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Footprinting of tRNA^{Phe} transcripts from *Thermus thermophilus* HB8 with the homologous phenylalanyl-tRNA synthetase reveals a novel mode of interaction

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ABSTRACT

The phosphates of the tRNA^{Phe} transcript from *Thermus thermophilus* interacting with the cognate synthetase were determined by footprinting. Backbone bond protection against cleavage by iodine of the phosphorothioate-containing transcripts was found in the anticodon stem–loop, the D stem–loop and the acceptor stem and weak protection was also seen in the variable loop. Most of the protected phosphates correspond to regions around known identity elements of tRNA^{Phe}. Enhancement of cleavage at certain positions indicates bending of tRNA^{Phe} upon binding to the enzyme. When applied to the three-dimensional model of tRNA^{Phe} from yeast the majority of the protections occur on the D loop side of the molecule, revealing that phenylalanyl-tRNA synthetase has a rather complex and novel pattern of interaction with tRNA^{Phe}, differing from that of other known class II aminoacyl-tRNA synthetases.

INTRODUCTION

For an understanding of the catalytic mechanism of phenylalanyl-tRNA synthetase (FRS) it is important to determine its interactions with cognate tRNA^{Phe} at the level of both FRS and tRNA^{Phe}. Several experimental approaches based on *in vivo* suppression assays, kinetic studies of aminoacylation, footprinting and other methods have been used to determine the nucleotides contributing to either recognition of tRNA^{Phe} by FRS or tRNA^{Phe} identity (1). Using mutational analyses of yeast tRNA^{Phe} transcripts five single-strand nucleotides, i.e. G20, G34, A35, A36 and A73, as well as nucleotides in the TψC loop (U59 and U60), the central core and the variable pocket (G10, C25, A26, G44 and U45), were identified as being involved in recognition by the enzyme (2–4). However, interaction with the enzyme is not limited to these bases, as is obvious from the complex protection pattern in footprints with ethylnitrosourea (5,6). In addition, for human FRS the G30-C40 and A31-U39 base pairs in the anticodon stem seem to be important for *in vitro* recognition, since the k_{cat}/K_m values of aminoacylation drastically decrease upon alterations of these base pairs (7). Also, in *Escherichia coli* certain nucleotides apart from U20 and A73 in the anticodon stem and the T loop were found to determine the tRNA^{Phe} suppressor identity (8). Protection against alkylation was seen at G24 (9). For tRNA^{Phe} transcripts from *Thermus thermophilus* the importance of G34, A35, A36 and A73, but not U20, has been demonstrated (10).

FRS from *T.thermophilus* belongs to class II of aminoacyl-tRNA synthetases (aaRSs), since the three characteristic sequence motifs are present in the α-subunits of this $\alpha_2\beta_2$ heterotetrameric enzyme (11,12). Recently the three-dimensional structure of this enzyme was solved (13). The catalytic domain of the α-subunits resembles the structural elements of the other known class II aaRSs. Also, a similar domain was found in the β-subunits. Due to its less conserved primary structure this domain may not be catalytically active and was designated the pseudocatalytic domain. In addition, the β-subunits are composed of several other structural elements in a modular manner. Two of these modules are supposed to interact with tRNA. Domain II of the β-subunit is similar to the anticodon binding domain of aspartyl-tRNA synthetase (DRS) (14,15), whereas C-terminal domain VIII structurally resembles the RNA binding motif of a family of small nuclear ribonucleoproteins (16,17). Since the anticodon of tRNA^{Phe} contains important recognition elements for FRS, one of these domains probably interacts with the anticodon; the other may stabilize the tRNA in the acceptor stem region. However, no structural data on the FRS–tRNA^{Phe} complex are as yet available.

Attempts have been made to study the interaction of FRS with tRNA by biochemical approaches. From labeling of *E.coli* and yeast FRS with periodate-oxidized tRNA^{Phe} several different enzyme regions were assumed to be close to the CCA end, i.e. Lys131 and Lys436 of the large subunit of the yeast cytoplasmic enzyme, as well as Lys2, Lys60 and Lys106 of the large subunit of the *E.coli* enzyme (18,19). These results are quite puzzling, since the localized residues are widely distributed over the aligned sequences of the large subunits of FRS from different species. Moreover, none of these residues correspond to the

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region comprising the catalytic fold in the small α-subunit supposed to be the active center of FRS from *T.thermophilus*, where the CCA end should be located.

The footprinting technique, based on cleavage of transcripts with statistically incorporated phosphorothioates by iodine has previously been applied to study interactions of $tRNA^{Ser}$ from *E.coli* and $tRNA^{Asp}$ from yeast with their corresponding aaRSs (20,21). It has the advantage that the reactive iodine is small and permits identification of phosphates which are in close proximity to the enzyme. Also, conformational changes upon binding of tRNA to an aaRS can be detected, since phosphates in bent regions are more accessible to iodine. This may result in enhancement of cleavage, as seen with $tRNA^{Asp}$ (21). The active enzyme conformation is maintained during the reaction, because the iodine reacts very rapidly with the phosphorothioates. Using the iodine footprinting method close contacts of tRNA phosphate groups with the enzyme can be seen, but not base-specific interactions. Ethylnitrosourea, which is another reagent used for footprinting studies on RNA molecules, reacts with both oxygens of a phosphate group (5). In contrast, iodine only reacts with the single oxygen of a phosphate group replaced by a sulfur (22).

We have used the iodine footprinting technique to study the interaction of $tRNA^{Phe}$ with FRS at the level of the RNA. Additional investigations are in progess to determine interactions at the level of the enzyme.

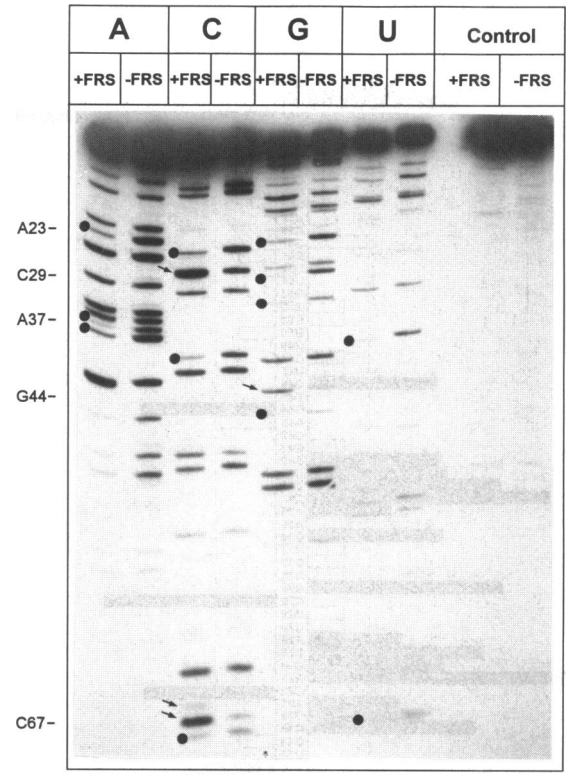

Figure 1. Autoradiography of the radioactively labeled, phosphorothioate-containing $tRNA^{Phe}$ transcripts after cleavage by iodine in the presence (+FRS) and absence (–FRS) of FRS from *T.thermophilus*. The footprinting experiments were performed using transcripts statistically substituted with ATP[αS] (A lanes), CTP[αS] (C lanes), GTP[αS] (G lanes) and UTP[αS] (U lanes), No iodine was used for the control reactions. The bullets indicate reduced, the arrows increased band intensities when comparing the lanes in the presence and absence of FRS. The numbering of the bands in the left panel corresponds to the 5′ phosphate groups of the nucleosides and follows the nomenclature of the tRNA databank.

MATERIALS AND METHODS

Preparation of tRNA transcripts

The plasmid for transcription of the $tRNA^{Phe}$ gene from *T.thermophilus* was constructed using oligodeoxyribonucleotides according to the sequence determined for $tRNA^{Phe}$ from *T.thermophilus* (23) as previously described (24). In the recently determined sequence of the *pheU*-gene of *T.thermophilus* (25) a C was found at position 65, which is in contrast to the U determined for $tRNA^{Phe}$ itself. It cannot be decided whether this difference is due to a sequencing mistake or the result of a post-transcriptional modification. The transcripts were obtained, after cleavage of the plasmid by *Bst*NI, in the presence of 5% of each nucleoside phosphorothioate using T7 RNA polymerase purified from overproducing *E.coli*, as previously reported (21). The proper transcripts were eluted from a preparative 16% acrylamide–urea gel and precipitated. Labeling with [α-^{32}P]ATP at the 3′-end was performed in 50 mM Tris, pH 8.0, 3 mM pyrophosphate, 3 mM $MgCl_2$, 5 mM dithiothreitol by nucleotidyltransferase. The band corresponding to the labeled transcript was eluted from a preparative 16% acrylamide–urea gel and the RNA precipitated and dissolved in footprinting buffer (10 mM HEPES, pH 7.4, 10 mM NaCl, 10 mM $MgCl_2$).

Preparation of the aaRS overproduced in *E.coli*

FRS from *T.thermophilus* was prepared from 200 g of a recombinant *E.coli* strain carrying the plasmid pPheST1 (12). The cells were broken by N_2 decompression (Parr Instruments, Moline, USA). The supernatant after centrifugation was heated to 70°C for 30 min. Purification of the thermally treated supernatant after a second centrifugation was similar to that described for elongation factor Tu from *T.thermophilus*, involving anion exchange chromatography on Q Sepharose Fast Flow and gel permeation chromatography on Sephacryl S200 HR (26). DRS from *T.thermophilus* was prepared as previously reported (27).

Determination of K_m

The Michaelis constant of the FRS–$tRNA^{Phe}$ complex was measured in a reaction mixture containing 100 mM HEPES–NaOH buffer, pH 7.2, 30 mM KCl, 10 mM $MgCl_2$, 2 mM ATP, 12.5 μM L-[^{3}H]phenylalanine (sp. act. 1880 c.p.m./pmol), 0.1 mg/ml bovine serum albumin, 10–20 nM FRS from *T.thermophilus* and $tRNA^{Phe}$ transcripts from *T.thermophilus* in the concentration range 0.02–1.5 μM at 37°C. The K_m values were determined from initial rates according to the Lineweaver–Burk plot.

Footprinting

The aminoacyl-tRNA synthetases were dialyzed against footprinting buffer (see above) using Centricon 50 (Amicon). The FRS and DRS concentrations in the footprinting assays were adjusted to 2.5 μM, corresponding to 5 μM active sites (two active sites per molecule). The transcript concentration was 1 μM. The reaction time for iodine cleavage was 2 min. All other conditions were as previously described (21). Quantification of the bands was

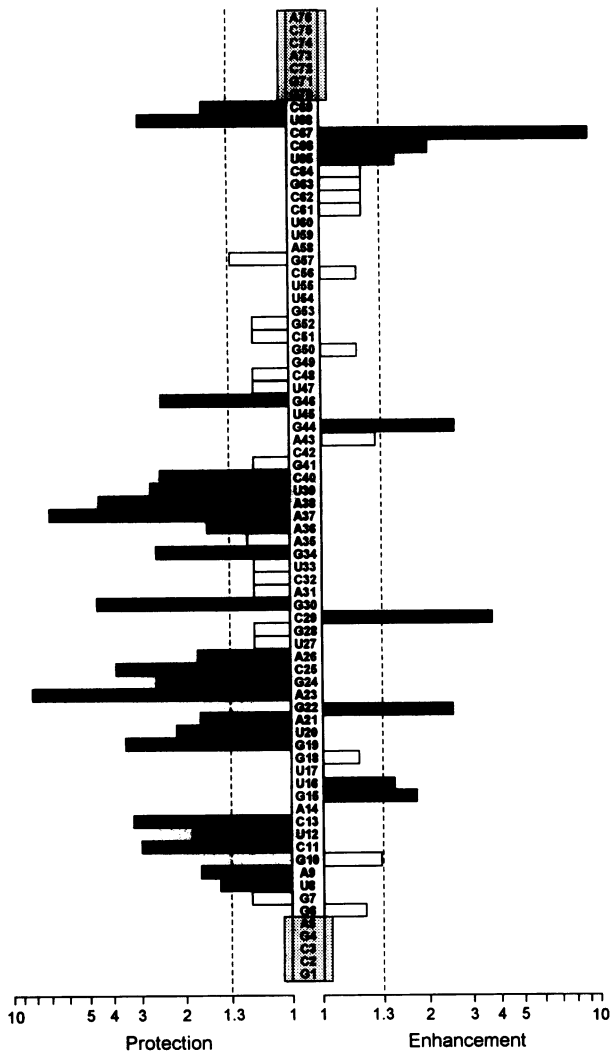

Figure 2. Quantification of protection or enhancement of cleavage by iodine of the phosphodiester bonds of $tRNA^{Phe}$ transcripts from *T.thermophilus* when complexed with FRS. Band intensities in the presence and absence of the enzyme were compared. The protection or enhancement factors are expressed as their proportion and represented by filled bars on a logarithmic scale. (As an example, a protection factor of 2 means 50% reduced band intensity in the presence of FRS.) Open bars represent values which were not in the significant range, being below a factor of 1.3, marked by dashed lines. The shaded boxes indicate tRNA regions which could not be analyzed by the applied technique.

performed using a Bio-Imager (Fuji). The protection or enhancement factors were calculated as the proportion of the band intensities in the presence and absence of the enzyme. Protection or enhancement was considered weak, moderate or strong when the factors were 1.3–3, 3–5 and 5–10 respectively.

RESULTS

The K_m of FRS for $tRNA^{Phe}$ transcripts was determined to be 150 nM, which is in the same range as previously reported for native $tRNA^{Phe}$ from *T.thermophilus* (10,28). The K_m for the tRNA transcripts used for the footprinting experiments, with about 5% of the nucleotides statistically substituted by phosphorothioates, was found to be comparable (20). Under the experimental

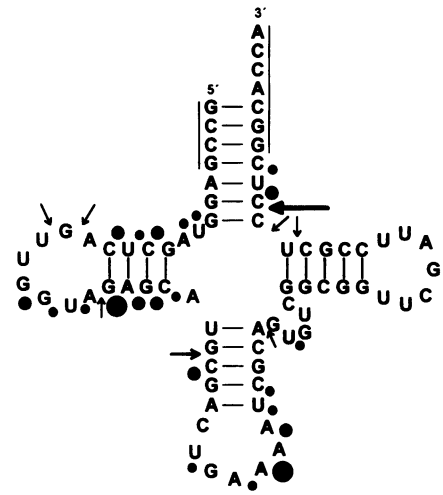

Figure 3. Cloverleaf representation of the $tRNA^{Phe}$ transcript from *T.thermophilus*. Small, middle and large bullets mark weak, moderate and strong cleavage protection and arrows enhancement of cleavage by iodine corresponding to factors of 1.3–3, 3–5 and 5–10 respectively (see Fig. 2). The lines indicate tRNA regions which could not be analyzed by the applied technique.

conditions for the footprinting ~85% of the $tRNA^{Phe}$ transcripts were calculated to be in complex with the enzyme. Since FRS is a symmetrical, functionally dimeric molecule (13), its mode of interaction with both tRNA molecules is supposed to be the same. Negative cooperativity in the binding of tRNA to FRS (28) may, therefore, not influence the pattern of the footprints.

The footprinting experiments were performed with phosphorothioate-containing $tRNA^{Phe}$ transcripts from *T.thermophilus* and FRS or DRS (to prove the specificity of the interaction) of *T.thermophilus* purified from overproducing *E.coli* strains. The intensities of several bands changed in the presence of FRS compared with reaction in the absence of the enzyme (Fig. 1). Strong and moderate protections against cleavage by iodate were found in the anticodon stem–loop (G30, A37 and A38), the D stem–loop (C11, C13, G19, A23, G24 and C25) and in the acceptor stem (U68). The protected phosphates are located in the 5′-position of the nucleosides indicated (Figs 2 and 3). In addition, weak protection was also seen in the variable loop. At a few positions enhancement of cleavage was seen, which indicates increased accessibility for iodine of the corresponding phosphates. Strong enhancement occurred at position C67 in the acceptor stem and moderate enhancement at position C29 in the anticodon stem. The cleavages at positions G15, U16, G22 G44, U65 and C66 were weakly enhanced. Due to the limited resolution of the gels no footprinting information was obtained for the 5′- and 3′-ends of the transcript (G1–A5 and G70–A76). No protection or enhancement was seen in the experiment using DRS instead of FRS (data not shown), indicating the specificity of the interactions found.

Weak bands occuring in the control lanes with labeled transcripts, which were not treated with iodine, are indicative of intrinsic instability of the RNA. When RNA corresponding to the full-length transcript was eluted from the gel and immediately run on another gel the same degradation products again occurred (data not shown). Similar instabilities were also found with other tRNA molecules (29,30).

A band compression was found between C48 and C51, which made it difficult to read the sequence of the transcript. To prove that the plasmid DNA used as the template for transcription had the correct sequence we determined the nucleotide sequence of the DNA insert comprising the tRNA gene. At the level of DNA a similar compression occurred at the corresponding region. This, however, could be resolved by using inosine triphosphate for the sequencing reactions, which clearly demonstrated the correct sequence (data not shown).

DISCUSSION

Contact regions of $tRNA^{Phe}$ with FRS

Most of the protected phosphates occur in regions where the identity elements of $tRNA^{Phe}$ were found, i.e. in the D stem–loop, the anticodon loop and the variable loop, indicating that the backbone in these regions is also in contact with FRS. The region around position 20 shows clear protection, although the nucleotide at position 20 is, in contrast to *E.coli* and yeast $tRNA^{Phe}$, not a recognition element in *T.thermophilus* $tRNA^{Phe}$. This indicates that the D stem–loop is in close contact with FRS, but a specific base at this position is not essential for the interaction in *T.thermophilus*. Accordingly, $tRNA^{Phe}$ transcripts from yeast with a G instead of a U at position 20 can, under suitable buffer conditions, also be aminoacylated by FRS from *T.thermophilus* (10). Fully modified $tRNA^{Phe}$ from yeast, however, is a worse substrate for FRS from *T.thermophilus*, reflecting less efficient recognition by the thermostable enzyme.

The regions of interaction identified by the phosphorothioate footprinting method are not in full accord with those found in the yeast system to be protected from alkylation by ethylnitrosourea (6). There are at least three possible reasons that could explain the differencies observed. (i) Due to the R_P configuration of the phosphorothioate groups in transcripts (31) the oxygens replaced by sulfur atoms point towards the inside of the major grove. Only their protection is detected. Using ethylnitrosourea footprinting, however, no constraints are set by the configuration, which may result in a different protection pattern. (ii) The mode of recognition of $tRNA^{Phe}$ by FRS from various species is slightly different in some details (see above). This can be concluded from differences in the recognition elements of $tRNA^{Phe}$ from *E.coli* (4), *T.thermophilus* (10), yeast (2) and human placenta (7). It is, therefore, possible that the alkylation protection pattern of yeast $tRNA^{Phe}$ does not fully represent that of $tRNA^{Phe}$ from *T.thermophilus*. (iii) Ethylnitrosourea footprinting requires non-physiological pH conditions which may affect the native structure of the complex and result in a different contact pattern.

Conformational changes occur upon binding to FRS

At a few positions enhancement of cleavage was seen. Although it cannot be ruled out that different protein environments influence the reactivity of iodine, the cleavage enhancement is probably due to bending of the tRNA upon binding to the enzyme, thus making some particular phosphothioester bonds more accessible to iodine. This indicates that tRNA is a flexible molecule, whose conformation changes when it binds to the enzyme. The bending does not only occur at the elbow of the tRNA molecule in the D loop, but also at the acceptor and anticodon stems. It can be concluded that $tRNA^{Phe}$ has a conformation in complex with the enzyme which is different from its free state conformation. Cleavage enhancement

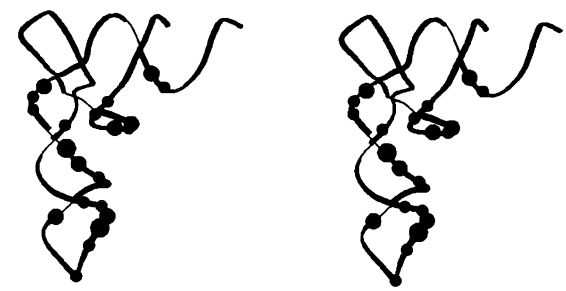

Figure 4. Stereoscopic representation of the backbone of $tRNA^{Phe}$ from yeast (34). In this model the phosphates with weak, moderate and strong cleavage protection in the presence of FRS according to Figure 3 are represented by bullets of increasing size. Phosphates with cleavage enhancement corresponding to regions where the tRNA may be bent are marked by thin lines.

was also found with other known aaRS complexes (20,21) and the corresponding conformational changes were confirmed by their crystal structures (32,33).

Mode of tRNA interaction in different class II aaRSs

When the positions of $tRNA^{Phe}$ identified by the iodine footprinting technique as interacting with FRS are applied to the three-dimensional model of $tRNA^{Phe}$ from yeast (34,35), it becomes obvious that most of them are located on the D loop side of the molecule (Fig. 4). This applies for the regions at positions 11–13, 23–26 and 34–40. However, some protections are also seen on the opposite side, which is valid for the regions at positions 19–21, 30 and 68–69. The whole protection pattern indicates a rather complex interaction of the enzyme with tRNA, which differs remarkably from that of the other known class II aaRSs. Thus FRS is another example that the mode of the interaction with tRNA by aaRSs of different subclasses is not the same, even if they belong to the same class. This is confirmed by the observation that aaRSs use, in addition to the acitive site domain, several modules in different arrangements for stabilization of tRNA. In the large β-subunit of FRS at least two different RNA binding domains were found (13). One is similar to the anticodon binding domain of DRS, while the other is similar to the C-terminal domain of monomeric yeast mitochondrial FRS (36) and resembles the RNA binding motif of a family of small nuclear ribonucleoproteins (16,17). However, it is still unclear which domain binds what region of tRNA. In the DRS–$tRNA^{Asp}$ complex of yeast almost all interactions occur on the TψC loop side, corresponding to the major grove side of the helical amino acid acceptor stem of the tRNA (14,15,33). The results of the iodine footprinting (21) agree fairly well with the situation found in the crystal structure. In the case of seryl-tRNA synthetase, where the anticodon does not contribute to recognition, as was confirmed by iodine footprintings (20), binding of the long variable arm stem by an N-terminal coiled-coil enzyme domain plays a major role in the recognition process (32,37,38). In addition to this variable arm, contacts are made with the TψC loop. The latter are found with neither DRS nor FRS.

It has been assumed that the two classes of aaRSs differ by their mode of tRNA binding. According to this model, all class II aaRSs are supposed to approach tRNA from the same side. This, however, is only valid for interaction of the acceptor end of the

tRNA with the active site domain. The other contacts are maintained by structurally rather different modules in various regions of the tRNA, which may be characteristic for each subclass, but are not class defining.

Orientation of $tRNA^{Phe}$ on FRS

It has been suggested that the different mode of tRNA interaction with FRS could explain the peculiar primary attachment site of the amino acid at the 2'-OH of the ribose (13). In fact, from the iodine cleavage protection pattern it appears that FRS has most of its contacts with $tRNA^{Phe}$ on the opposite side compared with the DRS–$tRNA^{Asp}$ complex. It was proposed that the tRNA is turned by 150° compared with the DRS–$tRNA^{Asp}$ complex, with its anticodon towards a domain in the N-terminal portion of the β-subunit of FRS, which is similar to the anticodon binding domain of DRS (13). This is an attempt to explain the differences in the aminoacylation site. However, superimposition of the catalytic domain in the α-subunit of FRS on the corresponding structure of the DRS–$tRNA^{Asp}$ complex indicates (data not shown) that the anticodon of the tRNA is in close proximity to the C-terminal domain in the β-subunit of FRS, where an RNA binding motif similar to that found in a family of ribonucleoproteins is present (16,17). Deletion of this domain by genetic egineering resulted in a drastically reduced aminoacylation activity (our unpublished results). From our docking experiments, taking into account the novel interactions found by footprinting in this work, the latter orientation seems more likely. Resolution of the three-dimensional structure of co-crystals of $tRNA^{Phe}$ and FRS from *T.thermophilus* (39) will reveal the mode of their interaction.

Evolution of FRS

It has been shown that minihelices derived from the acceptor stem of $tRNA^{Asp}$ from yeast are good substrates for aminoacylation by the corresponding DRS (40). No appreciable aminoacylation, however, was detected under the same conditions when a minihelix of $tRNA^{Phe}$ from *T.thermophilus* and the corresponding FRS were used (data not shown). This may be an indication that the interactions of the active site domain with the acceptor stem, represented by the minihelix, are not sufficiant for aminoacylation by FRS. Additional contacts, perhaps provided by the two RNA binding domains mentioned above, are probably necessary to stabilize tRNA in the position needed for aminoacylation. If this assumption is valid, the rather complex interactions of FRS with $tRNA^{Phe}$ indicate that this enzyme is not one of the primordial aaRSs (41). In contrast to the primitive aaRSs, it may have aquired additional modules for positioning of tRNA. Thus FRS may resemble, in terms of evolution, a more advanced aaRS than DRS.

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