

Tyrosyl-tRNA Synthetases

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Abstract

Tyrosyl-tRNA synthetase (TyrRS) comprises an N-terminal domain, which has the fold of the class I aminoacyl-tRNA synthetases, followed by idiosyncratic domains, which differ in eubacteria, archaeobacteria and eukaryotes. The eubacterial TyrRSs have recruited an RNA binding domain which is found in a large family of proteins. The crystal structures of the TyrRSs from *Bacillus stearothermophilus* (Bst-TyrRS) and *Thermus thermophilus* (Tth-TyrRS) have been solved, free, or in complex with tyrosine, or with tyrosyl-adenylate (Tyr-AMP). A quaternary complex between Tth-TyrRS, tRNA^{Tyr}, tyrosinol and ATP has been solved at 2.8 Å resolution. The dimer of Bst-TyrRS is symmetrical in the crystals but asymmetrical in solution. It unfolds through a folded compact monomeric intermediate, by dissociation of the subunits ($K_D = 84$ pM). A C-terminal domain is loosely linked to an intermediate α -helical domain through a fully flexible peptide. The tRNA binding site straddles the two subunits of TyrRS, which interacts with tRNA^{Tyr} according to a class II mode. The conserved sequences of class I, HIGH and KMSKS, are involved in the catalysis of tyrosine activation. The HIGH sequence is not involved in the transfer of tyrosine from Tyr-AMP to tRNA^{Tyr}, and the KMSKS sequence is involved in this transfer only through the initial binding of tRNA^{Tyr}. Other residues (Thr40, Lys82 and Arg86 in Bst-TyrRS), are involved in both steps of the catalytic reaction, by interacting first with ATP then with residue Ade76 of tRNA^{Tyr}. The identity elements of tRNA^{Tyr} comprise nucleotidic base Ade73, the anticodon, and either base-pair Gua1:Cyt72 in eubacteria or Cyt1:Gua72 in archaeobacteria and eukaryotes. The residues of TyrRS which interact with tRNA^{Tyr} or recognize its identity elements have been identified by extensive mutagenesis and kinetic studies of Bst-TyrRS and from the structure of the Tth-TyrRS-tRNA^{Tyr} complex. The two approaches are in excellent agreement. TyrRS catalyses the activation of tyrosine and its transfer to tRNA^{Tyr} by stabilizing the transition states for these two reaction steps, through interactions with ATP, Ade76, and the identity elements of tRNA^{Tyr}. The role of base pair 1:72 in the recognition of tRNA^{Tyr} results in a species specificity and makes TyrRS a potential target for antibiotics. This specificity relies on a short segment (<41 residues) of TyrRS and can be swapped between species. The specific recognition between TyrRS and tRNA^{Tyr} depends on the correct balance between the cellular

concentrations of synthetases and tRNAs. Moreover, a residue (Glu152) of Bst-TyrRS is involved in the rejection of noncognate tRNAs but not in the interaction with tRNA^{Tyr}, and thus is a purely negative determinant of specificity. Inhibitors of TyrRS have been discovered and characterized: tyrosinol, tyrosinyl-adenylate and tyrosyl-aryl dipeptides (e.g., Tyr-Tyr); however, they cross the bacterial envelope very inefficiently. Tyr-Gly dipeptides, derivatized with a sugar, have been isolated from microorganisms or synthesized, and shown to inhibit bacterial growth. The specificity of TyrRS towards the amino acid has been modified by screening or selecting mutants from random libraries which were targeted to residues of the tyrosine binding pocket. The species specificity of TyrRS towards tRNA^{Tyr} and the absence of an editing mechanism towards the amino acid, have made it possible to create a 21st triplet of synthetase, tRNA and amino acid, and thus to extend the genetic code. TyrRS has additional functions in some organisms: it charges plant viral RNAs; acts as a kinase or a cytokine; plays a role in the splicing of the group I introns (as an RNA chaperone), in the maintenance of the mitochondrial genome, in yeast sporulation and in the quality control of tRNAs in the cell nucleus. TyrRS has been used in the synthesis of analgesic neuro-dipeptides.

Evolution of TyrRS

The tyrosyl-tRNA synthetases (TyrRS) of all the phylogenetic kingdoms comprise an α/β domain, which has the mononucleotide binding fold of the class I aminoacyl-tRNA synthetases (aaRS) and an idiosyncratic insertion between the two halves of the fold (connective peptide CP1).¹ In eubacteria, the α/β domain is followed by an α -helical domain, which is specific for TyrRS, and by a C-terminal domain, which belongs to a numerous family of RNA-binding domains.² In the other two phylogenetic kingdoms, the α/β domain is followed by a C-terminal (in archaeobacteria) or intermediate (in eukaryotes) domain, which is common to TyrRS and to the tryptophanyl-tRNA synthetases (TrpRS) of all three kingdoms, and labelled C-W/Y. Finally, the *Homo sapiens* TyrRS (Hsa-TyrRS) comprises a C-terminal domain, homologous to the endothelial monocyte activating protein II (EMAP II), which is also found in eubacterial PheRS and MetRS.³ TyrRS and TrpRS appeared before the separation between eukaryotes and eubacteria, as the other aaRSs.^{4,5}

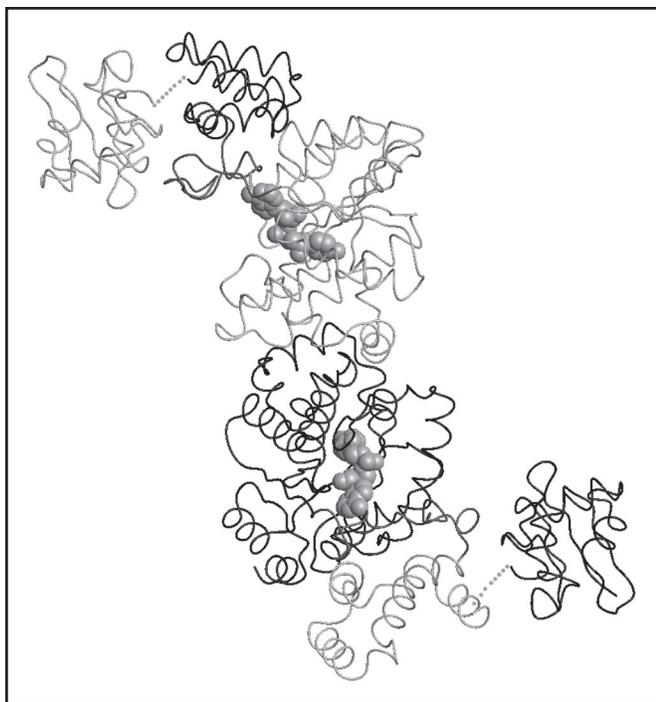


Figure 1. Structure of TyrRS from *Bacillus stearothermophilus*. The α/β -, α -helical, and C-terminal domains of each subunit are represented in different intensities of grey. Tyr-AMP is represented in a space-filling mode in the α/β domain of each subunit. The structure of the full-length Bst-TyrRS was reconstituted from the structures of the α/β and α -helical domains, solved by X-ray crystallography (pdb 3TS1),⁹ and from that of the C-terminal domain, solved by NMR methods (pdb 1JH3).¹⁵ The flexible peptide (residues 320-328) which links the α -helical domain to the C-terminal domain is visible neither in the crystal nor in the NMR structure and it was represented by a dashed line.

Structure of TyrRS

The crystal structure of TyrRS from *Bacillus stearothermophilus* (Bst-TyrRS) has been solved at 2.3 Å resolution (Fig. 1). The structures of complexes with tyrosine, tyrosinol, tyrosinyl-adenylate and tyrosyl-adenylate (Tyr-AMP) have also been reported. In the absence of tyrosine, ATP binds in the tyrosine binding pocket of TyrRS. In the presence of tyrosinol, which cannot be activated, ATP binds very weakly to the enzyme.⁶⁻⁹ Bst-TyrRS is a homodimer, possessing a two fold crystal symmetry. Each subunit of Bst-TyrRS comprises three sequential domains in the crystal structure. An α/β domain (residues 1-220), which contains the dimerization interface, and an α -helical domain (248-319) form a well ordered N-terminal domain (1-319). This domain is followed by an apparently disordered C-terminal domain (320-419). The properties of a Bst-TyrRS fragment, corresponding to the N-terminal domain, have shown that it is dimeric, it has the same crystal structure as the N-terminal domain in the full-length Bst-TyrRS, it forms Tyr-AMP with normal kinetics, but it does not bind tRNA^{Tyr} (refs. 8, 10). The properties of a Bst-TyrRS fragment, corresponding to the C-terminal domain, were characterized in solution by biophysical techniques and compared to those of the full-length Bst-TyrRS and of its N-terminal fragment. These characterizations have shown that the C-terminal domain is not in a disordered state; on the contrary, that it is in a stable, condensed, defined state of folding; that it has similar structures in its isolated form and in the

full-length Bst-TyrRS; and that its disorder in the crystals is due to the absence of tertiary interactions with the N-terminal domain.^{11,12}

When the sequences of the known eubacterial TyrRSs are aligned and programs of secondary structure prediction applied, it becomes apparent that their C-terminal domains possess a precise and conserved secondary structure.¹³ The three-dimensional structure of the C-terminal fragment from *B. stearothermophilus* has been determined by NMR spectroscopy (Fig. 1).^{14,15} This C-terminal domain belongs to the super-family of the ribosomal protein S4 and its structure is new among the aminoacyl-tRNA synthetases.¹⁶⁻¹⁸

Recently, the structure of TyrRS from *Thermus thermophilus* (Tth-TyrRS) has been solved. According to the crystal form, none, one or two C-terminal domains are visible, in orientations that are different and determined by the crystal packing. The C-terminal domain of Tth-TyrRS is connected to the preceding α -helical domain by a linker peptide which is fully flexible in the absence of tRNA. In contrast, the C-terminal domain is stabilized in a fixed orientation in the structure of the complex between Tth-TyrRS and tRNA^{Tyr}, and the linker peptide becomes more ordered.¹⁹

Stability of Eubacterial TyrRSs

The unfolding of the dimeric N-terminal fragment (residues 1-317) of Bst-TyrRS with urea was monitored by spectrofluorometry, circular dichroism and size exclusion chromatography. These experiments have shown the existence of an equilibrium between the native dimeric state of the N-terminal fragment, a folded monomeric intermediate and the unfolded polypeptide. From them, the stability of the association between the subunits ($K_D = 84$ pM) and the stability of the monomeric intermediate were deduced. One third of the fragment stability comes from its dimeric nature, and the unfolding of the monomeric intermediate is about 3 times more cooperative than the dissociation of the subunits.²⁰ As its monomer is inactive,²¹ TyrRS could have been formed by the association of two folded but inactive monomers during evolution.

To analyze how the stability of eubacterial TyrRSs has evolved, an ordered family of nine different hybrid proteins between TyrRS from the mesophilic bacterium *Escherichia coli* (Eco-TyrRS) and TyrRS from the thermophilic bacterium *B. stearothermophilus* (Bst-TyrRS) was constructed. The stability and activity of these hybrids were measured and their variations were analyzed when the position of the fusion point moved along the sequence. The results have shown that the two sequences can replace each other locally and still give stable and active TyrRS enzymes. The higher stability of Bst-TyrRS is due to cumulative changes of residues spread along the sequence, and compensatory changes of residues have occurred between the two sequences during evolution.²²

The evolution of a particular tertiary interaction in Bst-TyrRS, at the crossing between helices H₃ and H₅ near the active site, was further studied. The residues involved, Ile52 and Leu105, are replaced with Leu and Val respectively in Eco-TyrRS. The corresponding mutations were constructed in Bst-TyrRS and their effects on stability measured. Mutation I52L destabilizes the association between the two subunits of Bst-TyrRS, even though residue Ile52 is located more than 20 Å away from the subunit interface. This observation has suggested that the same approach could allow one to study directly and quantitatively the effects of mutations, located around the active site, on the transfer of information through the subunit interface. Mutation L105V destabilizes the

monomeric intermediate of unfolding. The two mutational pathways, going from the wild type Bst-TyrRS to the double mutant I52L-L105V, through one or the other of the two single mutants, are not equivalent. One of the pathways comprises two neutral steps for stability (I52L enabling L105V) whereas the other pathway comprises a destabilizing step (L105V) followed and compensated by a stabilizing step (I52L). Thus, the effects of mutations I52L and L105V on stability depend on the structural context. These results are compatible with the theory of the context effect in the evolution of proteins.²³

Structure of the Complex between TyrRS and tRNA^{Tyr}

Heterodimers between subunits of Bst-TyrRS carrying different mutations were constructed by *in vitro* methods and used to show that the binding site for one molecule of tRNA^{Tyr} is shared between the two subunits of one TyrRS molecule (Fig. 2).^{24,25} A structural model of the complex between Bst-TyrRS and tRNA^{Tyr} was constructed and refined by successive cycles of predictions, mutagenesis to test these predictions, and molecular modeling. At the beginning, some basic residues of Bst-TyrRS were assumed to form salt bridges with phosphate groups of tRNA^{Tyr}. The crystal structures of Bst-TyrRS and of tRNA^{Phe} from yeast, obtained in their free states, were used for the modelings. Four residues of Bst-TyrRS involved in the binding of ATP, 13 residues belonging to the binding site of tRNA^{Tyr}, and 1 residue (Glu152) involved only in the rejection of noncognate tRNAs were thus identified.²⁵⁻²⁹ The structural model of the complex between Bst-TyrRS and tRNA^{Tyr} was published before any complex between a synthetase and a tRNA was solved by crystallography.^{25,26} In this model, the acceptor arm of tRNA^{Tyr} interacts with the catalytic α/β domain of one subunit and its anticodon arm interacts with the C-terminal domain of the other subunit. TyrRS approaches tRNA^{Tyr} by the side of its variable loop and the major groove of its acceptor stem, i. e. by the side opposite to that which was later shown to prevail among the class I synthetases.

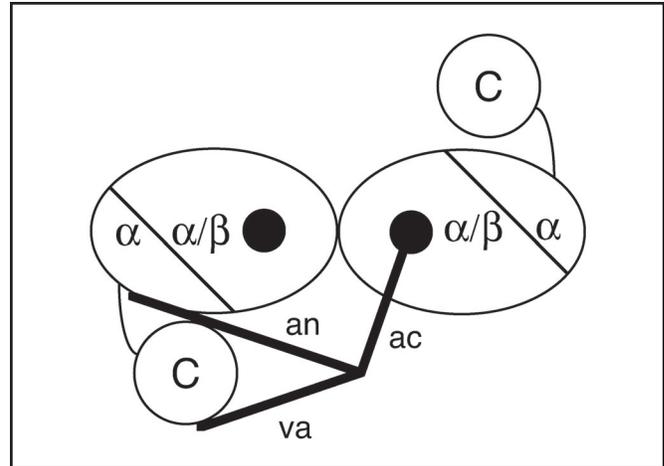


Figure 2. Schematic representation of the complex between eubacterial TyrRS and tRNA^{Tyr}. The α/β , α -helical, and C-terminal domains are schematized. The α -helical and C-terminal domains are linked by a flexible peptide. The active site is represented as a black disk in the α/β domain. The acceptor arm (ac) of tRNA^{Tyr} interacts with the α/β domain of one TyrRS subunit and its variable (va) and anticodon (an) arms interact with the α -helical and C-terminal domains of the other subunit (see text for references).

The crystal structure of the quaternary complex between Tth-TyrRS, its cognate tRNA^{Tyr}, tyrosinol and ATP has recently been solved at 2.9 Å resolution (Fig. 3).¹⁹ In this structure, the C-terminal domain is stabilized in a fixed orientation by binding in the elbow between the long variable arm and the anticodon stem of the tRNA. The tRNA binds across the two subunits of the dimeric enzyme and the mode of recognition resembles that of a class II synthetase, as predicted from the protein engineering studies on Bst-TyrRS. A comparison between the model structure of the Bst-TyrRS-tRNA^{Tyr} complex and the crystal structure

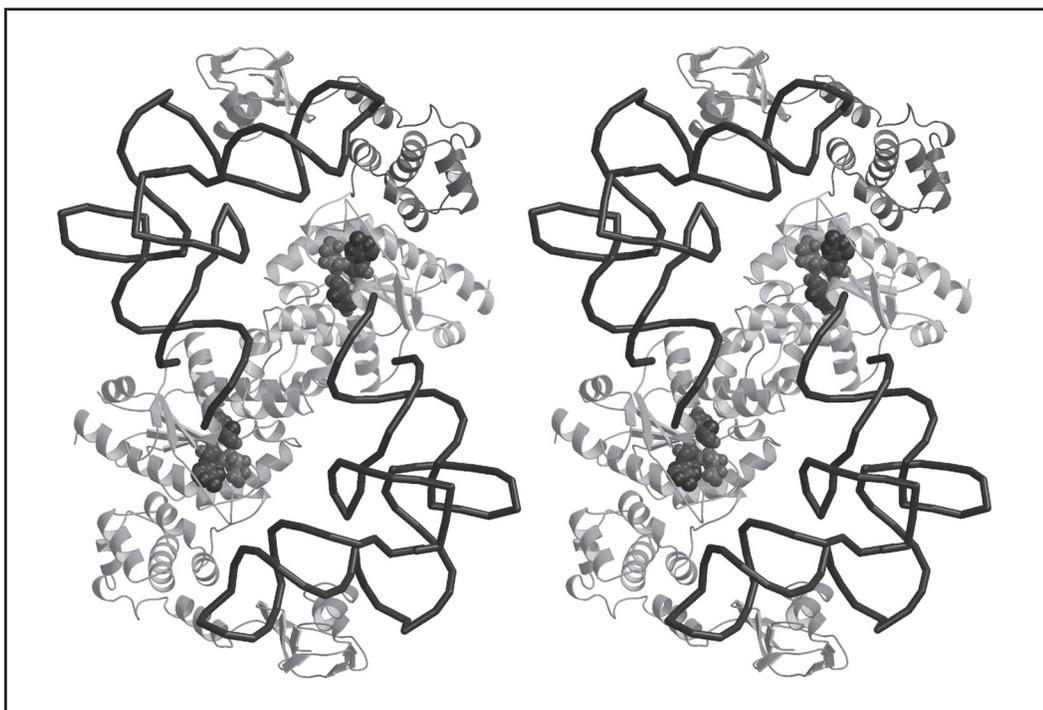


Figure 3. Stereo view of the complex between *T. thermophilus* TyrRS, tRNA^{Tyr}, tyrosinol and ATP, in the crystal structure at 2.9 Å resolution. Tyrosinol and ATP are shown in a space filling mode. Figure kindly provided by S. Cusack (see ref. 19).

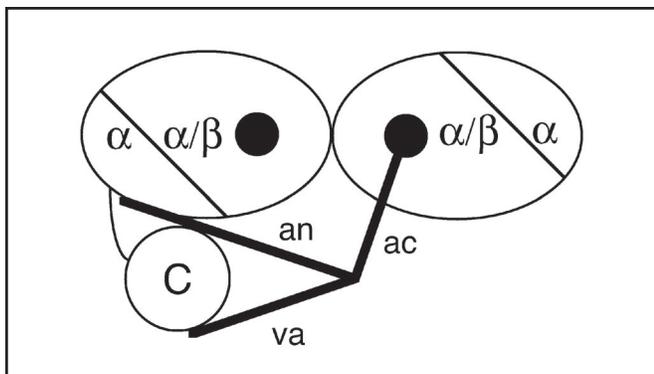


Figure 4. Schematic representation of the complex between a mutant heterodimer of TyrRS and tRNA^{Tyr}. The heterodimer is formed by the association of a full-length subunit of TyrRS and a truncated subunit, deleted for the C-terminal domain. This heterodimer contains two potential sites for the formation of Tyr-AMP, but only one site for the binding and charging of tRNA^{Tyr}. Only one molecule of Tyr-AMP is formed by each heterodimeric molecule of TyrRS, because of its half-of-the-sites reactivity. If Tyr-AMP is formed in the full-length subunit of the heterodimer, tyrosine cannot be transferred from Tyr-AMP to tRNA^{Tyr}.

of the Tth-TyrRS-tRNA^{Tyr} complex has shown that they are practically identical (at least for the interaction between the acceptor arm of tRNA^{Tyr} and the N-terminal domain of TyrRS, the only one that could be modeled in the *B. stearothermophilus* system).³⁰

Asymmetry of TyrRS in Solution

In the crystal structure of Bst-TyrRS, the two subunits are symmetrical through a two-fold rotational axis. The dimer binds two molecules of tyrosine and forms two molecules of Tyr-AMP.⁹ The dimer of Tth-TyrRS binds two molecules of tRNA^{Tyr} in the crystals of the complex.¹⁹ In solution, Bst-TyrRS shows half-of-the-sites reactivity: the enzyme binds only one molecule of tyrosine or tRNA^{Tyr}, and forms only one molecule of Tyr-AMP per molecule of dimer (for a review, see refs. 127, 32). Heterodimers between the full-length Bst-TyrRS and its N-terminal fragment were constructed in vitro (Fig. 4). Such heterodimers comprise two sites for the formation of Tyr-AMP but only one site for the binding of tRNA^{Tyr}. They can transfer tyrosine to tRNA^{Tyr} only when Tyr-AMP is formed at the truncated subunit. It was observed that they charge tRNA^{Tyr} at approximately half the rate of the parental Bst-TyrRS in the steady state. The following problem was therefore pinpointed. The stability of the enzyme bound Tyr-AMP combined with half-of-the-sites activity could lead to all of the enzyme accumulating as an inactive complex with Tyr-AMP bound at the full length subunit. Since the observed rate of charging does not decrease with time, there must be a mechanism for preventing random activation at one or the other active site, or selectively removing Tyr-AMP from the full-length subunit.²⁵ The symmetry of Bst-TyrRS in solution was investigated by introducing different mutations in the full-length and in the truncated subunits of heterodimers. The results have shown that each dimer is active at only one site in solution, but that the site used is randomly distributed between the two subunits. No detectable interconversion is found between active and inactive sites over time. The wild type enzyme is thus inherently assymetrical in solution, even in the absence of substrate.^{32,33}

Kinetics for the Formation of Tyr-AMP

The synthesis of Tyr-AMP from tyrosine and ATP, and then the transfer of tyrosine from Tyr-AMP to tRNA^{Tyr} have been studied in great detail for Bst-TyrRS. For such studies, Bst-TyrRS mutants were constructed and their properties were compared to those of the wild-type synthetase in experiments of both steady-state and presteady state kinetics. The data on the synthesis of Tyr-AMP have been reviewed extensively.³⁴⁻³⁶ The strategy has mainly consisted in mutating residues of Bst-TyrRS that contact Tyr-AMP in the crystal structure of the complex.⁹ The lack of structure for the TyrRS-ATP and TyrRS-Tyr-AMP-PP_i complexes, and the lack of well defined bonds between Bst-TyrRS and the adenine ring of Tyr-AMP at first hampered the elucidation of the mechanism for Tyr-AMP synthesis.³⁷ However, two types of analyses made further progress possible.

1) Chemical studies have shown that the formation of Tyr-AMP from tyrosine and ATP results in the inversion of the α -phosphorus, that no covalent enzyme-bound intermediate occurs and that the mechanism involves an "in-line" displacement, with the tyrosyl carboxylate acting as a nucleophile and the pyrophosphate being the leaving group. Such a reaction scheme implies a penta-coordinated transition state.³⁸ By using this chemical information, Leatherbarrow and coworkers constructed a model of the penta-coordinated intermediate between ATP and tyrosine.³⁹ This model suggested that Thr40 and His45 are involved in the binding of ATP. Residue His45 belongs to a sequence motif, HIGH, which is conserved among the class I aaRSs.^{40,41}

2) A systematic mutagenesis of the positively charged residues of Bst-TyrRS has shown that the changes of four residues in its N-terminal domain, Lys82, Arg86, Lys230 and Lys233, strongly affect the rates of Tyr-AMP synthesis and pyrophosphate exchange.²⁵ This result was unexpected because these four residues are far from Tyr-AMP in the crystal structure of Bst-TyrRS. Two of these residues, Lys230 and Lys233, belong to a pentapeptide which is conserved among the sequences of the class I aaRSs.^{41,42} The mutagenesis results have thus established the role for this motif, KMSKS, in the reaction of amino-acid activation.

Detailed kinetic studies have subsequently shown the role of residues Thr40, His45, Lys82, Arg86, Lys230, Lys233 and Thr234 in the binding of the β - and γ -phosphates of ATP, and in the binding of pyrophosphate at different steps in the formation of Tyr-AMP (see Chapter 30).³⁶

Kinetics for the Transfer of Tyrosine

Detailed kinetic studies have been performed on the transfer of tyrosine from the Tyr-AMP intermediate to the 3'-end of tRNA^{Tyr} by Bst-TyrRS. Four species of tRNA^{Tyr} were assayed as substrates for Bst-TyrRS and found to be equivalent in the in vitro charging reaction: native *E. coli* tRNA^{Tyr}, native *B. stearothermophilus* tRNA^{Tyr}, *B. stearothermophilus* tRNA^{Tyr} expressed in *E. coli*, and *B. stearothermophilus* tRNA^{Tyr} transcribed in vitro. Therefore, the modifications of tRNA^{Tyr} are not essential for its recognition by Bst-TyrRS in vitro.⁴³

The free energies of two molecular species, coming after the intermediate TyrRS-Tyr-AMP in the reaction pathway of Bst-TyrRS, have been determined by experiments of presteady state kinetics: those of the initial complex TyrRS-Tyr-AMP-tRNA^{Tyr} and of the transition state complex TyrRS-[Tyr-tRNA^{Tyr}-AMP][‡]. These determinations have shown that the activation energies of the two chemical steps in the reaction pathway, (a) the activation of tyrosine and (b) its transfer to tRNA, are of equal intensity: 15.4 kcal·mol⁻¹. Therefore,

the optimization of the global catalysis by TyrRS during evolution has probably involved a selective pressure on both steps.⁴³ To test this assumption, the rate constants for the transfer of tyrosine from Tyr-AMP to tRNA^{Tyr} by Bst-TyrRS mutants were measured. Mutations of Thr51 were chosen because this residue contributes to the rate constant for the activation of tyrosine, and to the binding of Tyr-AMP. The rate constant for the transfer of tyrosine decreased when the stability of the TyrRS·Tyr-AMP complex increased, due to mutations of Thr51. The stabilization of the TyrRS·Tyr-AMP complex by a mutation of Thr51 therefore accelerated the activation step but slowed down the transfer step of the aminoacylation reaction, consistent with the tested assumption.⁴⁴

The role of the conserved HIGH sequence in the catalysis of the transfer step was analyzed using the H45A and H48A mutants of Bst-TyrRS. This analysis has shown that the HIGH sequence is involved in the activation step but not in the transfer step, i. e. the kinetic parameters for the transfer of tyrosine to tRNA^{Tyr} are not affected by the mutations. In contrast, residue Thr40 which is present in a subset of the class I aaRSs, and Lys82 and Arg86 which are present only in TyrRSs, stabilize the transition states for both reaction steps.⁴⁵ The role of the conserved KMSKS sequence in the catalysis of the transfer step was analyzed similarly. Although Lys230 and Lys233 of Bst-TyrRS are involved in the initial binding of tRNA^{Tyr} and act synergistically, none of the residues in the KMSKS sequence affect the rate constant of the transfer step.⁴⁶ From an evolutionary viewpoint, the results on the HIGH and KMSKS motifs of Bst-TyrRS have suggested that the stabilization of the transition state for the first step of the aminoacylation reaction has preceded that for the second step. They are consistent with the proposal that the capacity of the aaRSs to catalyse the activation of the amino acid has preceded their capacity to attach the amino acids to the 3'-OH end of tRNAs.⁴⁵

During the construction of the structural model for the complex between Bst-TyrRS and tRNA^{Tyr}, rotations around the bonds which bracket phosphates-74, -75 and -76 were allowed, to bring the 2'-OH and 3'-OH of adenosine 76 (Ade76) in position to attack the carbonyl carbon of Tyr-AMP. In this model, Ade76 is located in the vicinity of residues Thr40, Lys82 and Arg86.²⁶ The kinetic data on mutations T40A, K82A and R86A are consistent with such a model and suggest that residues Thr40 and Lys82 each form one hydrogen bond (H-bond) with Ade76, whereas Arg86 forms two H-bonds with it (Table 1). Mutations T40A, K82A and R86A have a small effect on the initial binding of tRNA^{Tyr} (0.5 to 1.0 kcal·mol⁻¹) and a much larger effect on the stability of the transition state (1.7-3.5 kcal·mol⁻¹). These data suggest that a conformational change could take place during the formation of the transition state for the transfer reaction, and that the main role of Thr40, Lys82 and Arg86 could be to position Ade76 of tRNA^{Tyr} correctly for an attack on Tyr-AMP.⁴⁵

Residues Thr40, Lys82 and Arg86 have similar roles in the first and second steps of the aminoacylation reaction. They contribute little binding energy to the formation of the initial state complex TyrRS·Tyr-ATP, and a more important energy to the stabilisation of the transition state complex TyrRS·[Tyr-ATP][‡]. The three residues contribute little energy to the interaction with Ade76 of tRNA^{Tyr} during the formation of the initial state complex TyrRS·Tyr-AMP·tRNA^{Tyr} and an important energy to the stabilization of the transition state complex TyrRS·[Tyr-tRNA^{Tyr}-AMP][‡]. The three residues interact with the β- and γ-phosphates of ATP in the first step, and probably with

Table 1. Interaction energies of side chains of Bst-TyrRS with tRNA^{Tyr}

Residue	Interaction Energies of Side Chains in Complex with: ^a	
	Tyr-AMP · tRNA	[Tyr-tRNA-AMP] [‡]
Thr40	+	+++
Lys82	+	+++
Arg86	+	++++
Gln173	0	++++
Lys230	++	++
Lys233	+	+
Lys230 + Lys233	++	++

a. Apparent stabilization energy from the side chain. These energies are calculated when going from the TyrRS·Tyr-AMP complex to the indicated complex.⁴⁵⁻⁴⁷ Notations, in kcal·mol⁻¹: 0= -0.5 to +0.5; + = 0.5 to 1.0; ++ = 1.0 to 1.5; +++ = 1.5 to 2.0; ++++ = > 2.0.

the ribose portion of Ade76 in the second step. Globally, the data are compatible with a catalytic mechanism in which TyrRS catalyses the activation of tyrosine and its transfer to tRNA^{Tyr} by bringing together the substrates in the right orientation for the reaction to occur, and by utilizing the binding energy provided by noncovalent interactions to preferentially stabilize the transition states.^{34,45}

Several catalytic mechanisms have been evaluated for the transfer reaction. Residue Gln195 forms a H-bond with the carbonyl oxygen of Tyr-AMP and Gln173 forms a H-bond with the protonated ammonium group of Tyr-AMP in the crystal structure of the Bst-TyrRS·Tyr-AMP complex. Thr40, Lys82 and Arg86 probably interact with Ade76 during the second step of the reaction. Kinetic experiments in the presteady state have shown that mutation Q195A has no effect on the binding or rate constants for the transfer reaction. Mutations of the four other residues, Thr40, Lys82, Arg86 and Gln173, into Ala affect little or not the formation of the initial complex TyrRS·Tyr-AMP·tRNA^{Tyr} and strongly destabilize the transition state complex TyrRS·[Tyr-tRNA^{Tyr}-AMP][‡]. Thus, the stabilization of the transition state by TyrRS involves side chains (Thr40, Lys82 and Arg86) which are important for the formation of the new bond between tyrosine and Ade76, and a side chain (Gln173) which is probably important for the cleavage of the acyl-phosphate bond. On the basis of these data, First and coworkers have suggested that the transfer reaction occurs through a concerted mechanism in which the cleavable acyl-phosphate bond is elongated and strained, and the bond between the carbonyl carbon and the 3'-OH or 2'-OH of ribose 76 is partially formed.⁴⁷ The role of various residues in the transfer reaction is summarized in (Table 1.)

Identity Elements of tRNA^{Tyr}

Eubacteria. The identity elements of tRNA^{Tyr} have been analyzed systematically in eubacterial systems, in particular by transplantation into a noncognate tRNA. The in vitro properties of chemically modified *E. coli* tRNA^{Tyr} molecules (Eco-tRNA^{Tyr}) and the in vivo properties of mutant derivatives of the amber suppressor Eco-tRNA^{Tyr}(CUA) have provided a first delineation of the

Table 2. Effects of mutations in the identity elements of Eco-tRNA^{Tyr} on charging by Eco-TyrRS^a

Mutation	$K_M(\text{tRNA}^{\text{Tyr}})$	k_{cat}	k_{cat}/K_M
Native	0.75	1.03	1.40
wt (transcript)	1.00	1.00	1.00
G34C	1.64	0.068	0.043
U35G	15.6	0.076	0.0048
$\Delta(\text{U46, U47})^b$	10.0	0.31	0.031
$\Sigma(\text{A44.1, A44.2})^b$	na ^c	na	0.0031
A73G	2.84	0.076	0.027
A73C	3.44	0.45	0.13
A73U	4.06	0.26	0.064

a. Relative values.^{51,52} The double mutation (G1C, C72G) also decreases the rate of aminoacylation.⁵³

b. Δ , deletion; Σ , insertion.

c. na, not available.

Table 3. Comparison of the regions in contact with tRNA^{Tyr} in the model structure for Bst-TyrRS and in the crystal structure for Tth-TyrRS^{19,26,29,59}

TyrRS Region	Bst-TyrRS	Tth-TyrRS	tRNA Region
N-terminus	T17	V23	Ribose of Gua1
Cluster 1	N146, K151, E152	148-154	Acceptor stem
Cluster 2	W196, R207, K208	198-211	Acceptor stem
Cluster 3	R368, R371	371-393	Variable arm
Cluster 4	R407, R408, K410, K411	420-423	Anticodon stem

nucleotide residues through which Eco-tRNA^{Tyr} is recognized by Eco-TyrRS.^{31,48,49} A long variable stem and loop may not be absolutely required for recognition by Eco-TyrRS since a mutant Eco-tRNA^{Cys}, carrying the native Gua1:Cyt72 base pair, the amber anticodon CUA, and mutation U73A, inserts tyrosine into dihydrofolate reductase in vivo.⁵⁰ The equivalence of native and in vitro transcribed Eco-tRNA^{Tyr} molecules for in vitro tyrosylation by Eco-TyrRS has been established. Steady-state kinetics for the charging of Eco-tRNA^{Tyr} variants by Eco-TyrRS in vitro have shown that the recognition elements of Eco-tRNA^{Tyr} include Gua34, Uri35, the orientation of the long variable loop (dependent on the unpaired residues Uri46 and Uri47), and Ade73. Base pair Gua1:Cyt72 is recognized with a weak preference (Table 2).⁵¹⁻⁵³

Archaeobacteria. Mutagenesis and transplantation experiments have shown that the identity elements of *Methanococcus janaschii* tRNA^{Tyr} (Mja-tRNA^{Tyr}) comprise Ade73 as the most important element, base pair Cyt1:Gua72, and the anticodon with mainly Gua74. This set of identity elements is complete.^{54,55}

Saccharomyces cerevisiae. The native and the in-vitro transcribed cytoplasmic *S. cerevisiae* tRNA^{Tyr} (Sce(cyt)-tRNA^{Tyr}) have similar kinetics of tyrosylation by Sce(cyt)-TyrRS in vitro. Mutagenesis and transplantation experiments have shown that the identity elements of Sce(cyt)-tRNA^{Tyr} comprise Ade73, the base pair

Table 4. Apparent destabilization of the initial complex ($\Delta\Delta G_S$) and of the transition state complex ($\Delta\Delta G_T$) for the charging of Eco-tRNA^{Tyr} by Bst-TyrRS^a

Mutation	$\Delta\Delta G_S$	$\Delta\Delta G_T$
T17A	-	0
N146A	++++	0
K151N ^b	0	++++
E152A ^c	-	-
W196A ^d	++	+++
R207Q	na ^g	+++
K208N	na	+++
F323A ^e	++	++++
R368Q ^f	na	++++
R371Q	na	+++
R407Q	na	+++
R408Q	na	+++
K410N	na	+++
K411N	na	+++

a. The values are deduced from steady state kinetics.^{25-27,29} Notations, in kcal·mol⁻¹: - = -1.0 to -0.5; 0 = -0.5 to +0.5; + = 0.5 to 1.0; ++ = 1.0 to 1.5; +++ = 1.5 to 2.0; ++++ = > 2.0.

b. Mutations K151A and K151Q suggested that K151N induces a conformational change.²⁶

c. Mutations E152D and E152Q had similar effects to those of E152A.²⁷

d. Mutation W196Q had a mild effect and W196F had no effect.²⁶

e. Mutations F323L, -Y and -W showed that the hydrophobic character of Phe323 was essential.²⁹

f. Mutation S356A in TyrRS from *Acidithiobacillus ferrooxidans* (equivalent to S366A in Bst-TyrRS) induces a variation $\Delta\Delta G_S = 1.2$ kcal·mol⁻¹ (ref. 134).

g. na, not available.

Cyt1:Gua72, and the anticodon with mainly Gua34. This set is complete.⁵⁶ The change of pseudo-Uri35 into several modified pyrimidines has shown that the two N-H groups of this residue are required for an optimal interaction with Sce(cyt)-TyrRS.⁵⁷

Recognition of tRNA^{Tyr} and Its Identity Elements by TyrRS

The main regions of Bst-TyrRS and Tth-TyrRS which contact their cognate tRNA^{Tyr} are summarized in (Table 3). For the *B. stearothermophilus* system, these contacts have been deduced from mutagenesis experiments and structure modelings.^{25-27,29} For the *T. thermophilus* system, they have been deduced from the crystal structure of the complex.¹⁹ There is an excellent agreement between the two complementary approaches. The regions of contact include the four clusters of basic residues which have been found by mutagenesis studies.^{25,26,58} The contributions of the contact residues to the stabilities of the initial and transition state complexes have been evaluated from the kinetic parameters for the tyrosylation of tRNA^{Tyr} by Bst-TyrRS mutants, in steady state experiments (Table 4).

The interactions and the mechanism by which TyrRS recognizes the identity elements of tRNA^{Tyr} have been deduced from the above data and the tyrosylation of tRNA^{Tyr} variants by TyrRS

Table 5. Recognition of the identity nucleotides of tRNA^{Tyr} by Bst-TyrRS and Tth-TyrRS^{19,26,29}

Nucleotide	Group	Bst-TyrRS	Tth-TyrRS
A73	N ₆ -H ₂	Ala150, mcC=O ^a	Glu154, mcC=O
A73	N ₁	Lys151	
A73	C ₂ -H	Trp196, C _{δ1} -H	
A73	N ₃	Trp196, C _{δ1} -H	Arg198
G1	base	Trp196, packing	Leu202, packing
G1	N ₃		Arg198
C72	N ₄		Glu154
C71	N ₄		Glu154
G34	N ₁ , N ₂		Asp259
Psi35	N ₃		Asp423
Psi35	N ₁		Tyr342, mcC=O
Psi35	base	Phe323, aromatic ^b	Tyr342, aromatic

a. mc, main-chain.

b. Although Phe323 in Bst-TyrRS aligns with Ala346 in Tth-TyrRS, it could play a role similar to that of Tyr342 in Tth-TyrRS.

in the *E. coli* system, in steady state kinetics experiments (Table 5). In particular, the recognition of the discriminator base Ade73 involves residues of the connective peptide CP1, and the recognition of the anticodon involves a residue located in the peptide linking the α -helical and C-terminal domains. Comparison of the kinetic parameters K_M and k_{cat}/K_M for the charging of Eco-tRNA^{Tyr} variants has suggested that the identity elements form stronger interactions with Eco-TyrRS in the complex of the transition state TyrRS-[Tyr-tRNA^{Tyr}-AMP][‡] than in the complex of the initial state TyrRS-Tyr-AMP-tRNA^{Tyr}. Their recognition by TyrRS would thus stabilize the transition state for the transfer reaction and decrease the corresponding activation energy (Table 2). Conversely, the kinetic parameters for the charging of tRNA^{Tyr} by Bst-TyrRS variants leads to the same conclusions. For example, mutation K151N of Bst-TyrRS does not affect K_M (tRNA^{Tyr}) but decreases strongly k_{cat}/K_M for the charging reaction. Similarly, mutation W196A affects more k_{cat}/K_M than K_M (tRNA^{Tyr}).^{25,26} The mutations of the discriminator nucleotide Ade73 of Eco-tRNA^{Tyr} affect more k_{cat}/K_M than K_M (tRNA^{Tyr}) for the aminoacylation by Eco-TyrRS.⁵² Thus, Ade73 appears to be fully recognized by TyrRS only in the transition state for the charging of tRNA^{Tyr} (ref. 59). These conclusions were deduced from experiments of steady state kinetics for a reaction which comprises two steps.⁶⁰ However, the two steps have the same activation energy (at least for Bst-TyrRS) and the presence of tRNA^{Tyr} does not affect the activation of tyrosine.³²

The observation that mutations of the tRNA^{Tyr} anticodon, or mutations of Bst-TyrRS residues which interact with the anticodon arm affect the k_{cat} parameter for tyrosylation, suggests that there is a transmission of information between the distal regions of either tRNA^{Tyr} or TyrRS, and either the acceptor end of the tRNA or the active site of the synthetase. This transmission could take place through either the tRNA or the synthetase, with or without a conformational change.

Residue Gua35 is a minor identity element of Mja-tRNA^{Tyr} (ref. 55). Residues Asp286 and Lys288 of Mja-TyrRS belong to a motif of the C-terminal domain, PXDLK, which is conserved in the eukaryotic and archaeobacterial TyrRSs. Mutations D286A and

K288A do not affect the tyrosylation of a minihelix^{Tyr} but they do affect the charging of a full-length tRNA^{Tyr}. Moreover, mutations K288A in Mja-TyrRS and U35G in Mja-tRNA^{Tyr} have nonadditive effects on the charging reaction.⁵⁴ These data suggest an interaction between Lys288 and the anticodon, contrary to the authors' conclusion.

Lee and RajBhandary were the first to deduce that the specific recognition of base pair Cyt1:Gua72 relative to Gua1:Cyt72 in Sce(cyt)-tRNA^{Tyr}, implies that this base pair is recognized by Sce(cyt)-TyrRS in the major groove of the helical acceptor stem.⁶¹

Species Specificity

The presence of base pair Gua1:Cyt72 in eubacteria and Cyt1-Gua72 in archaeobacteria and eukaryotes as identity elements, results in a species specificity. It was observed 30 years ago that the *S. cerevisiae* mitochondrial tRNA^{Tyr} (Sce(mit)-tRNA^{Tyr}) can be tyrosylated by Eco-TyrRS in vitro and in vivo.^{62,63} In contrast, an amber suppressor tRNA(CUA), which carries base Ade73 and base pair Gua1:Cyt72 as the eubacterial tRNA^{Tyr}, cannot be charged by Sce(cyt)-TyrRS in vivo.⁶⁴ However, it can be charged both in vivo and in vitro if it carries Cyt1-Gua72.⁶¹ Similar conclusions were obtained by comparing the charging of microhelices^{Tyr}, derived from *S. cerevisiae* or *E. coli* tRNA^{Tyr}, by Eco-TyrRS and the TyrRS from the lower eukaryote *Pneumocystis carinii*. These microhelices^{Tyr} carried either Gua1:Cyt72 or Cyt1-Gua72 in addition to Ade73. In particular, the charging of the *E. coli* minihelix^{Tyr} by Eco-TyrRS is weakly affected by the change of base pair Gua1:Cyt72 into Cyt1-Gua72.⁵³ As regards higher eukaryotes, a recombinant Hsa-TyrRS charges Hsa-tRNA^{Tyr} but not Bst-tRNA^{Tyr}, whereas the reciprocal is true for Bst-TyrRS.⁶⁵ The systematic characterization of the identity elements for tRNA^{Tyr} in the *M. jannashii* and *S. cerevisiae* systems has firmly established the molecular basis of this species specificity,^{55,56} which might be an important property for the use of TyrRS as a target for new antibiotics.

Mycobacterium tuberculosis TyrRS can charge Eco-tRNA^{Tyr} in vivo and in vitro, but does not function in the *S. cerevisiae* cytoplasm. The comparison of its sequence with those of other eubacterial and eukaryotic TyrRSs revealed that the species specificity of TyrRS towards tRNA^{Tyr} is encoded non only in the identity elements of the tRNA (Cyt1-Gua72 versus Gua1-Cyt72) but also in TyrRS.⁵⁸ For example, the replacement of a peptide of 41 residues in Eco-TyrRS (residues 129-172, corresponding to 126-166 in Bst-TyrRS) by the homologous peptide from Hsa-TyrRS (125-162) enables the charging of Sce(cyt)-tRNA^{Tyr} by Eco-TyrRS. The reciprocal result, charging of Eco-tRNA^{Tyr} by an engineered Hsa-TyrRS, is also true.⁶⁶ In Bst-TyrRS, this peptide comprises residues Asn146, Ala150, Lys151 and Glu152, which are important either for the specific recognition of tRNA^{Tyr} or for the rejection of noncognate tRNAs.^{26,28}

Sequence comparisons have shown that three residues which stabilize the transition state for the formation of Tyr-AMP by Bst-TyrRS (Cys35, His48 and Lys233) are not present in Hsa-TyrRS. Moreover, Hsa-TyrRS needs potassium ions for activity, contrary to Bst-TyrRS. Presteady state kinetics experiments have shown that the two enzymes have identical activation energies for the synthesis of Tyr-AMP, despite the differences between their active sites and their requirements for the K⁺ ion. The differences between the two enzymes could be exploited for the rational design of antibiotics (J. Austin and E. A. First, submitted).

Discrimination between tRNAs

Correct aminoacylation depends not only on identity elements in tRNAs and their recognition by their cognate aaRSs, but also on competitions between different aaRSs for a given tRNA, or different tRNAs for a given aaRS.⁶⁷ Several studies with the tyrosine system have substantiated this concept of competition.

When Eco-GlnRS is overproduced in vivo, it incorrectly acylates the amber suppressor tRNA^{Tyr}(CUA) with glutamine. This mis-aminoacylation is abolished if the intracellular concentration of the cognate Eco-tRNA^{Gln} is increased.⁶⁸ It is also abolished if Eco-TyrRS is overproduced in vivo. This last effect can be reproduced in vitro: TyrRS competes with GlnRS for tRNA^{Tyr}(CUA) charging with glutamine. These experiments have shown that the competition between the two aaRSs occurs at the aminoacylation step and suggested that it depends on their relative affinities for the tRNA.⁴⁹ Similarly, although the mutant amber suppressor Eco-tRNA^{Tyr}(G3:U70, CUA) is quantitatively aminoacylated with both alanine and tyrosine in vitro, competition between Eco-AlaRS and Eco-TyrRS prevents aminoacylation with alanine in vivo. As the concentration of the AlaRS increases, the identity of tRNA^{Tyr}(G3:U70, CUA) is switched from a tyrosine to an alanine tRNA.⁵¹

The overproduction of Eco-TyrRS or Bst-TyrRS is toxic for *E. coli* and results in the destabilization of cellular proteins. The toxicity increases with the growth temperature. The causes of this phenomenon were analyzed by varying the cellular concentrations of TyrRS and its activity of tRNA^{Tyr} charging, through genetic means. These experiments have shown that the toxicity of the overproduced TyrRS results from its interaction with tRNAs and probably from the ensuing mis-incorporation of amino acids into essential proteins. It was also shown that the balance between the cellular concentrations of the aaRSs and tRNAs is essential for the cell viability, by contributing to the precision of the translation of the genetic code.³¹ Similarly, Scc(cyt)-TyrRS is not toxic for *E. coli* when it is produced from a vector of low copy number and at low temperatures (22 °C to 30 °C). However, it becomes toxic at high cellular concentrations or growth temperatures. The toxicity is due to the charging of Eco-tRNA^{Pro} with tyrosine.⁶⁹

During genetic translation, each aaRS specifically aminoacylates its cognate tRNAs and rejects the 19 other species of tRNAs. A decrease in the specificity of this reaction can lead to mis-incorporations of amino acids into proteins and be deleterious to the cell. Residue Glu152 of Bst-TyrRS is close to phosphate groups 73 and 74 of tRNA^{Tyr} in the structural model of their complex. Eleven changes of Glu152 were created by mutagenesis to determine whether this residue contributes to the recognition of tRNA^{Tyr} and to the discrimination between tRNAs by Bst-TyrRS. The mis-aminoacylations of tRNA^{Phe} and tRNA^{Val} with tyrosine in vitro (on a scale going from 1 to 30) and the toxicity of Bst-TyrRS in vivo (on a scale going from 1 to 10⁷) increased in a correlated way when the nature of the side chain in position 152 varied from negatively charged to neutral then to positively charged. The aminoacylation of tRNA^{Tyr} was not affected by the mutations. The toxicity of the mutations was abolished by a second mutation in TyrRS, which prevents the binding of tRNA^{Tyr}. These results have shown that the role of Glu152 in the discrimination between tRNAs is purely negative and that it acts by electrostatic repulsion of the noncognate tRNAs.^{27,28} Such a role is supported by the structure of the Tth-TyrRS-tRNA^{Tyr} complex.¹⁹

Inhibitors

As TyrRS is an essential cellular protein, inhibitors could be used as antibiotics. Stabilized mimics of Tyr-AMP, such as tyrosinyl adenylate, are potent inhibitors of TyrRS but their polarity prevents their transport across the bacterial cell wall.^{6,70} Several triazine dyes inactivate Bst-TyrRS irreversibly. They are excluded from the tyrosine binding site and occupy the ATP-binding site. These dyes are not specific for TyrRS and also inactivate TrpRS and MetRS.^{71,72} Tyrosyl aryl dipeptides, which inhibit the aminoacylation activity of *Staphylococcus aureus* TyrRS (Sau-TyrRS) (IC₅₀ = 0.5 μM) have been identified. A crystal structure of Sau-TyrRS complexed with one of the inhibitors, the dipeptide Tyr-Tyr, shows occupancy of the tyrosine binding pocket and interactions of the inhibitor with key catalytic residues.⁷³

A potent inhibitor, specific for bacterial TyrRSs and designated SB-219383, has been isolated from a *Micromonospora* species. SB-219383 shows competitive inhibitory activity against Sau-TyrRS ($K_i = IC_{50} = 0.6$ nM for Sau-TyrRS; IC₅₀ = 22 μM for mammalian TyrRS) and weak anti-bacterial activity against some *Streptococcal* strains in vitro (MIC = 32 μg/ml).⁷⁴ SB-219383 can be described as a Tyr-Gly dipeptide, in which the C_α-position of the Gly moiety is derivatized with a bicyclic sugar.⁷⁵ Several derivatives of SB-219383, which retain high inhibitory activities, have been synthesized. SB-239629 (IC₅₀ = 3 nM) is a monocyclic derivative of SB-219383, obtained by cleaving its bicyclic sugar; SB-243545 (IC₅₀ = 0.3 nM) is a butyl ester derivative of SB-239629; in SB-284485 (IC₅₀ = 4 nM), the bicyclic sugar of SB219383 is replaced with fucose. The crystal structures of complexes between Sau-TyrRS and each of these four inhibitors have been solved, down to 2.2 Å resolution for some of them. The bicyclic sugar of SB-219383 and its monocyclic derivative in SB-239629 globally occupy the binding site of TyrRS for ribose. The butyl group of SB-243545 has revealed the existence of a new binding pocket in TyrRS, which involves displacements of the HIGH and KMSKS class I motifs. The fucose moiety of SB-284485 forms more H-bonds with TyrRS than the ribose moiety of Tyr-AMP. These structures have revealed the existence of five different binding sites in TyrRS (for tyrosine, α-phosphate, ribose, adenine, butyl and pyrophosphate) which could be further explored for the design of inhibitors.⁷⁶ Other pyranosyl and carbocyclic analogs of SB-219383 have been synthesized to reduce its overall polarity and thus improve its penetration through the bacterial cell wall. One of the compounds shows as high an inhibitory activity against Sau-TyrRS as SB-219383 and an improved antibacterial activity against *Moraxella catarrhalis* and *Streptococcus pyogenes* (MIC = 8 μg/ml).^{77,78}

From random libraries displayed at the surface of phage M13, peptides that bind to *Haemophilus influenzae* TyrRS (Hin-TyrRS) have been isolated. Most of these peptides are specific inhibitors of the Hin-TyrRS activity and appear to preferentially bind to the TyrRS active site. One of the corresponding synthetic peptides showed a competitive inhibition towards tyrosine ($K_i = 80$ nM) and a mixed inhibition towards ATP ($K_i = 60$ nM). Another showed noncompetitive inhibitions towards both tyrosine and ATP ($K_i = 300$ -500 nM). These two peptides were used in a binding assay to detect small inhibitory molecules, in the μM to nM range, that bind to the same sites.⁷⁹

Charging of Noncognate or Nonnatural Amino Acids

The mechanism by which Bst-TyrRS specifically recognizes tyrosine was studied by mutagenesis of residues close to the active site, according to the crystal structures. The carboxylate of Asp176 makes a hydrogen bond with the hydroxyl group of tyrosine.⁹ Mutation of Asp176 results in an inactive enzyme. Asn123 and Trp126 do not interact directly with tyrosine but appear to make H-bonds with Asp176. Mutations N123A and N123D strongly affect the kinetics of tyrosine activation, and in particular k_{cat} and $K_M(\text{Tyr})$. In contrast, W126F and W126L do not affect these kinetics with respect to ATP, and modestly increase $K_M(\text{Tyr})$. The specificity for Tyr against Phe, determined from the ratio k_{cat}/K_M in the pyrophosphate exchange reaction ($1.2 \cdot 10^5$ for the wild type TyrRS), decreases 4 fold for N123A but increases 2 fold for W126L and 7 fold for W126F. Thus, the wild type enzyme can be improved for discrimination between Tyr and Phe.⁸⁰

An advantage of TyrRS, when looking for the charging of nonnatural amino acids, is that it does not possess an editing mechanism. The tyrosine analog azatyrosine, L- β -(5-hydroxy-2-pyridyl)-alanine, can convert the Ras-transformed phenotype to normal phenotype, presumably by its incorporation into cellular proteins in place of tyrosine and its inability of being phosphorylated. To understand better this abnormal incorporation, Eco-TyrRS mutants, capable of charging tRNA^{Tyr} with azatyrosine, were isolated. A library of mutant Eco-*tyrS* genes was constructed by error prone PCR. Mutant clones (about 1400) were screened for the incorporation of L-[³H]tyrosine or L-[³H]azatyrosine into trichloro-acetic acid precipitable materials. One mutant, carrying the F130S change, showed a 17 fold higher activity for azatyrosine incorporation than the wild-type Eco-TyrRS. According to the Bst-TyrRS structure, Phe130 interacts with Asp182, which receives a H-bond from the hydroxyl of the bound Tyr-AMP (the equivalents of Phe130 and Asp182 in Eco-TyrRS are Ile127 and Asp176 respectively in Bst-TyrRS). The discrimination between tyrosine and azatyrosine, measured in vitro by k_{cat}/K_M for the charging of crude *E. coli* tRNA, decreased from 36 to 19 when going from the wild-type to the F130S mutant.⁸¹

In yeast, TyrRS is the aaRS which has the highest discrimination factor between the cognate and noncognate amino acids.⁸² Sce(cyt)-TyrRS mutants, capable of charging noncanonical amino acids, have been constructed by site-specific mutagenesis of putative active site residues, identified by analogy with Bst-TyrRS. The mutant Sce(cyt)-TyrRS(Y43G), equivalent to Bst-TyrRS(Y34G), was able to utilize 3-substituted tyrosine analogs as substrates for aminoacylation. The catalytic efficiency k_{cat}/K_M of Sce(cyt)-TyrRS(Y43G) for aminoacylation with tyrosine was decreased 400 fold as compared to the wild-type. The ability to utilize 3-iodo-L-Tyr was newly generated in this mutant. The mutant TyrRS could serve for site-specific incorporation of new amino acids into proteins.⁸³ In Bst-TyrRS, Tyr34 is a donor of a H-bond to the Oⁿ atom of tyrosine.

The TyrRSs from *E. coli*, *B. subtilis* and *S. cerevisiae* cytoplasm can charge their homologous tRNA^{Tyr} with D-tyrosine.^{70,84,85} The resulting D-Tyr-tRNA^{Tyr} is hydrolyzed by a D-Tyr-tRNA^{Tyr}-deacylase, which has been identified and characterized in *E. coli* and *S. cerevisiae*.^{85,86} In both organisms, the specificity of the deacylase is not restricted to tRNA^{Tyr}. In the absence of deacylase, some D-amino acids are toxic to the organism.⁸⁷ Thus, although TyrRS does not possess an incorporated editing mechanism, the deacylase provides one for some D-amino acids.

Expanding the Genetic Code

An expansion of the genetic code must satisfy three conditions:

1. An aaRS N° 21, which specifically charges a tRNA N° 21 to the exclusion of the 20 homologous tRNAs, must be introduced into an organism.
2. A tRNA N° 21, which is specifically charged by aaRS N° 21 to the exclusion of the 20 homologous aaRSs, and which uses a codon differing from the existing codons, must be introduced into the same organism.
3. aaRS N° 21 must specifically recognize, activate and transfer an amino acid N° 21, to the exclusion of the 20 other amino acids.

RajBhandary and coworkers have constructed two couples of aaRS and tRNA which satisfy conditions 1 and 2. One of them is based on Sce(cyt)-TyrRS and an amber suppressor tRNA(CUA), which carries the identity elements Cyt1-Gua72 and Ade73 of the eukaryotic tRNA^{Tyr}. As described above, plasmids expressing high levels of Sce(cyt)-TyrRS cannot be stably maintained in *E. coli*, because they mischarge Eco-tRNA^{Pro}, which also comprises Cyt1-Gua72 and Ade73. The *Sce-tyrS1* gene, coding for Sce(cyt)-TyrRS, was mutagenised by error prone PCR and three mutants were isolated which could be stably expressed in *E. coli*. The Sce(cyt)-TyrRS mutants quantitatively aminoacylate the tRNA(CUA) in vivo, and show a better discrimination in vitro for the tRNA(CUA) and against Eco-tRNA^{Pro} (ref. 69).

Schultz and coworkers have built a couple which satisfies the three conditions above and is based on Mja-TyrRS and Mja-tRNA^{Tyr}. Mja-TyrRS efficiently aminoacylates an amber suppressor Mja-tRNA^{Tyr}(CUA), but does not aminoacylate any *E. coli* tRNA.^{54,88} The recognition of Mja-tRNA^{Tyr}(CUA) by the *E. coli* aaRSs, which is low, was further decreased by the following means. A library of Mja-tRNA^{Tyr}(CUA) mutants was constructed then panned through a negative selection (absence of aminoacylation by the *E. coli* aaRSs; no barnase activity) then a positive selection (aminoacylation by Mja-TyrRS, β -lactamase activity) to select a Mja-tRNA^{Tyr}(CUA, mut) variant.⁸⁹ To alter the amino acid specificity of Mja-TyrRS, five residues which are located in the vicinity of the C ξ -atom of tyrosine, chosen from the crystal structure of the Bst-TyrRS-Tyr-AMP complex, were first changed into Ala and then randomized to create a library of Mja-TyrRS mutants. The mutants of this library which were able to suppress a nonsense mutation in a nonessential position of the chloramphenicol acetyl transferase gene, in the presence of O-methyl-L-tyrosine but not in its absence, were selected. As a result, a mutant derivative of Mja-TyrRS could incorporate O-methyl-L-tyrosine into proteins by translation of an amber codon, with a fidelity higher than 99 % (ref. 90).

Binding and Charging of tRNA^{Tyr} Mimics

Sce(cyt)-TyrRS charges the viral RNA of the Brome Mosaic Virus (BMV), or recombinant derivatives of this RNA, with tyrosine. The 3'-OH end of the viral RNA folds into a structure which contains a pseudo-knot and partially mimicks tRNA^{Tyr} (for a review, see ref. 91). Its aminoacylation depends on nucleotide Ade4 (structural homolog of Ade73 in tRNA^{Tyr}) and base pair Cyt116-Gua5 (homolog of Cyt1-Gua72). There is no equivalent of the tRNA^{Tyr} anticodon. Chemical attack experiments, performed on a transcript of 196 nucleotides which can be charged with tyrosine, have indicated that the amino acid acceptor branch of the viral RNA is protected by Sce(cyt)-TyrRS against cleavage by iodine, as well as a hairpin domain which might be located

perpendicular to the acceptor branch. This domain, which has no canonical anticodon loop or tyrosine anticodon, could act as an anchor for interaction with TyrRS, leading to a better efficiency of charging.⁹² Whether or not this hairpin domain makes specific interactions with the synthetase remains unknown.

The mitochondrial TyrRSs from *Neurospora crassa*, and from *Podospora anserina*, Ncr(mit)-TyrRS and Pan-(mit)-TyrRS respectively, function in the aminoacylation of the cognate mitochondrial tRNA^{Tyr} and in the splicing of the group I introns. The splicing activity of the mitochondrial TyrRS seems limited to these two particular organisms. Ncr(mit)-TyrRS is encoded by the *cyr-18* gene. It binds to the catalytic core of the group I introns and assists the intron RNA in forming a catalytically active structure. Ncr(mit)-TyrRS splices different group I introns which have little sequence conservation. This sequence comparison has suggested that Ncr(mit)-TyrRS probably recognizes conserved features of secondary and tertiary structure in the intron RNAs. Experiments of chemical attack of the intron RNAs and of Ncr(mit)-tRNA^{Tyr}, and molecular modeling studies have suggested that Ncr(mit)-TyrRS recognizes a tRNA-like structure of the catalytic core of the group I introns (reviewed in refs. 93; 94).

Comparison of the sequences of Ncr(mit)-TyrRS, Pan(mit)-TyrRS, and other bacterial TyrRSs has shown that the two mitochondrial TyrRSs comprise the four clusters of positively charged residues which are involved in the recognition of tRNA^{Tyr} (Table 3). Other regions are conserved between Ncr(mit)-TyrRS and Pan(mit)-TyrRS but absent from the other bacterial or mitochondrial TyrRSs. The construction of mutations in Ncr(mit)-TyrRS has helped to further establish its similar modes of interaction with the intron RNAs and tRNA^{Tyr}, and to characterize the role of its different regions in splicing.⁹⁵⁻⁹⁷ An N-terminal region (residues 41-59), which is absent from the eubacterial TyrRSs and predicted to form an amphipatic α -helix, is required for the splicing activity. It appears to act indirectly, by stabilizing the structure of another TyrRS region which is in direct contact with the intron RNA. The properties of insertion mutations have shown that the aminoacylation activity is not required for the splicing activity. Mutations of the N-terminal extension or of the C-terminal domain have shown that Ncr(mit)-TyrRS favors splicing by different sets of interactions with different group I introns. Thus different functional modes could have evolved from an interaction based on the recognition of a tRNA-like structure.

Eukaryotic TyrRSs and Their Cellular Localization

Several eukaryotic TyrRSs have been studied: from human origin,⁶⁵ bovine liver,⁹⁸ rabbit,⁹⁹ mouse liver,¹⁰⁰ wheat germ,¹⁰¹ *S. cerevisiae*,¹⁰² *P. carinii*,⁵³ etc. The dimeric state of the TyrRSs from bovine liver,^{98,103} wheat germ,¹⁰¹ and yeast^{104,105} has been directly established.

Eco-TyrRS, when fused with a mitochondrial import signal, is able to restore respiration of a strain which is defective for this function because of a mutation in Sce(mit)-TyrRS.⁶³ The essential character of Sce(cyt)-TyrRS was shown by gene disruption in a diploid strain.¹⁰⁶ The essential character of the Hsa-TyrRS is suggested by the observation that the sera of patients with auto-immune diseases (rheumatoid arthritis and systemic lupus erythematosus) contains antibodies against TyrRS (and other aaRSs), contrary to the sera of healthy patients. The sera of the patients who are ill also contain anti-idiotypic antibodies of IgG type against the auto-antibodies.¹⁰⁷

The tRNAs are synthesized in the nucleus and then exported to the cytosol where they are aminoacylated and play their function in translation. In *S. cerevisiae*, there is a pool of nuclear TyrRS whose import depends on a nuclear localization sequence. The inactivation of this sequence by mutation does not affect the catalytic activity of TyrRS but results in a reduction of its nuclear pool, causes a defect in the export of tRNAs to the cytosol, and results in the nuclear accumulation of tRNA^{Tyr}, tRNA^{Met} and tRNA^{Ala}. The inactivation of TyrRS by a thermosensitive mutation results in the accumulation of tRNA^{Tyr}, tRNA^{Met} and tRNA^{Ile} in the nucleus, at the nonpermissive temperature. Thus, some tRNAs could be exported from the nucleus to the cytosol through an aminoacylation dependent pathway.^{108,109}

Other Properties and Functions of TyrRS

The rabbit liver TyrRS has a TyrRS kinase activity, as the homologous ThrRS has a ThrRS kinase activity.¹⁰⁰ The C-terminal domain of the human Hsa-TyrRS is 50% identical to the C-terminal domain of MetRS from *C. elegans*, at the level of the amino acid sequence, 49% identical to the EMAP II protein, and 43% identical to the Arcp1 protein from *S. cerevisiae*. These comparisons suggested that the C-terminal domain of Hsa-TyrRS could have a cytokine activity and direct the tRNAs to the active site of the enzyme.⁶⁵ It was later shown that Hsa-TyrRS can be split into two distinct cytokines. In cell culture under apoptotic conditions, the full length Hsa-TyrRS is secreted and cleaved by an extracellular protease into an N-terminal fragment which is catalytically active for tRNA charging, and a C-terminal fragment. The N-terminal fragment is an interleukin-8 (IL8)-like cytokine, and the C-terminal fragment is an EMAP II-like cytokine. The IL8 activity of the N-terminal fragment depends on a Glu-Leu-Arg motif, which is found in α -chemokines, and is conserved in the TyrRSs from mammals but not from lower eukaryotes. A synthetic heptapeptide, whose sequence is present in the C-terminal domain, has EMAP II-like activity for mononuclear phagocytes and polymorphonuclear leucocytes, but not the homologous peptides from lower eukaryotes. Therefore, the cytokine activities of the split Hsa-TyrRS depend on motifs that are idiosyncratic to the mammalian systems.^{110,111}

The yeast nuclear mutation *mgm104-1* leads to slow growth on glucose medium and temperature sensitive loss of mitochondrial DNA. The *tyrS1* nuclear gene, coding for Sce(cyt)-TyrRS, can complement the *mgm104-1* mutation for these phenotypes when present in two or more copies within the cells. The *tyrS1* and *mgm104* genes are different since *tyrS1* has no mutation in the *mgm104-1* mutant allele. These data suggest that *tyrS1* has an additional function, which is directly or indirectly involved in the maintenance of the mitochondrial genome.¹⁰⁶ Sce(cyt)-TyrRS strongly interacts with the Knr4 protein of *S. cerevisiae*, as demonstrated by a genetic two-hybrid system and a biochemical pull-down experiment using a GST-TyrRS fusion protein. The Knr4 protein is involved in the regulation of the cell wall assembly in *S. cerevisiae*. The efficiency of spore formation is drastically reduced in diploid cells, homozygous for a temperature sensitive mutation of the *tyrS1* gene or a disruption of the *knr4* gene. The physical interaction between the two corresponding proteins might be required for di-tyrosine formation during the sporulation process.¹¹²

Bst-TyrRS has been used in hemisynthesis. For example, tyrosine and leucinamide are condensed by TyrRS in the presence of ATP to give tyrosyl-leucinamide, L-Tyr-L-Leu-NH₂. TyrRS has no strict specificity for the amino acid derivative used as a

substrate and even D-amino acids can be incorporated into peptides in this enzymatic reaction.¹¹³ It has thus been possible to synthesize an analgesic neuropeptide, called kyotorphin, H-Tyr-Arg-OH, from tyrosine and arginine. Radioisotope-labeled oligopeptides could be synthesized by this type of reaction and used in receptor binding assays.¹¹⁴

TyrRS and the Classification of 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.¹¹⁵ In the *E. coli* system, Fraser and Rich¹¹⁶ have found that the primary site of aminoacylation of tRNA^{Tyr} by TyrRS is located at the 2'-OH rather than the 3'-OH of the ribose (85% vs 15% of the molecules respectively), as the majority of the class I aaRSs. Sprinzl and Cramer¹¹⁷ have found that both 2'-OH and 3'-OH can be aminoacylated (63% vs 37%). Note that both the 2'-OH and 3'-OH of Ade76 are in proximity of the carbonyl carbon of Tyr-AMP in the model of the Bst-TyrRS-tRNA^{Tyr} complex.²⁶ In the *S. cerevisiae* system, tRNA^{Tyr} can be quantitatively charged both at the 2'-OH and 3'-OH of the Ade76 ribose. The kinetic parameter $K_M(\text{tRNA}^{\text{Tyr}})$ of the aminoacylation reaction is the same for tRNA^{Tyr}-C-C-2'dA, tRNA^{Tyr}-C-C-3'dA, and tRNA^{Tyr}-C-C-A. However, V_{max} is about 15 times slower for tRNA^{Tyr}-C-C-2'dA than for the two other tRNA^{Tyr} species. Thus, tRNA^{Tyr} is aminoacylated preferentially at the 2'-OH group through a kinetic effect.¹¹⁸ The fact that V_{max} but not K_M is affected by the presence of 2'-deoxy-Ade76 is consistent with the conclusion that Ade76 is recognized by Bst-TyrRS mainly in the transition state for the transfer reaction.⁴⁵ The class I aaRSs are mostly monomeric whereas the class II aaRSs are oligomeric. TyrRS and TrpRS are exceptions to this rule since they are obligatory dimers and belong to class I.¹¹⁹

The fact that Bst-TyrRS interacts with tRNA^{Tyr} according to a class II mode was clearly stated as early as 1993, on the basis of the existing data on the Bst-TyrRS-tRNA^{Tyr} interaction.⁵⁹ Because TyrRS belongs unambiguously to class I, its interaction with tRNA^{Tyr} according to a class II mode was accepted with difficulty. For example, other models or modes of interaction have been proposed.^{34,44,120-122} The crystal structure of the TyrRS-tRNA^{Tyr} complex in the *T. thermophilus* system has completed the demonstration.¹⁹ Although tRNA^{Tyr} can be aminoacylated at either the 2'-OH or the 3'-OH of Ade76, it is preferentially aminoacylated at the 2'-OH for rate reasons, at least in yeast (see above). Thus, TyrRS and PheRS bind their cognate tRNAs according to a class II mode and yet, aminoacylate it preferentially at the 2'-OH, as the canonical aaRSs of class I.

The experimental data showing that TyrRS is an exception among the aaRS, as regards their classification, was developed during the years 1986-1989.^{25,26,28,45,58,59,61,123-128} Since then, other exceptions have been observed. For example, there are two types of LysRS, depending on the organism, one belonging to class I and the other to class II.¹²⁹ In some organisms, tRNA^{Cys} can be charged by ProRS, which belongs to class II, although its cognate CysRS normally belongs to class I.¹³⁰⁻¹³² It will be interesting to find out how TrpRS interacts with tRNA^{Trp}, given its structural homology with TyrRS.¹³³

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Note Added in Proof

Reports on the structure of human TyrRS¹³⁵ and on the complex between archaeal TyrRS and tRNA have recently been published.¹³⁶

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