# Domains of phenylalanyl-tRNA synthetase from *Thermus thermophilus* required for aminoacylation

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**Abstract The contribution of entire domains or particular amino acid residues of the phenylalanyl-tRNA synthetase (FRS) from *Thermus thermophilus* to the interaction with $tRNA^{Phe}$ was studied. Removal of domain 8 of the β subunit resulted in drastic reduction of the dissociation constant of the FRS·$tRNA^{Phe}$ complex. Neither the removal of arginine 2 of the β subunit, which makes the only major contact between domains β1–5 and the tRNA, nor the replacement of the conserved proline 473 by glycine had an influence on the aminoacylation activity of the FRS. Thus, the body comprising domains 1–5 of the β subunit may not be essential for efficient aminoacylation of $tRNA^{Phe}$ by the FRS and rather be involved in other functions.**



*Key words:* Phenylalanyl-tRNA synthetase; *Thermus thermophilus*; Aminoacylation; $tRNA^{Phe}$; Protein-RNA interaction

## 1. Introduction

The phenylalanyl-tRNA synthetase (FRS) belongs to class II of aminoacyl-tRNA synthetases (aaRSs) [1]. With the exception of the monomeric yeast mitochondrial FRS, all other known FRSs have a heterotetrameric quaternary structure resembling a functionally dimeric $(\alpha\beta)_2$ enzyme [2]. The α subunits comprise the catalytic aminoacylation site with the three sequence motifs characteristic for class II aaRSs. In addition a N-terminal domain composed of two long antiparallel α helices stabilizes the binding of the $tRNA^{Phe}$ to the enzyme [3]. The β subunits exhibit a complicated modular arrangement of as many as eight different domains. Two of them have typical helix-loop-helix motifs (domains β1 and 5) and may be responsible for the specific interaction of the FRS with DNA sequences (manuscript submitted). Another domain (β6) structurally resembles the catalytic domain of the α subunit. The function of this pseudocatalytic domain, however, is obscure. Domain β3, whose function is also unknown, is structurally similar to the Scr homology 3 (SH3) domain of eukaryotic tyrosine kinases. It is, therefore, an open question how many and which domains of the FRS are absolutely required for the aminoacylation of $tRNA^{Phe}$. Other functions accomplished by particular domains of this enzyme should be considered as well.

The anticodon of $tRNA^{Phe}$ is one of the major recognition elements for the FRS [4–6]. Two domains of the β subunit were good candidates to accomplish this anticodon recognition, i.e. domain β2, which structurally resembles the anticodon binding domain of the bacterial aspartyl-tRNA synthetase, and domain β8, which is similar to the RNA binding domain of the spliceosomal protein U1A. The results of our previously reported footprinting experiments of $tRNA^{Phe}$ transcripts with the FRS suggested an orientation of the $tRNA^{Phe}$ with the anticodon pointing towards domain β8 rather than domain β2 [7].

In this work we report that domain β8 is essential for aminoacylation. Furthermore we provide evidence that several domains of the β subunit are dispensable for aminoacylation. Thus, our experimentally obtained biochemical results on the FRS-$tRNA^{Phe}$ interaction complete the recently published structural data of the FRS·$tRNA^{Phe}$ complex [3].

## 2. Materials and methods

### 2.1. Bacterial strains, plasmids, recombinant DNA technology and overexpression

*Escherichia coli* strain SG13009 [8] was grown in Luria-Bertani (LB) nutrient medium [9] as previously reported [10]. For deletion mutagenesis of *pheT* the plasmid pPheST1 [11] was hydrolyzed with *Pst*I and *Not*I, whose cleavage sites are located downstream of the *pheT* gene, thus enabling the unidirectional deletion of the 3′ portion of *pheT* by the exonuclease III/nuclease S1 technique (Fig. 1). The extents of the deletions were mapped by restriction analyses of the resulting plasmids (expressing FRSβΔ759–785 and FRSβΔ742–785) with suitable combinations of endonucleases. The exact end points of the deletions were determined by DNA sequencing. For site-directed mutagenesis [12] and expression the plasmid pALPheST20 [10] based on the vector pKKM13+ [13] was used with oligodeoxyribonucleotides 5′-GGAGAAGGGCAC-CATAGAACCCCCT for FRSβΔR2, 5′-TCTTGGAAGGCGAGTTATTTGTCCGGGAGG for FRSβΔ681–785, and 5′-GCGGGGAGGGCCAAACCGATGGT-CTCGTAGC for FRSβP473G. Standard recombinant DNA methods were performed according to the protocols of Ausubel et al. [14] and Sambrook et al. [15].

### 2.2. Enzyme purification and characterization

The FRS variants were purified as described for the native FRS [10]. The molecular masses of the FRS and its variants were determined by size exclusion chromatography as previously reported [16]. The thermal stability of the proteins was assessed by their temperature-dependent change in UV absorbance using 0.5 μM protein solutions [16].

The aminoacylation activity of the FRS and its variants was determined by a filter binding assay in a total volume of 60 μl of 100 mM sodium cacodylate, pH 7.6, 10 mM KCl, 8 mM $MgSO_4$, 5 mM 2-mercaptoethanol, 2 mM ATP, 20 μM [$^{14}$C]phenylalanine (50 Ci/mol) and 2 μM purified *T. thermophilus* $tRNA^{Phe}$ (overproduced in *E. coli*; aminoacylation capacity 1580 pmol phenylalanine/$A_{260}$ $tRNA^{Phe}$) at 65°C. The reaction was started by the addition of 1–8 nM FRS. Aliquots of 10 μl were withdrawn at 30, 60, 90, 120, and 150 s, pipetted on Whatman 3MM papers, and the aminoacylated tRNA was precipitated in 10% trichloroacetic acid. The filter-bound radioactivity, after washing twice with 10% trichloroacetic acid and once with ethanol, was counted. The protein concentration was determined by the Bio-Rad (Hercules, CA, USA) assay. One unit of FRS activity was defined as the amount of enzyme needed to attach 1 nmol of [$^{14}$C]phenylalanine to the tRNA in 1 min.

For the determination of kinetic data, the active sites were titrated

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according to Fersht et al. [17]. The concentration of the substrate [$^{14}$C]phenylalanine (125 Ci/mol) was varied in the range of 2–14 μM. When the dependence of the substrate tRNA$^{Phe}$ was investigated, its concentration was varied in the range of 0.1–1 μM. The data were analyzed using Eadie-Hofstee and Lineweaver-Burk plots [18].

The formation of the phenylalanyladenylate was tested by the determination of radioactively labeled ATP in the equilibrium of an exchange reaction with [$^{32}$P]pyrophosphate, non-labeled ATP, and phenylalanine as substrates [19]. In a total volume of 300 μl containing 100 mM Tris, pH 7.6, 10 mM $MgCl_2$, 2 mM ATP, 2 mM KF, 2 mM [$^{32}$P]pyrophosphate (0.5 Ci/mol), and 2 mM phenylalanine the reaction was started with 8–40 nM of the FRS or its variants. Aliquots of 40 μl were drawn after 2, 4, 6, 8, 12, and 15 min of incubation at 50°C and immediately mixed with 70 μl of 12% perchloric acid and 300 mM pyrophosphate. 100 μl of the solution was filtered through charcoal filters (Schleicher & Schüll, Germany), the filters were rinsed with 5 ml water and 5 ml ethanol, dried, and the radioactivity was counted.

For the determination of kinetic data in dependence of the substrate phenylalanine its concentration was varied in the range of 2–12 μM. When the dependence of the substrate ATP was investigated, its concentration was varied in the range of 0.5–8 mM. The data were analyzed using Eadie-Hofstee and Lineweaver-Burk plots [18].

### 2.3. Determination of tRNA$^{Phe}$ transcript binding by the FRS

α[$^{32}$P]GTP (3000 Ci/mmol)-labeled T7 RNA polymerase transcripts [20] of *T. thermophilus* tRNA$^{Phe}$ were obtained as previously described [7] using plasmid $pT_7TthF$-5 (P. Hoffmüller, unpublished), which contains the *pheU* gene from *T. thermophilus* [21] linked to a T7 promoter in pUC19 [22].

To determine the approximate binding constant, in a filter binding assay [14] 2 nM tRNA$^{Phe}$ transcript in 20 mM Tris, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 0.1 mM DTT, 0.1 mM PMSF, and 5% glycerol were mixed with 50 fM–10 μM FRS in a total volume of 400 μl and incubated for 15 min at 40°C. The solution was filtered through nitrocellulose filters (Schleicher & Schüll, Germany), washed with 400 μl of buffer, dried, and the radioactivity was counted.

## 3. Results and discussion

### 3.1. Involvement of the C-terminal domain 8 of the FRS β subunit in tRNA$^{Phe}$ binding

To determine the possible involvement of the C-terminal domain 8 of the FRS β subunit in tRNA binding, a series of mutants with deletions in the corresponding 3′ portion of the *pheT* gene were constructed. Based on the three-dimensional structure of the FRS [2] an additional FRS variant completely lacking the entire domain 8 was constructed by site-directed mutagenesis. This was accomplished by the introduction of a stop codon in the *pheT* gene yielding in this way the FRS variant FRSβΔ681–785 with their β subunits consisting of only 680 N-terminal amino acids. All FRS variants were overproduced in *E. coli*, purified and checked for their ability to form phenylalanyladenylate and to aminoacylate tRNA$^{Phe}$ (Fig. 1). Adenylate formation was affected by none of the variants. Aminoacylation of tRNA$^{Phe}$ still occurred when the 27 C-terminal amino acids were missing (variant FRSβΔ759–785). However, the removal of 44 C-terminal amino acids (variant FRSβΔ742–785) or the whole domain 8 (variant FRSβΔ681–785) resulted in the complete loss of aminoacylation activity. By size exclusion chromatography the molecular mass of variant FRSβΔ681–785, which lacks domain 8, was demonstrated to correspond to the heterotetramer (data not shown). Though thermal denaturation of this variant occurred at a slightly lower temperature than that of the wild-type FRS ($< 5$°C; Fig. 2), we suppose that the removal of domain 8 had no major effect on the overall structure of the remaining protein. This was also confirmed by the $K_M$ and $k_{cat}$ values for the two substrates phenylalanine and ATP determined by the pyrophosphate exchange reaction, which are in the same range as those of the intact FRS (Table 1). This indicates that the catalytic center was not affected by the truncation. However, the affinity of tRNA$^{Phe}$ transcripts to this variant was drastically reduced (Fig. 3). The apparent dissociation constant determined by a filter binding assay was, compared to the wild-type FRS, about three orders of magnitude lower, which is at the level of unspecific binding. It thus can be concluded that the inactivity in aminoacylation of this variant was due to the weak and uncoordinated binding of the tRNA$^{Phe}$ to the enzyme. This further implies that the C-terminal domain 8 is responsible for the interaction with the anticodon as the major determinant of tRNA$^{Phe}$, which is confirmed by the X-ray structure of the FRS·tRNA$^{Phe}$ complex [3]. Anticodon base-specific interactions occur via hydrogen bonds with aspartates 696 and 729, serine 742 and arginine 780, by hydrophobic interactions with tyrosine 731 and by van der Waals contacts with leucine 697 and alanine 698. Upon the removal of 44 C-terminal amino acids, some of these important amino acids are missing in variant FRSβΔ742–785. Moreover, the architecture of the whole domain was probably destroyed, which explains the inability of this truncated protein to aminoacylate

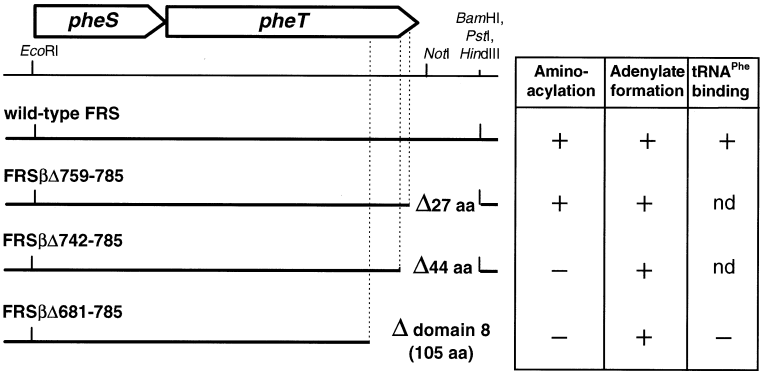


Fig. 1. Effect of truncation of the C-terminal portion of the FRS β subunit. At the upper left panel the extensions of the FRS-encoding genes *pheST* with respect to the restriction map of the overexpressing plasmid are shown. The wild-type gene and the extents of three different gene deletions are indicated by the gaps in the bold lines below. The positions of missing and the number of removed amino acids (aa) are denoted above these lines and in the gaps, respectively. In the right panel the qualitative properties of the corresponding FRS variants are indicated: +, property detectable; −, property not detectable; nd, not determined.

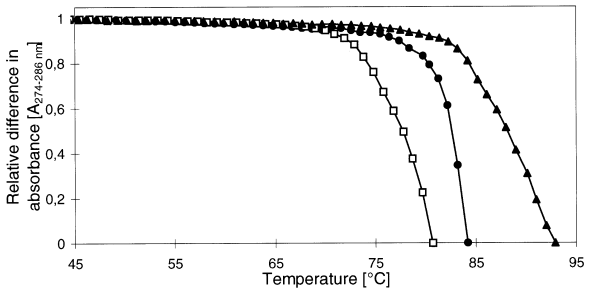


Fig. 2. Thermal stability of the FRS (●), FRSβΔR2 (▲) and FRSβΔ681–785 (□) determined by the change of UV absorance in dependence on the temperature.

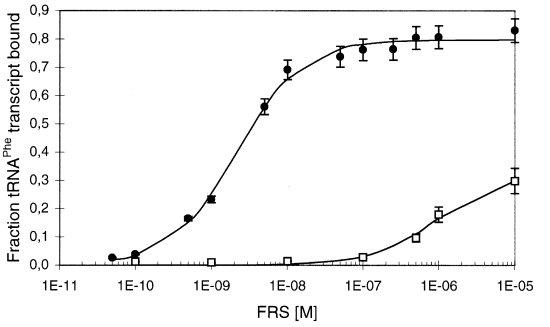


Fig. 3. Binding of the $tRNA^{Phe}$ to the FRS (●) and a FRS variant lacking domain 8 (FRSβΔ681–785) of the β subunit (□) determined in a filter binding assay by titration of increasing protein concentrations to the $tRNA^{Phe}$. The turning points of the curves indicate the approximate dissociation constants.

$tRNA^{Phe}$. Surprisingly, the removal of 27 C-terminal amino acids, corresponding to an α helix and the adjacent C-terminal arm of domain 8, did not abolish aminoacylation activity. This indicates that the domain was not destroyed by this truncation and tRNA still can efficiently be bound to the FRS variant. Possibly the specificity of the anticodon recognition may be affected.

### 3.2. Domains 1–5 of the FRS β subunit may be dispensable for $tRNA^{Phe}$ aminoacylation

Since the acceptor domain of $tRNA^{Phe}$ interacts with the α subunit and the anticodon is bound by domain 8 of the β subunit, it was questionable whether the whole body comprising domains 1–5 of the β subunit makes a contribution to the interaction with $tRNA^{Phe}$. Several lysine residues (2, 60, 106) in domains 1 and 2 of the *E. coli* FRS were reported to be labeled with periodate-oxidized $tRNA^{Phe}$ [23,24]. This led to the conclusion that these domains are in close contact with the acceptor end of the tRNA. However, in the three-dimensional structure of the *T. thermophilus* FRS·$tRNA^{Phe}$ complex only the side chain of arginine 2 corresponding to lysine 2 in the *E. coli* enzyme was found to form salt bridges with the sugar-phosphate backbone of the acceptor helix at A73 and C74 [3]. No other contacts exist between domains 1–5 and $tRNA^{Phe}$, apart from two putative hydrogen bonds. Since genetically produced FRS variants with a truncated N-terminus of the β subunit were unstable and could not be studied, we constructed a variant just lacking arginine 2. Neither the aminoacylation activity nor the catalytic efficiency of this variant (FRSβΔR2) was substantially affected (Table 1). This indicates that the presence of a basic residue at this position is not an absolute requirement for the efficient aminoacylation of $tRNA^{Phe}$, as also reflected by the non-conservation of this residue among the FRSs from different species. Interestingly, the variant FRSβΔR2 exhibited higher thermal stability with different denaturation kinetics compared to the wild-type FRS (Fig. 2).

Domains 1–5 of the β subunit form a body which is spatially apart from the rest of the FRS molecule. In the short linker between domains 5 and 6 a proline is remarkable (position 473), which is conserved among the FRSs from different organisms. Due to the restricted rotation angles this proline may be involved in the positioning of the body comprising domains 1–5 with respect to the remainder of the molecule. To study the possible importance of such a fixed body for the stabilization of the tRNA we replaced the proline by a flexible glycine (FRSβP473G). However, neither aminoacylation activity nor catalytic efficiency of this variant was significantly affected (Table 1). This is in agreement with structural data of the FRS·$tRNA^{Phe}$ complex, where, in contrast to other domains of the molecule, no major movement of domains 1–5 upon binding of the $tRNA^{Phe}$ occurs compared to the free state of the FRS. The conservation of this proline residue may be important for an additional function of the FRS (see below), e.g. the correct positioning of the two symmetrically arranged helix-loop-helix motifs located in domains 5 of the functionally dimeric FRS [2].

In conclusion, there is no evidence that the body comprising domains 1–5 of the β subunit is required for the efficient aminoacylation of $tRNA^{Phe}$ by the FRS. The few contacts reported between domains 1–5 and $tRNA^{Phe}$ may be not essential [3]. This is in agreement with the composition of the yeast mitochondrial FRS, which lacks the domain β1 and β3–7 but is fully active in aminoacylation. The part of the molecule comprising domains 1–5 of the other FRSs may also be involved in a function not related to aminoacylation, which is not relevant in mitochondria. We have clear evidence that the

Table 1
Catalytic constants of the FRS and its variants for aminoacylation and ATP-[$^{32}$P]$PP_i$ exchange with $tRNA^{Phe}$, phenylalanine and ATP as substrates

| | Aminoacylation | | | | | ATP-[$^{32}$P]$PP_i$ exchange | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $tRNA^{Phe}$ | | Phenylalanine | | | Phenylalanine | | ATP | | |
| | $K_M$ (μM) | $k_{cat}/K_M$ ($10^5$ $s^{-1}$ $M^{-1}$) | $K_M$ (μM) | $k_{cat}/K_M$ ($10^5$ $s^{-1}$ $M^{-1}$) | $k_{cat}$ ($s^{-1}$) | $K_M$ (μM) | $k_{cat}/K_M$ ($10^5$ $s^{-1}$ $M^{-1}$) | $K_M$ (μM) | $k_{cat}/K_M$ ($10^5$ $s^{-1}$ $M^{-1}$) | $k_{cat}$ ($s^{-1}$) |
| FRS | 0.32 | 12.6 | 2.1 | 1.9 | 0.40 | 3.57 | 146.6 | 1080 | 0.48 | 52.35 |
| FRSβΔ681–785 | na | na | na | na | na | 1.87 | 308.8 | 1589 | 0.36 | 57.75 |
| FRSβΔR2 | 0.40 | 10.6 | 2.3 | 1.8 | 0.42 | nd | nd | nd | nd | nd |
| FRSβP473G | 0.35 | 10.1 | 2.1 | 1.7 | 0.35 | nd | nd | nd | nd | nd |

na, not applicable; nd, not determined.

FRS from *T. thermophilus* specifically binds DNA (to be published elsewhere). Since the enhanced expression of one human FRS subunit seems causally be related to tumorigenic myeloid leukemia [25], it is a tantalising assumption that the FRS, apart from its aminoacylation activity, interferes with a fundamental process of cell proliferation which is related in bacteria and in humans, e.g. by the regulatory action involving its domains β1–5.

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