adenylate; Bst-, from *Bacillus stearothermophilus*; Eco-, from *Escherichia coli*.

Tyrosyl-tRNA synthetase (TyrRS) and the classification of the synthetases

TyrRS belongs to class I of the aminoacyl-tRNA synthetases (aaRS) since its catalytic domain has the dinucleotide binding fold and its sequence contains the conserved motifs HIGH and KFGKT [6, 7]. Fraser and Rich [8] have found that the primary site of aminoacylation of $tRNA^{Tyr}$ by TyrRS from *Escherichia coli* (Eco-TyrRS) is located at the 2'-OH rather than the 3'-OH of ribose 76 (85% *vs* 15% of the molecules, respectively), as the majority of the class I aaRSs. Sprinzl and Cramer [9] have found that both 2'-OH and 3'-OH can be aminoacylated (63 % *vs* 37%). Thus, an uncertainty remains on the strict specificity of TyrRS towards 2'OH as the primary site of charging.

The class I aaRSs are mostly monomeric whereas the class II ones are oligomeric. TyrRS and TrpRS are exceptions to this rule since they are dimeric and belong to class I. MetRS is monomeric in the lower eukaryotes and dimeric in the prokaryotes but the

monomer is active. Bst-TyrRS is smaller than *E coli* CysRS (419 *vs* 461 residues), which is the smallest monomeric aaRS [10, 11].

The structures of TyrRS and GlnRS indicate different binding modes of the tRNAs

The class I aaRSs have been subdivided according to several criteria. TyrRS has been ranged in the same subclass as GlnRS [11] or in a different subclass [12, 13]. Detailed comparisons of the structures of TyrRS and GlnRS have shown that the two synthetases cannot interact with their cognate tRNAs according to the same mechanism [14–16]. The three main reasons are the following.

1) When one superposes the dinucleotide binding folds of Eco-GlnRS and Bst-TyrRS, residue Asp78 of TyrRS occupies the same position as does nucleotide A76 of $tRNA^{Gln}$ in the GlnRS:$tRNA^{Gln}$ complex. This superposition implies different modes of binding for the acceptor ends of the respective tRNAs [16].

2) Each class I aaRS possesses an inserted domain between the first and the second half of the dinucleotide binding fold, called connective peptide 1 [12]. In GlnRS, this domain contains 108 residues (positions 103 to 211; helices α-D to α-F and strands β-4 to β-8) and binds a hairpinned conformation of the acceptor end of $tRNA^{Gln}$ [17]. In TyrRS, this domain contains only 40 residues (123 to 163; helices α-H6 to α-H9) and forms part of the interface between the subunits of the dimer [4, 5, 18].

3) The dinucleotide binding fold of TyrRS possesses a sixth β-strand (β-A), which is antiparallel and formed by residues 15 and 16 at the N-terminus of the enzyme. This additional strand lies adjacent to strand β-F at the carboxyl end of the fold [5]. GlnRS also possesses a sixth β-strand (β-11) at this position of the fold but it is parallel, it follows strand β-10 in the sequence and the two strands are linked *via* an unusual left-handed connection (residues 264–316), called connective peptide 2, which has no equivalent in TyrRS. Strand β-11 is part of a motif (α-K, β-11, α-L) of GlnRS, which interacts with the inside corner of the L of $tRNA^{Gln}$ and serves to globally position this substrate on the surface of the enzyme [12, 17].

Identity elements of $tRNA^{Tyr}$

Studies on mischarging mutants of *E coli* $tRNA^{Tyr}su+3$ and their revertants have shown that the discriminator base, A73, is recognized by Eco-TyrRS *in vivo* but not the bases of the three first pairs of the acceptor stem, 1.72, 2.71 and 3.70 (reviewed in [14, 19]). The recognition necessitates that bases 1 and 72 pair, by either Watson-Crick or non-canonical hydrogen bonds, and adopt a geometry compatible with the helical structure of the acceptor stem. Thus, the change of the wild type pair, G1.C72, into A1.C72 abolishes the recognition [14]. A long variable stem and loop is not required for recognition by Eco-TyrRS since a mutant *E coli* $tRNA^{Cys}$, carrying the amber anticodon CUA and a change of nucleotide U73 into A, inserts tyrosine into dihydrofolate reductase *in vivo* [20].

In vitro studies on the aminoacylation of mutant *E coli* $tRNAs^{Tyr}$ by Eco-TyrRS have shown that the central base of the anticodon, U35, is the major element of recognition. The discriminator base, A73, the first base of the anticodon, G34, and the orientation of the extra stem and loop are important recognition elements [21–23]. Mutation G34C, which changes $tRNA^{Tyr}$ into the amber suppressor $tRNA^{Tyr}su+3$, decreases 24 times the kinetic parameter k_{cat}/K_m for tyrosylation [21]. Therefore, the mutations that do not affect the activity of $tRNA^{Tyr}su+3$ *in vivo*, are unlikely to affect the activity of the wild type $tRNA^{Tyr}$ (*ie* $tRNA^{Tyr}su^-$); in contrast, the mutations that abolish the activity of $tRNA^{Tyr}su+3$, may have a less drastic effect on the wild type $tRNA^{Tyr}$.

Comparison of $tRNA^{Tyr}$ with $tRNA^{Gln}$ and $tRNA^{Asp}$

The recognition of the discriminator base, A73, the lack of recognition of the bases in the three first pairs of the acceptor stem and the necessity to have a strong base pair in positions 1.72 are characteristics for the recognition of $tRNA^{Tyr}$ by TyrRS that are similar to those for the recognition of the acceptor arm of $tRNA^{Asp}$ by yeast AspRS [24, 25]. The glutaminyl system of *E coli* has very different characteristics.

In the structure of the GlnRS:$tRNA^{Gln}$ complex, the acceptor end of $tRNA^{Gln}$ forms a hairpinned structure, base pair U1.A72 is melted and GlnRS forms specific hydrogen bonds with the N2-H_2 groups of bases G2 and G3 [17]. *In vitro* and *in vivo* studies have shown that a tRNA becomes a better substrate for GlnRS when one goes from a strong base pair (C.G or G.C) in position 1.72 to a weak base pair (U.A, G.A or G.U) than to an unpaired couple (C.A) [26, 27]. The formation of the complex is rather insensitive to the nature of the base in position 73, which can be G or A in an *in vitro* charging assay, or any base in an *in vivo* suppression assay. In contrast, the aminoacylation of $tRNA^{Gln}$ is very sensitive to the nature of the bases in pairs 2.71 and 3.70, *in vivo* and *in vitro* [19, 26]. We conclude from this comparison that TyrRS and GlnRS interact differently with the acceptor arms of their cognate tRNAs.

In vitro studies have shown that mutation U35G of *E coli* $tRNA^{Tyr}$ increases 16 times the K_m and

decreases 13 times the V_{max} for its aminoacylation by Eco-TyrRS [22]. Thus, this mutation affects K_m and V_{max} to similar extents contrarily to the mutations in the anticodons of $tRNA^{Gln}$ and $tRNA^{Asp}$, which mainly affect k_{cat} [24, 26].

The binding site of $tRNA^{Tyr}$ is shared between the two subunits of TyrRS

The possibility that the binding site for one molecule of $tRNA^{Tyr}$ is not contained within one subunit of the TyrRS dimer but straddles both subunits was first envisioned by Blow *et al* [28] after comparing the dimensions of the yeast $tRNA^{Phe}$ and Bst-TyrRS molecules. This hypothesis was compatible with the behaviours of the TyrRS:$tRNA^{Tyr}$ complex in experiments of chemical attack of TyrRS [29] and of neutron scattering [30]. It was unambiguously proven by the study of heterodimers between mutant subunits of Bst-TyrRS. Very different mutations were used [31–35]. In most of these experiments, one of the TyrRS subunits carried a deletion of residues 318–417, which might have introduced a structural asymmetry in the remainder of the molecule [4]. However, in one of these experiments at least, the two subunits were full-length and only carried a point mutation, different in each of the two subunits [31]. The results have shown that the binding site of $tRNA^{Tyr}$ recruits residues from the N-terminal domain (residues 1-319) of one subunit and the disordered C-terminal domain (320–419) of the other subunit.

It is interesting to note that the sequence of Bst-TyrRS is shorter than the one of Eco-SerRS (419 *vs* 430 residues) and that the binding site of $tRNA^{Ser}$ at the surface of the SerRS dimer is also shared between the two subunits in the crystal structure of their complex [36].

Structural model of the TyrRS:$tRNA^{Tyr}$ complex

We have constructed and refined a structural model of the complex between Bst-TyrRS and $tRNA^{Tyr}$ by successive cycles of predictions, mutagenesis of TyrRS to test these predictions, and molecular modeling. To construct this model, we used the crystal structures of free Bst-TyrRS and yeast $tRNA^{Phe}$. The five 3'-terminal nucleotides, CACCA3', of $tRNA^{Tyr}$ and $tRNA^{Phe}$ are identical. In a first step, we assumed that some basic residues of TyrRS form salt bridges with phosphate groups of $tRNA^{Tyr}$. We therefore changed 40 basic residues of Bst-TyrRS individually, by mutagenesis, and found that 13 mutations affected tyrosine activation or $tRNA^{Tyr}$ charging [31]. In a second step, we mutated eight residues of the N-terminal domain of TyrRS that were close to the acceptor arm of the tRNA in our first model and found that four mutations affected $tRNA^{Tyr}$ charging [37]. In subsequent steps, we constructed several mutations of selected residues to characterize the nature of their interactions with ATP or $tRNA^{Tyr}$ ([37-39]; R Nageotte, H Bedouelle, unpublished). So far, we have constructed 68 mutations of 48 different residues of TyrRS. We found four residues involved in the binding of ATP, 12 residues belonging to the $tRNA^{Tyr}$ binding site (six in the N-terminal domain and six in the C-terminal domain), and we identified one residue (Glu152) only involved in the rejection of non-cognate tRNAs, *ie* a purely negative determinant of the specificity for $tRNA^{Tyr}$.

In the model that has resulted from this extensive mutagenesis, the acceptor arm of $tRNA^{Tyr}$ interacts with the N-terminal domain of one TyrRS subunit and the anticodon arm is in a position suitable to interact with the disordered C-terminal domain of the other subunit. $tRNA^{Tyr}$ lies on TyrRS by one of its sides and there is a very good complementarity of shape between the acceptor stem of the tRNA and the surface of TyrRS. The CCA3' end of the tRNA is stretched to reach Tyr-AMP and its bases are oriented towards the solvent (fig 1). The bases of the acceptor stem are not recognized by TyrRS, except the discriminator base, A73. Most contacts with the acceptor stem are made through phosphate groups. Residue Glu152 is close to phosphate groups 73 and 74 of $tRNA^{Tyr}$ (fig 2) [37].

Most of the TyrRS mutations that define the binding site of $tRNA^{Tyr}$ change sequence and structure elements that are peculiar to TyrRS. Thus, residue Thr17 is located at one end of the antiparallel strand β-A; Asn146, Lys151 and Glu152 belong to the connective peptide 1; Arg368, Arg371, Arg407, Arg408, Lys410 and Lys411 belong to the disordered C-terminal domain. Trp196, Arg207 and Lys208 are exceptions. They are located at both ends of helix α-H11, which directly links strands β-E and β-F in the secondary structure. In the tertiary structure of TyrRS, this helix is located between the connective peptide 1 and the antiparallel strand β-A [5]. In the model, it runs along the acceptor stem of the tRNA (fig 1). In the GlnRS:$tRNA^{Gln}$ complex, only one end of the structurally equivalent helix, α-H, interacts with $tRNA^{Gln}$ [17].

Our surface scanning of TyrRS by mutagenesis revealed that four mutations, at residues Lys82, Arg86, Lys230 and Lys233, strongly impaired the formation of Tyr-AMP [31]. Such a result was unexpected because these four residues are far from Tyr-AMP in the crystal structure of Bst-TyrRS [5]. A detailed kinetic analysis has shown that they belong to two mobile loops that move and interact with the β- and γ-phosphates of ATP in the transition state for the formation of Tyr-AMP [38]. Two of these residues,

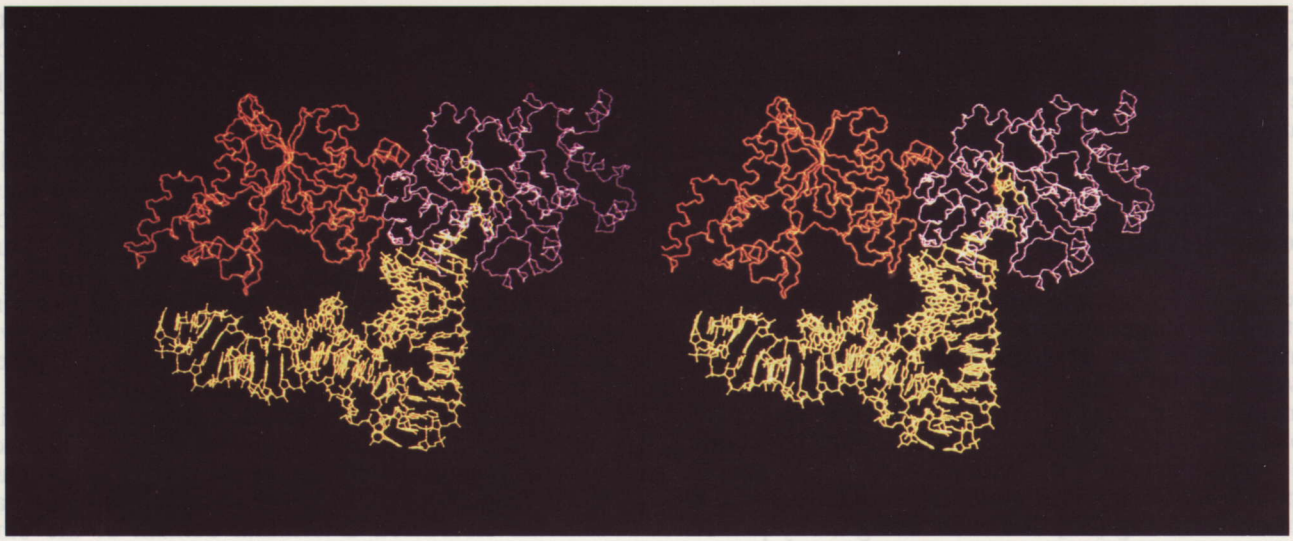

Fig 1. Structural model of the complex between tyrosyl-tRNA synthetase from *B stearothermophilus* and $tRNA^{Tyr}$ (stereo view). The two subunits of TyrRS are represented in purple and orange. Yeast $tRNA^{Phe}$, taken as a model of $tRNA^{Tyr}$, is in yellow. The position of tyrosine in the active site of the purple subunit is in red. The disordered C-terminal domain (residues 320–419) of TyrRS is not represented. Adapted from [31, 37].

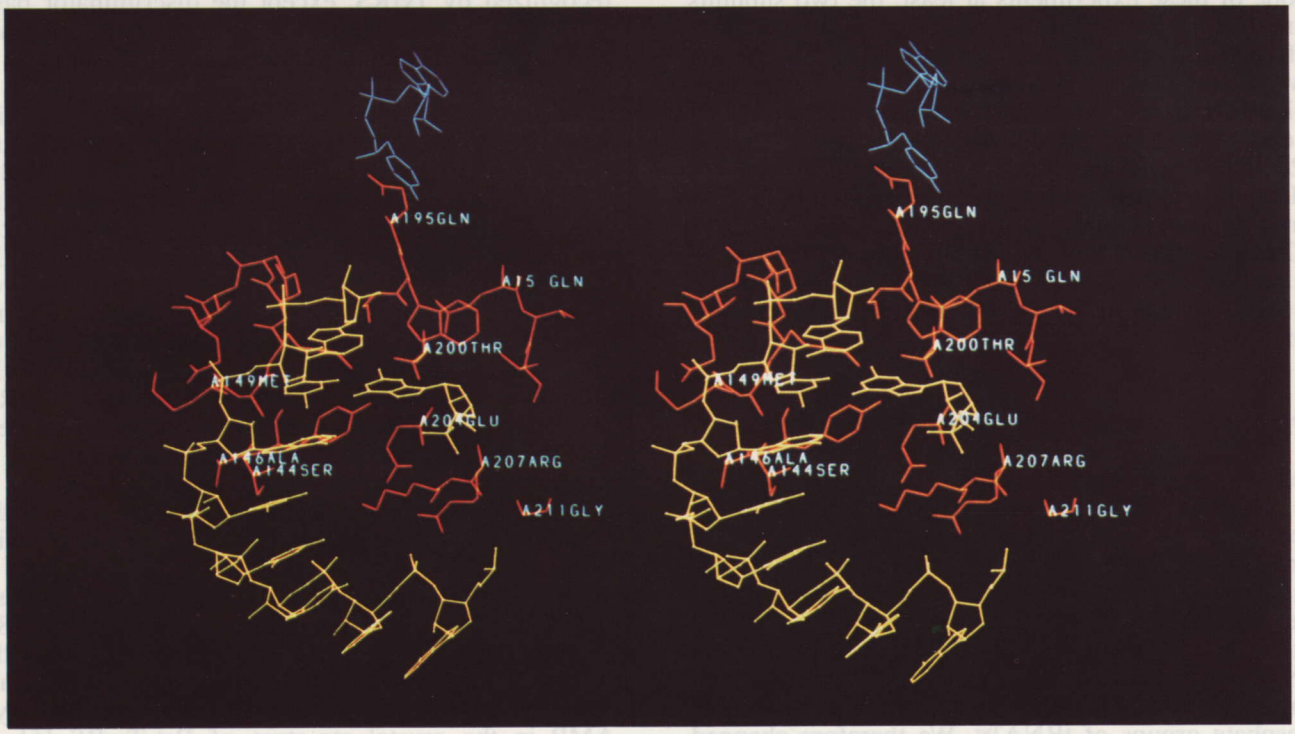

Fig 2. Interactions between the acceptor stem of $tRNA^{Tyr}$ and the N-terminal domain of TyrRS in the model of the TyrRS:$tRNA^{Tyr}$ complex (stereo view). The residues of TyrRS are represented in red, those of $tRNA^{Phe}$ (1 and 66–73) are in yellow and Tyr-AMP is in blue. Only the N- or 5'-terminal residue of each TyrRS or $tRNA^{Phe}$ fragment is numbered. The side chain of Asn146 is not visible beyond Cβ in the electron density map. Adapted from [37].

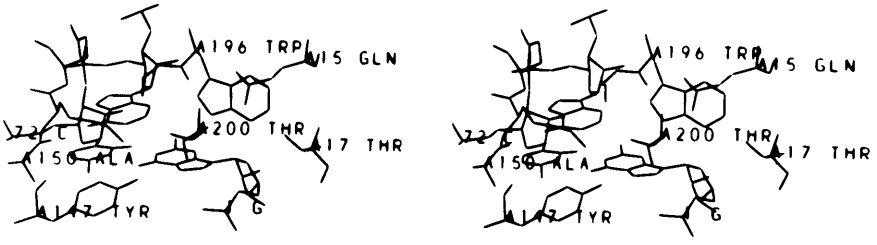

Fig 3. Interactions between the discriminator base, A73, of $tRNA^{Tyr}$ and TyrRS in the model of the TyrRS:$tRNA^{Tyr}$ complex (stereo view taken from [39]). Residues 1 and 72–73 of $tRNA^{Phe}$ are represented. Only the N- or 5'-terminal residue of each TyrRS or $tRNA^{Phe}$ fragment is numbered.

Lys230 and Lys233, belong to a pentapeptide which is conserved in the class I aaRSs [11]. Our results have established the role of this motif, KMSKS, in the reaction of amino acid activation ([31, 38]; see also [16, 40]).

Recognition of the discriminator base in the transition state for acyl transfer

The data on the mutations of $tRNA^{Tyr}$ and on its identity elements are compatible with the model of the TyrRS:$tRNA^{Tyr}$ complex described above. This model predicts a specific recognition of the discriminator base, A73, by the N-terminal domain, an interaction between the anticodon arm and the C-terminal domain, and an absence of recognition for the bases of the first three pairs of the acceptor stem.

In the model, the N6-H_2 group of adenine-73 is engaged in a hydrogen bond with the carbonyl oxygen of residue Ala150, and its C2-H group and N3 atom are in Van-der-Waals contacts with the Cδ1-H group of Trp196 (fig 3). These interactions are consistent with the values of the kinetic parameter k_{cat}/K_m for the tyrosylation of $tRNA^{Tyr}$ derivatives, mutated at position 73. The relative k_{cat}/K_m values are equal to 1 for the wild type A73, 0.13 for the mutant C73, 0.064 for U73 and 0.026 for G73 [22]. Bases A and C which have an NH_2 group in position C6, donor of a hydrogen bond, are thus better recognized than U and G which have an oxygen atom in position C6. Base A, which has only an hydrogen atom in position C2 is better recognized than C, U or G which have a more bulky oxygen atom or NH_2 group in position C2 (fig 4). The interactions of the model are also consistent with the levels of tyrosine incorporation in dihydrofolate reductase by derivatives of $tRNA^{Tyr}su+3$, mutated at position 73, in experiments performed *in vivo*: 95% for the wild type A73, 93% for the mutant C73, 30% for U73 and 8% for G73 [19].

The change of Lys151 into Asn does not modify the K_m for the tyrosylation of $tRNA^{Tyr}$ but decreases 150 times k_{cat}/K_m. Thus Bst-TyrRS and $tRNA^{Tyr}$ form additional contacts, in the vicinity of Lys151, when their complex goes from the initial state to the transition state [31]. Because Lys151 is close to the discriminator base, A73, in the model of the TyrRS:$tRNA^{Tyr}$ complex, we have suggested that these additional contacts, in the transition state, involve A73 [37]. The values of $1/K_m$ and k_{cat}/K_m for the tyrosylation of the $tRNA^{Tyr}$ derivatives, mutated at position 73, are consistent with this hypothesis. The relative values of $1/K_m$ are equal to 1 for the wild type A73, 0.36 for the mutant G73, 0.29 for C73 and 0.24 for U73 [22]. Thus, the values of $1/K_m$ vary less than those of k_{cat}/K_m (see previous paragraph). For example, $1/K_m$ varies four-fold when A73 is changed into G whereas k_{cat}/K_m varies 14-fold. These data on mutant $tRNAs^{Tyr}$ show that the interactions between base A73 and Eco-TyrRS are fully realized only in the transition state of the TyrRS:$tRNA^{Tyr}$ complex. Together, the kinetic data on the mutant Bst-TyrRSs and $tRNAs^{Tyr}$ indicate that the full recognition of A73 by TyrRS is only made in the transition state. A similar situation has been described in the case of the AlaRS:$tRNA^{Ala}$ complex [41].

The strong effect of mutation U35G of *E coli* $tRNA^{Tyr}$ on the K_m for its tyrosylation by Eco-TyrRS is consistent with the strong effects of point mutations (Arg368, Arg371, Arg407, Arg408, Lys410 and Lys 411) in the C-terminal domain of Bst-TyrRS on this same K_m parameter, and with the absence of $tRNA^{Tyr}$ binding by Bst-TyrRS, when the C-terminal domain is deleted [22, 31, 42].

TyrRS approaches $tRNA^{Tyr}$ on the side of the variable loop

The structures of the GlnRS:$tRNA^{Gln}$ and AspRS:$tRNA^{Asp}$ complexes show that the two synthetases

interact with different sides of their cognate tRNAs. The class I GlnRS interacts with the side of the D-loop and with the minor groove of the acceptor stem. The class II AspRS interacts with the side of the variable loop and with the major groove of the acceptor stem. Ruff *et al* [43] have proposed that these two different modes of approach are characteristic of the two classes of aaRSs and linked to the primary site of aminoacylation, either the 2'OH or the 3'OH of nucleotide A76.

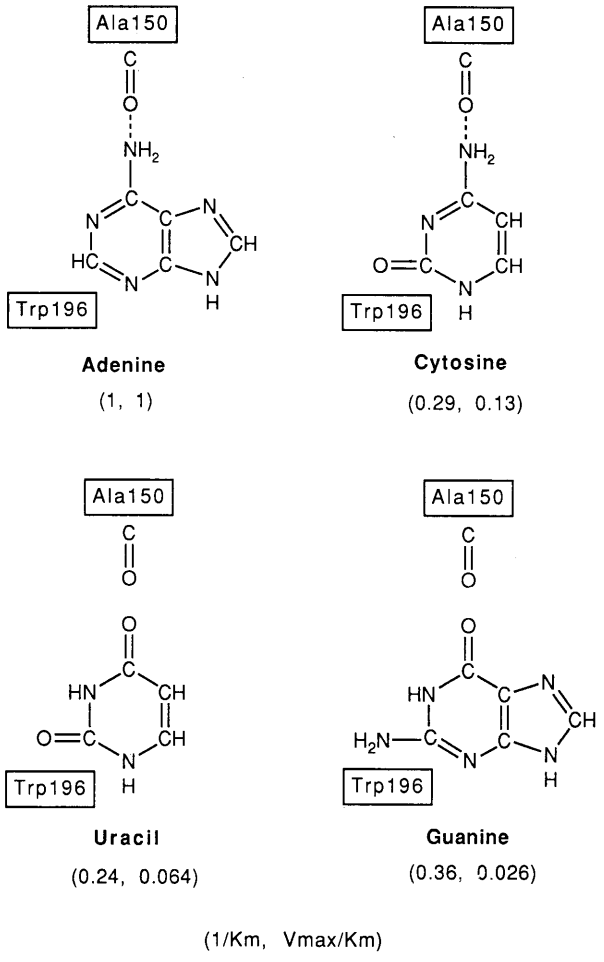

Fig 4. Comparison of the structural model of the TyrRS:tRNA^{Tyr} complex with mutagenesis data on the discriminator base, A73. In the model, the N6-H_2 group of A73 forms a hydrogen bond with the carbonyl oxygen of residue Ala150, and its C2-H group and N3 atom are in Van der Waals contacts with the Cδ1-H group of Trp196. For each change of A73, the relative values of $1/K_m$ and V_{max}/K_m for the charging of the mutant tRNA^{Tyr} by Eco-TyrRS are given [22].

Our mutagenesis data on the binding site of tRNA^{Tyr} at the surface of Bst-TyrRS imply that TyrRS approaches tRNA^{Tyr} on the side of the variable loop and of the major groove of the acceptor stem, approximately as in the AspRS:tRNA^{Asp} complex. The mutagenesis data on *E coli* tRNA^{Tyr} show that the orientation of the variable stem and loop is important for the interaction with Eco-TyrRS [22] and therefore also indicate that this interaction involves the side of the variable loop. Thus, TyrRS is an exception to the postulate that links the class of an aaRS and the side of the tRNA with which it interacts [11, 43].

Given the flexibility of the acceptor end of tRNAs [37] and the very different and distorted conformations it can take, it might be premature to strongly link the side of the tRNA that interacts with its cognate aaRS and its primary site of charging. In the AspRS:tRNA^{Asp} complex, nucleotides 73–75 follow the general course of the acceptor stem helix but base A76 is unstacked and projects in an opposite direction [25]. In the GlnRS:tRNA^{Gln} complex, the acceptor end of tRNA^{Gln} bends inwards and forms a hairpin; bases A76, C75 and G73 are stacked on each other but nucleotide C74 is looped out [17]. In the model of the TyrRS:tRNA^{Tyr} complex, the acceptor end of the tRNA is stretched and unwound, which orientates bases 74–76 towards the solvent and enables the 2'OH and 3'OH of nucleotide A76 to come close to the carbonyl carbon of Tyr-AMP, in line with tyrosine (fig 1; [37]).

Discrimination between tRNAs and competition between synthetases

In 1972, Yarus proposed that the precision of tRNA aminoacylation is enhanced by the existence of parallel systems of ligands *in vivo*. The idea is the following. Each synthetase interacts strongly with its cognate tRNAs and weakly with the non-cognate tRNAs. As the aaRSs and tRNAs are approximately in equimolar amounts within the cell, the strong affinity of the cognate molecules makes them unavailable for cross-reactions [44]. This model suggests several ways of modifying the precision of aminoacylation, for example by increasing the cellular concentration of one synthetase or by constructing mutant synthetases or tRNAs having increased affinities for non-cognate partners [21, 23, 45, 46].

What are the expected consequences of a decreased precision of aminoacylation? First, an erroneous incorporation of amino acids into proteins; then, a destabilization of proteins, due to these misincorporations, and eventually the inactivation of essential proteins. These direct consequences lead to the following predictions: a decreased precision of amino-

acylation will be toxic to the cell and this toxicity will increase with the growth temperature as do the destabilization and inactivation of proteins. These predictions have a useful corollary: toxicity may be used as a test for the *in vivo* precision of aminoacylation. We have tested these hypotheses in the case of TyrRS.

Overproduction of TyrRS is toxic to *E coli*

We have shown that the overproduction of an aaRS can be toxic to the host cell. We measured the toxicity of TyrRS by the ratios of the efficiencies with which producing bacteria plated in various growth conditions. We manipulated the productions of Eco-TyrRS and Bst-TyrRS by inserting the *tyrS* genes in plasmids with increasing copy numbers, under control of promoters with increasing strength. We found that both Eco-TyrRS and Bst-TyrRS were toxic to *E coli*. Their toxicities increased with their levels of production and were much higher (up to 2000-fold) at 37°C than at 30°C. The overproduction of TyrRS destabilized β-galactosidase, synthesized by the same cell. The cellular toxicity and the destabilization of β-galactosidase were due to interactions between TyrRS and tRNAs since they were abolished by mutation K410N or K411N of the C-terminal domain. We concluded that the overproduction of TyrRS induces a decreased precision of aminoacylation, either directly by mischarging of non-cognate tRNAs or indirectly by non-productive binding and sequestration of tRNAs [47].

Glu152, a purely negative determinant of the specificity for $tRNA^{Tyr}$

We have set up an *in vivo* approach to analyze the effects of mutations in an aaRS on the discrimination between tRNAs. This approach is based on the observation that a mutant aaRS, that discriminates less well between tRNAs, can be toxic to the host cell. We measure this toxicity by the ratios of the efficiencies with which the producing cells plate in induced and repressed conditions, at 30°C and 37°C.

We have inserted the *tyrS* gene from *B stearothermophilus* into a multicopy plasmid under control of promoter *tac*, which is repressed by the *lac* repressor and inducible with IPTG. We have constructed mutations of residue Glu152 of Bst-TyrRS in this genetic background. Glu152 is close to phosphate groups 73 and 74 of $tRNA^{Tyr}$ in the model of the TyrRS: $tRNA^{Tyr}$ complex. This contact was a major constraint during the construction of the model and it is a very specific prediction of this model (fig 3) [37].

The wild type Bst-TyrRS was not toxic in these experimental conditions. A mutant synthetase, TyrRS-(E152A), carrying the change of Glu152 into Ala, was slightly toxic at 30°C, by a factor of 5, and highly toxic at 37C°, by a factor of 12 000. There was a strong effect of temperature on this toxicity, by a factor of 6400. The toxicity of TyrRS(E152A) was abolished by additional mutations (K410N or K411N) that prevent the binding of $tRNA^{Tyr}$. The toxicity was therefore due to the interaction of TyrRS with tRNAs. Because TyrRS(E152A) was fully active for the tyrosylation of $tRNA^{Tyr}$ (with K_m decreased 2.5 times and k_{cat} unchanged in comparison with the wild type TyrRS), its toxicity could only be due to faulty interactions with non-cognate tRNAs. Otherwise stated, residue Glu152 is not involved in the interaction of TyrRS with the cognate $tRNA^{Tyr}$; its role is to reject non-cognate tRNAs, to prevent their binding or their mischarging [39].

We have introduced 10 different side chains at position 152 of Bst-TyrRS. Short (Gly, Ala, Ser) or positively charged (Lys, Arg) side chains were highly toxic. Uncharged, bulky side chains (Gln, Met, Trp, His) had intermediate toxicities. Negatively charged side chains (Glu, Asp) were not toxic. In general, the toxicity of the mutant TyrRSs was correlated with the *in vitro* mischarging of pure $tRNA^{Phe}$ and $tRNA^{Val}$ with tyrosine ([39]; R Nageotte, H Bedouelle, unpublished). These results show that the contribution of Glu152 to the discrimination between tRNAs operates through steric and electrostatic repulsions. Thus, our results are fully consistent with the predictions of the model about Glu152. Acidic residues involved in the discrimination between tRNAs [48–50] and purely negative discriminants [51] have also been found in other synthetases.

Mutation H45N of Bst-TyrRS abolishes the formation of Tyr-AMP and thus the tyrosylation of tRNAs. A doubly mutant TyrRS, that carried both mutations E152A and H45N, was strongly toxic at both 30°C and 37°C (20 000- and 300 000-fold respectively) and there was only a limited effect of temperature on this toxicity (15-fold). These results suggested that two different mecanisms of toxicity existed: the mischarging of non-cognate tRNAs for the single mutant TyrRS(E152A), and the non-productive binding and sequestration of tRNAs for the doubly mutant TyrRS(H45N, E152A) [39]. A mutant IleRS, defective for Ile-AMP formation, has also been shown to sequester tRNAs [52].

Evolution of recognition, discrimination and stability

The TyrRSs from prokaryotic or mitochondrial origins can generally charge *E coli* $tRNA^{Tyr}$ *in vitro*. Conversely, Eco-TyrRS can charge $tRNAs^{Tyr}$ from other prokaryotic or mitochondrial origins. In particular, the TyrRSs from *E coli* and *B stearothermophilus* charge

the $tRNAs^{Tyr}$ from both organisms with similar efficiencies and Bst-TyrRS charges these $tRNAs^{Tyr}$ with very similar steady-state kinetics (reviewed in [14]) [2, 53]. These heterologous chargings show that the elements of interaction between TyrRS and $tRNA^{Tyr}$ have been globally conserved during evolution. The *tyrS* genes from *B stearothermophilus*, *B subtilis* and *B caldotenax* can complement a thermosensitive mutation in the *tyrS* gene of *E coli in vivo*. Similarly, the *tyrS* gene from *E coli* can complement a mutation in the *tyrS* gene of yeast mitochondria, and the *tyrS* gene of *Podospora anserina* can complement a mutation in the corresponding gene of *Neurospora crassa* for aminoacylation and splicing of group I introns (reviewed in [14]) [54, 55]. These *in vivo* genetic complementations show that the elements of discrimination by TyrRS between the 20 species of tRNAs have also been globally conserved during evolution. These observations justify the mingling that we usually operate between the data obtained from *E coli* and *B stearothermophilus*, and the use of *E coli* $tRNA^{Tyr}$ as a substrate for aminoacylation in our characterizations of mutant Bst-TyrRSs [31, 37, 39].

The TyrRSs from *E coli* and *B stearothermophilus* are 58% identical in amino acid sequence [56]. To analyze the structural mechanisms by which TyrRS evolved, we have constructed a family of nine different hybrid proteins between the two enzymes. The N-terminal part of each hybrid came from Eco-TyrRS and its C-terminal part from Bst-TyrRS. We measured the stability and activity of these hybrids *in vitro* and *in vivo*, then analysed their variations when the position of the fusion point moved along the protein sequence. The results showed that the two sequences and structures can replace each other locally and still give a stable, active and discriminating TyrRS. The results also showed that the greater stability of Bst-TyrRS is due to cumulative changes of residues scattered along the sequence and suggested that Bst-TyrRS is more rigid than Eco-TyrRS at low temperature. The existence of exceptional hybrids, having lower activity or stability than both neighbouring hybrids, showed that compensatory changes of residues have occurred between the two sequences during evolution. These changes correspond to tertiary or quaternary interactions in the crystal structure of Bst-TyrRS, some of which involve the structural elements that are peculiar to TyrRS and involved in the binding of $tRNA^{Tyr}$, in particular the connective peptide 1 and helix α-H11 [57].

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