Structure-specific tRNA-binding protein from the extreme thermophile *Aquifex aeolicus*

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The genome of the bacterium *Aquifex aeolicus* encodes a polypeptide which is related to a small portion of a sequence found in one prokaryotic and two eukaryotic tRNA synthetases. It also is related to a portion of Arc1p, a tRNA-binding protein believed to be important for nuclear trafficking of tRNAs. Here we cloned, expressed and purified the 111 amino acid polypeptide (designated Trbp111) and showed by ultracentrifugation analysis that it is a stable dimer in solution. The protein was also crystallized in a monoclinic lattice. X-ray diffraction analysis at 2.8 Å resolution revealed a prominent non-crystallographic 2-fold axis, consistent with the presence of a symmetric homodimeric structure. Band-shift analysis with polyacrylamide gels showed that the dimer binds tRNAs, but not RNA duplexes, RNA hairpins, single-stranded RNA nor 5S rRNA. Complex formation with respect to tRNA is non-specific, with a single tRNA bound per dimer. Thus, Trbp111 is a structure-specific tRNA-binding protein. These results and other considerations raise the possibility that Trbp111 is a tRNA-specific chaperone which stabilizes the native L-shaped fold in the extreme thermophile and which has been incorporated into much larger tRNA-binding proteins of higher organisms.

Keywords: RNA–protein interactions/RNA recognition/RNA world/tRNA evolution/tRNA synthetase

Introduction

We describe here the isolation and characterization of a tRNA-specific binding protein from the extreme thermophile *Aquifex aeolicus*. Our interest was drawn to this protein because of the possibility that it played a role in the evolutionary development of the translation apparatus. An RNA world based on catalytic RNAs and devoid of proteins has been proposed for the origin of self-replicating systems (Pace and Marsh, 1985; Sharp, 1985; Gilbert, 1986; Altman *et al.*, 1989; Cech, 1993). Such a hypothesis tries to explain how an RNA can be both the carrier of information and an effector molecule. A system of this type could evolve into the present day protein-based environment where nucleotides are still used in information storage, but where catalysis is carried out mostly by

proteins. The tRNA molecule is an example of the connection between the RNA world and the theatre of proteins, because it has a central role in the translation of genetic information into proteins. It is generally thought that tRNAs arose early during the transition to the theatre of proteins (Weiner and Maizels, 1987). Analysis of tRNAs and their interactions with proteins is akin to examining living fossils, and might provide insights into early systems.

The cloverleaf base pairing of the tRNA sequence folds into an L-shaped structure, as described by Kim *et al.* (1972) and Robertus *et al.* (1974). This structure has two main domains: the acceptor stem-T ψ C minihelix with the amino acid attachment site and the anticodon-containing stem–loop motif. Studies of tRNA recognition by aminoacyl-tRNA synthetases showed that the minihelix typically provides sufficient information for correct attachment of its cognate amino acid (Francklyn and Schimmel, 1989; Schimmel *et al.*, 1993; Frugier *et al.*, 1994; Nureki *et al.*, 1994; Martinis and Schimmel, 1996; Saks and Sampson, 1996; Hamann and Hou, 1997). The minihelix motif is ancient, occurring as a tag for RNA genomes (Weiner and Maizels, 1987). Indeed, modern and ancient reverse transcriptases use the minihelix motif to prime DNA synthesis from an RNA template (Maizels, 1993; Kennell *et al.*, 1994). Thus, a minihelix-like molecule may have been the earliest substrate for aminoacylation by ribozymes. As the translation system developed, a second domain was added to give the two-domain L-shaped tRNA structure (Schimmel *et al.*, 1993). That the two domains of tRNA interact with separate ribosomal RNAs in the ribosome is consistent with distinct origins for each domain of tRNA (Noller, 1993).

The two domains of the tRNA are approximately recapitulated by the two major domains of their tRNA synthetases. The more ancient catalytic domain of synthetases has insertions that facilitate docking of the acceptor-T ψ C minihelix at the active site (Steitz *et al.*, 1990; Moras, 1993; Cusack, 1997). A second domain provides for interactions with the second domain of the tRNA structure, including the anticodon triplet (Carter, 1993; Schimmel and Ribas de Pouplana, 1995; Arnez and Moras, 1997; Cusack, 1997). This domain is far less conserved among synthetases and appears to have been added later. Domains that interact with specific regions of tRNA are of interest because they might provide insight into the differentiation and specialization of the tRNA-synthetase recognition system.

The increased availability of complete genomes from a wide range of organisms offers the opportunity to gain insight into the evolution of specific domains and to discover new proteins that might have played a role in the evolutionary history of tRNAs and their synthetases. Genomes from organisms believed to be close to the root

Fig. 1. Alignment of Trbp111 with the homologous region in four representative sequences. The sequences of *E.coli* MetRS (Barker *et al.*, 1982), the 12 kDa hypothetical open reading frame in the *IleX-Egbr* intergenic region of *E.coli* (EcOrf) (Blattner *et al.*, 1997), *H.sapiens* EMAPII (Kao *et al.*, 1992) and *S.cerevisiae* Arc1p (Simos *et al.*, 1996) are shown. Strongly conserved residues occurring in three or more sequences are highlighted.

of the tree of life allow us to look for evolutionary footprints of domains which have diverged and adapted to their specific environments. Based on the phylogenetic analysis of 16S ribosomal RNA sequences (Pitulle *et al.*, 1994; Brown and Doolittle, 1995; Bocchetta *et al.*, 1996), the extreme thermophilic bacterium *A.aeolicus* is part of one of the most deeply branching families of organisms. Although there is some disagreement as to its exact position in the evolutionary tree (Baldauf *et al.*, 1996), it is a fairly primitive organism that could provide information about early systems involved in protein synthesis.

The complete genome of *A.aeolicus* was sequenced recently (Deckert *et al.*, 1998). Our attention was drawn to a coding sequence for an 111 amino acid protein that is homologous to the C-terminal end of *Escherichia coli* MetRS and to at least three other proteins known to bind tRNAs in higher organisms. Thus, this coding sequence for a short protein in *A.aeolicus* suggested a molecule that may have had a role in the evolutionary development of tRNAs and tRNA synthetases. For that reason, we cloned and characterized it in some detail.

Results

Alignment of *A.aeolicus* Trbp111 with other proteins

Figure 1 shows an alignment of Trbp111 with four representative sequences from different protein families. Trbp111 from *A.aeolicus* has a 50% identity and 71% similarity to the A566–G676 C-terminal region of *E.coli* MetRS. Although this region is not essential for aminoacylation (Cassio and Waller, 1971), residues within it have been chemically crosslinked to bound tRNA^{Met} (Valenzuela and Schulman, 1986). Trbp111 has a 30% identity and 58% similarity to a hypothetical 12.3 kDa protein (EcOrf) of unknown function in the *IleX-Egbr* intergenic region at 69 min of the *E.coli* genome. In addition, it has 32% identity and 57% similarity to the putative RNA-binding region of *Saccharomyces cerevisiae* Arc1p. The sequence of Trbp111 is also related to part of the sequence of the endothelial monocyte activating polypeptide II (EMAP II) from *Homo sapiens* and other mammals. Finally, high similarity can be found when comparing Trbp111 to the C-terminal domain of *H.sapiens* tyrosyl-tRNA-synthetase. Thus, several proteins known to bind RNA have domains that are homologous to Trbp111.

Cloning and expression of *A.aeolicus* Trbp111

Expression of *A.aeolicus* Trbp111 in *E.coli* resulted in a stable protein product of ~12 kDa as determined by

SDS-PAGE and Tricine-SDS-PAGE. Purification of the thermostable protein lacking a His₆ tag was preferred because circular dichroism scans showed a slight difference in spectra between the tagged and untagged samples (not shown). Using the unmodified protein expressed from pTSMg32, it was easy to purify the thermostable product by heating the crude extract to 80°C for 20 min. During the purification, a large amount of RNA co-eluted with the protein. This RNA was of a similar size as tRNA and possessed limited methionine acceptor ability in a charging assay with *E.coli* MetRS (data not shown).

An RNase A step during purification was added to obtain protein free of contaminating RNA. A 4 l culture resulted in 30–50 mg of highly purified protein. Absorbance scans of the final purified protein sample showed no signs of contaminating nucleic acids, and presented a characteristic blue shifted absorption maximum (276 nm) due to the absence of tryptophans in the protein sequence (data not shown).

Structural characterization of Trbp111

Trbp111 is a thermostable protein when expressed in *E.coli*, as shown by a dramatic enrichment of the protein through heat treatment of the crude cell extract as the first step in the purification protocol. In addition, the circular dichroism spectrum of Trbp111 between 190 and 260 nm taken at various temperatures from 4 to 80°C did not show any significant changes, as expected for a thermostable product (data not shown).

The calculated molecular weight of Trbp111 is 12 095 g/mol. Using sedimentation velocity experiments (40 000 r.p.m.) at 4°C, we determined the molecular weight in solution to be 23.29 ± 0.23 kDa. [The calculated sedimentation coefficient (s) is 2.033 ± 0.002 s with a r.m.s. residual of 1.0024×10^{-2} .] This molecular weight corresponds to a dimer in solution.

Figure 2 shows overlays of the observed absorbance at different times during the sedimentation experiment with theoretical curves using three different assumptions for the molecular weight of the protein [12 (monomer), 23.29 (dimer, fitted size) and 36 (trimer) kDa]. The calculated curve for the monomer and trimer deviate considerably from the observed data, while the curve for the dimer provides the best fit.

The dimeric state of Trbp111 is consistent with observations of the dimeric states of the homologous domains of other proteins (Figure 1). Early studies of *E.coli* MetRS identified the homologous C-terminal domain as essential for dimerization (Cassio and Waller, 1971; Meinnel *et al.*,

Fig. 2. Determination of the oligomeric state of Trbp111 in solution by analytical ultracentrifugation at 4°C. Sedimentation velocity profile and fit to mol. wt of 23.29 kDa (solid lines) for Trbp111 (15 μ M, pH 7.0) corresponds to a dimer. Fit parameters are as follows: $s = 2.033 \pm 0.002$, r.m.s. residual = 1.00241×10^{-2} . Data sets were taken ~ 30 min apart. Inset (A) shows the first data set fit to the expected monomer mol. wt of 12 kDa, and inset (B) shows the first data set fit to the expected trimer mol. wt (36 kDa).

1990). In addition, Gale and Schimmel (1995) showed that a 309 amino acid C-terminal fragment of *E. coli* MetRS was a dimer in solution. In contrast, Quevillon *et al.* (1997) showed that a 175 amino acid C-terminal fragment from hamster p43 (that is believed to be the same protein as pro-EMAPII) encompassing the region homologous to Trbp111 is a monomer when expressed in *E. coli*. However, upon binding of tRNA they observed a higher order protein:tRNA complex which they interpret as being a 2:1 complex. This result suggested that the EMAPII fragment has the ability to form a dimer under at least some conditions.

Crystallization and preliminary X-ray characterization

Trbp111 crystallized in several space groups from various sets of conditions. Cryo-X-ray data were collected from a monoclinic form. Figure 3, top shows large monoclinic crystals of Trbp111 obtained from 18% PEG 10 000 (pH 7.2). The results of self-rotation function calculations revealed a strong non-crystallographic symmetry element at $\psi = 90^\circ$, $\phi = 90^\circ$, $\kappa = 180^\circ$, corresponding to the second top self-rotation function peak (Figure 3, bottom, peak height = 6.2, $\sigma = 0.5$). The top peak corresponds to the crystallographic dyad at $\psi = 0^\circ$, $\phi = 0^\circ$, $\kappa = 180^\circ$ (peak height = 8.9). This solution defines a dyad axis in the *a*-*c* plane and $\sim 6^\circ$ away from the *c*-axis. A second peak (peak height = 3.1) appears at $\psi = 90^\circ$, $\phi = 180^\circ$ (Figure 3, bottom). This peak corresponds to a second 2-fold axis, which could reflect the presence of two symmetric homodimers in the asymmetric unit. (Although the second peak is more diffuse than the one at $\psi = 90^\circ$, $\phi = 90^\circ$, their integrated intensities are comparable. The diffuse nature of the second peak is consistent with there being a looser packing of the homodimer around the second 2-fold axis.) Assuming two homodimers in the

asymmetric unit, the calculated V_m is $4.0 \text{ \AA}^3/\text{Da}$ and solvent content is 69%. Although these crystals diffract to $2.8 \text{ \AA}$ resolution, they are extremely sensitive to radiation as is commonly the case with high-solvent-content crystals [e.g. crystals of lysyl-tRNA synthetase with 74% solvent content, and diffraction to $2.1 \text{ \AA}$ (Onesti *et al.*, 1994)]. Rotation function searches for 3-fold ($\kappa = 120^\circ$) and 4-fold ($\kappa = 90^\circ$) non-crystallographic symmetry yielded no unique self-rotation function solution. These results are consistent with the presence of a symmetric homodimeric structure in the asymmetric unit, as seen with the Trbp111 dimer observed in solution.

tRNA binding to Trbp111

Because of the sequence relationships with specific domains of other RNA-binding proteins, and because a large amount of tRNA-sized material co-elutes during purification of Trbp111, we tested for the tRNA-binding activity of Trbp111. Radioactively labeled tRNA^{Met} was incubated with an increasing amount of Trbp111 and analyzed by a gel-mobility shift assay (Figure 4, top). Upon formation of a complex, the increased size of the bound tRNA^{Met} caused a decrease in its rate of migration. Only two labeled bands were observed, one corresponding to free and one to bound tRNA^{Met}. This observation suggested that only one tRNA binds per dimer of Trbp111. The amount of tRNA^{Met} that was shifted could be determined and compared with the total amount of tRNA^{Met}, to calculate the fraction bound to Trbp111.

The binding profile obtained was plotted on a logarithmic scale and simulated with a theoretical curve based on a simple binding equilibrium. The apparent dissociation constant (K_d) (assuming dimer binding to tRNA) was 32 nM (Figure 4, bottom). This compares to the tRNA-binding affinity of similar proteins like yeast Arc1p that bind to tRNA with an apparent dissociation constant of

Fig. 3. Crystallization of Trbp111. (Top) Monoclinic crystals ($0.35 \times 0.15 \times 0.1 \text{ mm}^3$) of *A.aeolicus* Trbp111. (Bottom) Contour plot showing the self-rotation function of monoclinic Trbp111 diffraction data at 15–4 Å resolution at the $\kappa = 180^\circ$ section. The mean value of the self-rotation function is 1.06 and σ is 0.5. The crystallographic 2-fold axis is apparent in the rotation function peak A ($\psi = 0^\circ$, peak height 8.9). A non-crystallographic dyad is at peak B ($\psi = 90^\circ$, $\phi = 90^\circ$, peak height 6.2) and peak C ($\psi = 90^\circ$, $\phi = 180^\circ$, peak height 3.1). The lowest contour level is at 1 σ above the mean, the highest contour is at the maximum of the self-rotation function.

5–10 nM (Simos *et al.*, 1998). In contrast, the tRNA-binding capacity of the homologous region in hamster EMAPII is much lower, with an apparent K_d of 40 μM (Quevillon *et al.*, 1997).

Stoichiometry of the complex

Results obtained by the gel-mobility shift assay suggested that only one species formed upon complex formation, even at the highest concentrations tested. We also analyzed the stoichiometry of complex formation by sedimentation equilibrium analysis on an analytical ultracentrifuge. Trbp111 and tRNA^{Met} were mixed and allowed to equilibrate at 10 000 r.p.m. The absence of tryptophans in Trbp111 simplified our analysis because we were able to model the absorbance data (at 260 nm) by fitting them to the molecular weight of the tRNA-containing species. Using this analysis, we determined the molecular weight of the tRNA-containing species to be 52.10 kDa (Figure 5, top). This result was evaluated by looking for a random distribution of residuals on either side of the fitted function, a method that can be used to determine the quality of fit

Fig. 4. Binding of tRNA^{Met} to Trbp111. (A) Gel-motility shift assay of Trbp111 binding to radioactively labeled tRNA^{Met}. Trbp111 was incubated at increasing concentrations with 1–2 nM [$5'$ - ^{32}P] tRNA^{Met} and electrophoresed on a native acrylamide gel at 4°C . The top band denotes formation of a complex. (B) Binding profile obtained after determination of fraction of tRNA^{Met} bound to Trbp111 (squares) at various concentrations. Data were simulated using the curve corresponding to a simple-binding equilibrium (solid line). The apparent dissociation constant for tRNA^{Met} binding was determined as 32 nM (dimer concentration).

for a particular function. Figure 5 (bottom) shows the fitted function as a solid line while the observed data are shown as open circles. No systematic deviation from the fitted function was observed, suggesting that we correctly modeled the system.

Additional experiments were performed to determine the molecular weight of tRNA alone using sedimentation velocity analysis. The molecular weight of tRNA^{Met} was determined to be 23.71 kDa (not shown; the calculated mass is 26.24 kDa). Given that we previously determined the molecular weight of Trbp111 to be 23.29 kDa (as a dimer), the sedimentation results are consistent with a 2:1 Trbp111:tRNA^{Met} complex. In addition, the fit of the sedimentation data was evaluated in terms of fits for other possible stoichiometries for the complex. No other feasible molecular weights for the complex provided a good fit of the data (not shown). This result mirrors observations by Quevillon *et al.* (1997) on human EMAPII, in which they reported a band-motility shift corresponding to a 2:1 complex with tRNA.

Competition of tRNA^{Met} with other tRNAs

As stated earlier, some methionine-accepting tRNA was purified with *A.aeolicus* Trbp111 that was expressed in *E.coli*. The results of Trbp111 binding to tRNA^{Met} by the criteria of gel-shift and sedimentation analysis are consistent with this observation. To see whether other tRNAs also bound to Trbp111, we pre-incubated it (at a dimer concentration of 75 nM) in separate experiments with varying amounts of either unlabelled tRNA^{Glu},

Fig. 5. Determination of tRNA^{Met}-binding stoichiometry to Trbp111 by analytical ultracentrifugation. (A) In a 150 μ l sample volume, tRNA^{Met} (400 nM) was mixed with 800 nM Trbp111 at 4°C. Sedimentation equilibrium profile of tRNA^{Met} in the presence of Trbp111 was fit to a single species model (solid line). The calculated mol. wt for this complex is 52.10 kDa with a $\chi^2 = 8.1214 \times 10^{-5}$. (B) Residual plot showing the difference between the experimental data and the fit for each observed point.

Fig. 6. Competition of tRNA^{Met} binding to Trbp111 by various tRNAs. An increasing concentration of either tRNA^{Met}, tRNA^{Tyr} and tRNA^{Glu} was preincubated with Trbp111 prior to incubation for 20 min with 1–2 nM [5'-³²P] tRNA^{Met}. The fraction of tRNA^{Met} bound was normalized to that for no binding of competitor. (Inset) Competition of tRNA^{Met} by modified (natural) and unmodified (transcript) tRNA^{Ile}.

tRNA^{Tyr} or tRNA^{Met}. The pre-incubation was followed by addition of [5'-³²P]tRNA^{Met}. The fraction of tRNA^{Met} bound was then determined by the gel-shift assay. All of the tested tRNAs were able to lower the amount of bound tRNA^{Met} to a similar extent (Figure 6). Competition by tRNA^{Val} had the same effect (not shown).

We also examined the role of tRNA modifications on the binding of tRNA to Trbp111. We tested the ability of an *in vitro* transcript of tRNA^{Ile} to compete with tRNA^{Met} and compared the transcript to modified tRNA^{Ile} that was purified directly from *E. coli*. No significant difference in binding between modified and unmodified tRNA^{Ile} was observed (Figure 6, inset). This result suggests that tRNA binding is achieved through some generally conserved structural elements in tRNA.

Binding affinity of Trbp111 for other RNAs

Because Trbp111 bound each tested tRNA, we were interested in analyzing its ability to bind RNAs that lacked the L-shaped tRNA structure (Figure 7A). We investigated single-stranded RNA [Poly(A)] and found that it was not a competitor with tRNA^{Met} (Figure 7B). We also tested an RNA duplex based on the acceptor stem tRNA^{Met} (C1–A72 Duplex^{Met}) (Figures 7A and B). In a separate experiment, we tested the same duplex with a C1–G72 mutation to ensure that the C1–A72 misspair was not responsible for the lack of binding (not shown). Neither of these constructs competed with tRNA^{Met} for binding to Trbp111.

In addition, we investigated specific subdomains of tRNA^{Met} to see if any part of the structure could compete with tRNA^{Met}. We tested micro^{Met}, a hairpin microhelix that reconstructs the acceptor stem of tRNA^{Met}, and an RNA oligonucleotide that reconstructs the anticodon stem and loop of tRNA^{Met} (AC stem-loop) (Figure 7A). Neither of these tRNA fragments interfered with Trbp111 binding to tRNA^{Met} (Figure 7B). Collectively, these results demonstrated that the intact tertiary structure of tRNA is necessary for recognition by Trbp111.

Finally, we were interested in the ability of Trbp111 to bind an RNA (other than tRNA) that has a complex tertiary structure. For this purpose, we used *Xenopus laevis* 5S rRNA. No significant inhibition by 5S rRNA of binding of tRNA^{Met} to Trbp111 was observed (Figure 7C). This result confirmed that Trbp111 is specific for the L-shaped tRNA structure per se.

Discussion

The intact L-shape of the tRNA molecule appears to be critical for binding by Trbp111. Isolated regions of the L-shaped tRNA do not compete for binding, suggesting that they must be linked together in order to interact with Trbp111. It is of interest to note that the T ψ C- (or UUC-) loop which is common to all tRNAs is present in the microhelix that does not bind (Figure 7A and B). Nor does the anticodon stem-loop structure bind to Trbp111 (Figure 7A and B). That common features in isolation do not confer RNA-binding activity re-inforces the idea that the L-shape itself is important for interaction with Trbp111. These results suggest that Trbp111 recognition of tRNA is based on the spatial arrangement of tRNA-specific structural motifs, such as conserved nucleotides and particular backbone atoms.

Previous work by Simos *et al.* (1996) characterized tRNA binding by yeast Arc1p. This protein has a C-terminal domain homologous to Trbp111 (Figure 1). Yeast Arc1p binds to yeast MetRS and increases the apparent affinity of the synthetase for tRNA^{Met}. The

Fig. 7. Competition of tRNA^{Met} with other RNAs. (A) Primary structure of tRNA^{Met} and various RNAs used in competition experiments. Relevant features of the tRNA structure are shown. (B) Competition of 1–2 nM [5'-³²P] tRNA^{Met} with RNAs that resemble individual domains of tRNA^{Met}. Competition by single-stranded Poly-A RNA is also shown. (C) Competition of 1–2 nM [5'-³²P] tRNA^{Met} with *X.laëvis* 5S rRNA.

N-terminal domain of the 376 amino acid Arc1p was identified as necessary for interacting with MetRS, while the C-terminal region that is homologous to Trbp111 was shown to bind to tRNA. Binding of human tRNA^{Met} to yeast Arc1p was inhibited by addition of other tRNAs and, to some extent, by the T ψ C-acceptor stem minihelix and by 5S rRNA. These results were obtained using up to a 100-fold molar-excess of competitor. In contrast, Trbp111 appears to bind tRNA in a more specific manner, because a higher excess (500- to 1000-fold) of various RNA molecules, including the microhelix which contains the T ψ C-loop, are unable to compete with tRNA^{Met} binding (Figure 7B).

Although other RNAs are able to compete with human tRNA^{Met} for binding to yeast Arc1p, none are able to compete as well as full-length tRNAs (Simos *et al.*, 1996). This result is consistent with tRNA binding by Arc1p being mostly structure specific, as is the case for Trbp111. Deletion analysis of Arc1p showed that the Trbp111-like region (domain C) and an adjacent middle domain (M) were both necessary for efficient tRNA binding (Simos *et al.*, 1998). In contrast, *A.aëolicus* Trbp111 has a comparable affinity for tRNA^{Met} but it lacks the alanine-lysine-rich M-domain of yeast Arc1p and it appears to

have a higher specificity for the tRNA structure. Thus, Trbp111 lacks both the M-domain and the auxiliary N-domain that allows Arc1p to dock to other proteins. This comparison suggests that Trbp111 is designed specifically for tRNA interactions per se.

The sedimentation equilibrium experiments revealed a 2:1 Trbp111:tRNA^{Met} complex. Together with the observation of free Trbp111 as a dimer both in solution and in the crystal, these results collectively suggest that tRNA binds directly to the Trbp111 dimer. Because the X-ray diffraction analysis of the monoclinic form showed that Trbp111 forms a symmetrical dimer, we speculate that the single tRNA-binding site in Trbp111 is formed at the dimer interface and across the dyad axis. (Whether Arc1p is a dimer and whether it binds a single tRNA per dimer is not known). In contrast, dimeric aminoacyl-tRNA synthetases [*Thermus thermophilus* SerRS (Biou *et al.*, 1994), *E.coli* HisRS (Arnez *et al.*, 1995) and yeast AspRS (Cavarelli *et al.*, 1993)] bind two tRNAs across the subunit interface at positions that are offset from the dyad axis and, thereby, preserve the 2-fold symmetry.

The region in other proteins which corresponds to Trbp111 may be adapted for different functions in different organisms. For example, the Trbp111-like region corres-

ponds to the C-terminal part of MetRS in many prokaryotes and in at least one eukaryote aminoacyl-tRNA synthetase [human TyrRS (Kleeman *et al.*, 1997)]. In addition, *E. coli* and other prokaryote PheRSs (Simos *et al.*, 1996; Quevillon *et al.*, 1997) have a region homologous to Trbp111. In *S. cerevisiae*, the C-terminal region of Arc1p has been implicated in nuclear transport of tRNA (Wolin and Matera, 1999) and in mammals p43 (a portion of which is an Arc1p and Trbp111 homolog) is part of the multi-synthetase complex (Kerjan *et al.*, 1994). Finally, a Trbp111-like region is found as the C-terminal region of a 310 amino acid protein (EMAPII) that has been identified as a cytokine in humans and rodents (Kao *et al.*, 1992). Thus, the heterogeneous distribution of the Trbp111 domain across a wide range of species and functions suggests that it is a structural scaffold that can bind tRNA but has also been adapted for non-tRNA-specific roles. Consistent with this interpretation, the affinity for tRNA of EMAPII is low (~40 μ M) compared with the affinity for tRNA of Arc1p or Trbp111 (~10–30 nM).

The sequence encoding Trbp111 was originally designated as the coding sequence for MetRSb. It was thought that the MetRSb protein (i.e. Trbp111) associated with MetRSa. The latter is homologous to the catalytic domain of the class I methionyl-tRNA synthetase. We cloned and analyzed MetRSa from *A. aeolicus* and found that it was a fully functional enzyme whose activity was not enhanced by the MetRSb protein (Trbp111) (not shown). Thus, we believe that Trbp111 is not a part of a functional MetRS but rather possesses a more general function involving tRNA binding. The ability to bind many different tRNAs is consistent with this interpretation. One possibility is that it acts in the high temperature environment of *A. aeolicus* as a tRNA-specific chaperone that stabilizes the L-shaped structure.

From an evolutionary standpoint, the occurrence of Trbp111 in a potentially primitive organism like *A. aeolicus*, and the presence of a Trbp111-like domain within proteins of higher organisms, raises the possibility that Trbp111 was developed early in evolution. Initially, the protein may have stabilized the emerging tRNA structure that is universally used for coded protein synthesis. Eventually, a Trbp111-like domain was incorporated into proteins that interact with tRNAs, such as specific tRNA synthetases (e.g. MetRS, TyrRS and PheRS) and Arc1p. In these ways, Trbp111 may have played a critical and longstanding role in the establishment and stabilization of the L-shaped tRNA structure as the cornerstone molecule of the translational apparatus (RajBhandary and Söhl, 1996).

Materials and methods

Cloning

The coding sequence for Trbp111 was PCR-amplified from *A. aeolicus* genomic DNA (a gift from Diversa Corp., San Diego, CA) using the primers CCATGGGATCCGCATGCATATGGAAGAAAAGGC and CCT-CCGCGTCTAGATTATCAACTGAGTTTAGCCC to incorporate *Nde*I and *Xba*I sites, respectively. This DNA fragment was then cloned behind a *taq* promoter (for expression in *E. coli*) into plasmid pTSMg22 to create pTrbp111-6H which adds a sequence encoding an N-terminal His₆ [based loosely on pQE60 (Qiagen, Santa Clarita, CA)] and pGEX (Promega, Madison, WI) (R.J. Turner and P. Schimmel, unpublished data). The fragment was also transferred to a plasmid (pTSMg32) without the 6_{His} encoding tag (R.J. Turner and P. Schimmel, unpublished data) to create plasmid pTrbp111.

Purification of *A. aeolicus* Trbp111

Escherichia coli TG1 cells (Amersham Corporation, Arlington Heights, IL) containing the pTrbp111 plasmid were grown to an OD₆₀₀ of 0.5–0.8 and induced with 0.5 mM isopropyl- β -D-thiogalactoside (IPTG) for 3–5 h. Cultures were harvested at 5000 r.p.m. for 20 min and frozen. Per liter of original culture, cells were resuspended in 25 ml of 20 mM Tris (pH 7.4), 20 mM NaCl, 1 mM EDTA and 5 mM β -mercaptoethanol. Disruption of cells was carried out using a French Press at 15 000 p.s.i. The crude extract was then cleared of cellular debris by centrifugation at 100 000 g for 30 min.

Clarified extracts were heated to 80°C for 20–25 min and centrifuged at 250 000 g for 60 min. The resulting supernatant was loaded onto a 10/10 Mono-Q column (Pharmacia, Piscataway, NJ) and eluted with a 0–100% 1 M NaCl gradient. Fractions containing Trbp111 were then pooled and incubated overnight at 20°C with 0.1 mg/ml RNase A to digest any bound RNA. This sample was subsequently re-loaded onto a 10/10 Mono-Q column and eluted with a 0–100% 1 M NaCl gradient. Resulting fractions (~200–400 mM NaCl) were pooled, concentrated and dialyzed against 20 mM Tris, 20 mM NaCl and 1 mM EDTA (pH 7.4). They were subsequently run on a Superose 12 column (Pharmacia), and concentrated using a Centrprep-3 concentrator (Amicon, Inc., Beverly, MA). This process resulted in >95% purity as estimated by SDS-PAGE and Tricine-SDS-PAGE (Schagger and von Jagow, 1987).

Circular dichroism (CD)

CD spectra were obtained using an AVIV Instruments CD Spectrophotometer Model 62A DS (Lakewood, NJ). All experiments were carried out in 10 mM Na phosphate, 100 mM Na fluoride buffer (pH 7.0). The sample was placed in a 0.5 mm cell and scans were taken between 190 and 260 nm. Temperature scans were obtained between 4 and 80°C with 10–20 min incubation between scans.

Labeling and purification of [5'-³²P]tRNA^{Met}

Purified tRNA^{Met} was obtained from Sigma (Sigma Chemical Company, St Louis, MO) and incubated with calf intestinal phosphatase for 30 min at 30°C to remove the 5' terminal phosphate. This was followed by a phenol:chloroform extraction (pH 4.5) and ethanol precipitation in the presence of 100 mM NaOAc (pH 5.2). The tRNA^{Met} was then treated with T4 polynucleotide kinase (New England Biolabs, Beverly, MA) for 30 min at 37°C in the presence of [γ -³²P]ATP (Silberklang *et al.*, 1979). The labeled RNA was extracted using a Centri-Spin 20 column (Princeton Separations, Adelphia, NJ). Radioactively-labeled tRNA^{Met} was electrophoresed on a 1.5 mm thick, 10% 19:1 acrylamide:bis-acrylamide native gel (Hoefer SE260 apparatus, Hoefer Scientific, San Francisco, CA) in 50 mM Tris-borate (pH 7.0), 0.0001% Triton X-100 and 5 mM MgCl₂. The labeled tRNA^{Met} was identified by autoradiography, excised from the gel, and eluted for 16 h at 37°C using 0.5 M NH₄OAc, 1 mM EDTA (pH 5.2). The eluted tRNA was ethanol-precipitated and resuspended in an appropriate volume of Tris-EDTA (pH 7.0).

Binding assays

Gel-motility shift assays were performed using a variation of the method described by Carey (1991). Trbp111, at increasing concentrations, was mixed at 4°C with 1–2 nM 5'-³²P-labeled tRNA^{Met} for 20 min. The reaction was carried out in a 20 μ l volume containing 50 mM Tris-borate (TB) buffer with 0.001% Triton X-100, 5 mM MgCl₂ and 0.2 U/ μ l RNasin (Promega Corp., Madison, WI). A 20% sucrose solution (10 μ l) containing tracer dyes was added immediately before loading. The final (20 μ l) mix was then loaded on a 1.5 mm thick, 10% 19:1 acrylamide:bis-acrylamide native gel (Hoefer SE260 apparatus) in 0.5 $\times$ Tris-borate, 0.0001% Triton X-100 and 5 mM MgCl₂. The gel was run at 130 V (10–30 mA) for ~3 h in the cold room (4°C) and dried onto a Whatmann type 3 filter sheet in a vacuum drier at 80°C for 75 min. (Electrophoresis was performed in a cold environment to prevent overheating of the gel. The CD spectrum of Trbp111 was unchanged between 4–80°C, suggesting that the conformation of Trbp111 is the same at 4 and 80°C.) The gel was exposed for 16 h on a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA). Radioactivity levels were quantified using ImageQuant software (Molecular Dynamics).

We used 1–2 nM tRNA^{Met} in the binding experiments shown, but obtained similar results using a much higher concentration (70 nM) of tRNA^{Met} (not shown). There was always a small amount of tRNA that remained unbound even at the highest concentration of protein (>500 nM). This unbound material is probably due to incorrectly folded or denatured tRNA^{Met}. The unbound tRNA was treated as background and subtracted from the total tRNA.

Competition assays were performed as described above except that

75 nM Trbp111 dimer was incubated with the competitor for 20 min at 4°C prior to incubation with radioactive tRNA^{Met}.

Oligomeric size determination by sedimentation velocity

Trbp111 (400 µl) at a concentration of 15 µM was placed in a double sector cell consisting of a 12 mm Epon centerpiece and quartz windows. The cell was mounted in an An60Ti rotor in a Beckman XL-I analytical ultracentrifuge and spun at 40 000 r.p.m. at 4°C. Absorbance and interference data were collected in continuous mode with a step size of 0.005 cm.

The absorbance data was analyzed and modeled using the program Svedberg, developed by Philo (1997). This program employs a direct fitting analysis. Multiple data sets were simultaneously fitted to the Lamm equation, using the sedimentation coefficient (s), the diffusion coefficient (D), and the loading concentration as variables (Philo, 1994). Using this method, we calculated the molecular weight of the species using the following equation:

$$MW = \frac{sRT}{D(1-\bar{v}\rho)} \quad (1)$$

Where $\bar{v}$ is the partial specific volume of the species (which can be calculated from the amino acid composition), ρ is the density of the buffer, R is the universal gas constant and T is the temperature. The partial specific volume ($\bar{v}$) was calculated as 0.7616 using the program Sednterp (Laue, 1992), which calculates hydrodynamic values based on chemical composition. The density of the buffer (50 mM Tris, 50 mM boric acid, 5 mM MgCl₂) was measured using a 10 ml pycnometer as 1.0016 g/l. Scans were collected continuously during the entire experiment. Nine data sets were used in the fitting process corresponding to every thirtieth scan (~30 min apart). Similar fits were performed with 20–40 data sets. Data were also fit keeping the molecular weight constant at expected monomer, dimer and trimer sizes for comparison purposes.

Determination of RNA–protein complex stoichiometry by sedimentation equilibrium

Sedimentation equilibrium experiments were performed in a double sector Epon Cell in an An60Ti rotor on a Beckman XL-I analytical ultracentrifuge. Sample (100–150 µl) was spun at 10 000 r.p.m. at 4°C until equilibrium was reached as determined by superimposition of scans 2–3 h apart. Equilibrium data at 10 000 r.p.m. were then analyzed by a non-linear least-squares analysis using a customized version of Origin Software (Microcal) provided by Beckman Instruments.

The tRNA^{Met} absorbs strongly at 260 nm with an extinction coefficient (ϵ) of 726 700 M⁻¹×cm. In contrast, Trbp111 has two tyrosines, two phenylalanines and no tryptophans. It has a small extinction coefficient of ~1550 L/mol×cm at 260 nm. Thus, the contribution to the absorption at 260 nm by Trbp111 in the presence of tRNA^{Met} is negligible. This allowed simplification by fitting for the molecular weight of one species (tRNA^{Met}). A change in the apparent molecular weight of tRNA^{Met} monitors complex formation.

We determined the molecular weight of tRNA (and a possible complex with Trbp111) by following the absorbance at 260 nm using the following equation (McRoie, 1993):

$$A = e^{\ln(A_0) + \{(\omega^2(1-\bar{v}\rho)/2RT)\}(x^2-x_0^2)} + E \quad (2)$$

This equation allows fitting for the molecular weight (M) where A is the absorbance (at 260 nm) at radius x , A_0 is the absorbance at the meniscus (radius = x_0), and ω is the angular velocity. The constants used for fitting were the universal gas constant ($R = 8.314 \times 10^7$ ergs/mol) and the temperature T . In addition, E is the base-line error correction factor. The density of the solvent was measured using a 10 ml pycnometer to be 1.0016 g/l. For a complex of Trbp111 with tRNA, the partial specific volume $\bar{v}$ was calculated to be the average for a complex of the two species (0.53 for RNA and 0.7616 for Trbp111) and results in a $\bar{v}$ of 0.6458. The fit was evaluated by inspection of the distribution and magnitude of the residuals, which are defined as the differences between the fitted function and the actual data points.

RNA oligonucleotides

Microhelix^{Met}: 'CGCCGGGUUCGAAUCCCGGCAACCA' was synthesized using a Pharmacia Gene Assembler Special as described by Musier-Forsyth *et al.* (1991). Other synthetic RNAs included Dup^{Met}CI-A72 ['CGCUACGACAGG' and 'CCCGUCGUAGCAACCA' (a duplex based on the tRNA^{Met} acceptor stem with a C1–A72 misspair engineered to resemble the tRNA^{Met} acceptor stem)], Dup^{Met}CI–G72, and AC stem-

loop (GUCGGGCUCAUAACCCGAC—based on the anticodon stem and loop of tRNA^{Met}). These oligonucleotides were kindly provided by Dr R.Alexander (The Scripps Research Institute).

Purified *E.coli* tRNA^{Ile} was provided by Dr T.Nomanbhoy (The Scripps Research Institute) and *E.coli* tRNA^{Ile} transcript was provided by Dr M.Farrow (The Scripps Research Institute). *Xenopus laevis* (oocyte type) 5S rRNA transcript (GCCTACGGCCACACCACCTGAAAGC-GCCCGATCTCGTCTGATCTCGGAAGCGATCCAGGGCCGGGCTTGGTTAGTACCTGGATGGGAGACCCGCTGGGAATACCGGGTGT-CGTAGGCTTT) was generously provided by L.Neely (The Scripps Research Institute). Finally, purified *E.coli* tRNA^{Met}, tRNA^{Tyr} and tRNA^{Glu} were obtained from Sigma (St Louis, MO).

Crystallization and preliminary X-ray characterization

Trbp111 was crystallized using the vapor diffusion method in a hanging drop setup at room temperature (Weber, 1997). Crystals were grown from a drop made of a mixture of equal volumes of protein solution set at 8.0 mg/ml and a reservoir solution containing 16–20% PEG 10 000, 0.1 M imidazole (pH 7.2). The drop was equilibrated against the reservoir solution for several days. Crystals of various morphologies appeared with dimensions of up to 0.5 × 0.2 × 0.2 mm. Initial X-ray diffraction data were collected in 1° oscillations on a MAR345 image plate (MAR USA, Evanston, IL) mounted on a high brilliance rotating anode generator, Rigaku FR-D (Molecular Structure Corporation, The Woodlands, TX), operating at 5 kW. The crystals diffract to 2.8 Å resolution with an apparent mosaicity of 0.8°. They belong to space group C2 with unit cell dimensions $a = 149.4$ Å, $b = 72.68$ Å, $c = 72.04$ Å, $\beta = 95.17^\circ$. X-ray data were 84% complete to 2.8 Å (79% complete in 3.01–2.80 Å resolution shell) and overall R -merge was 0.12. Initial assumption of two-molecules in the asymmetric unit resulted in a calculated V_m of 8.0 Å³/Da, and solvent content of 81%. A self-rotation search was performed using the Crystallography & NMR System (CNS) software package (Brünger *et al.*, 1998) to locate a possible non-crystallographic dyad axis relating the molecules in the asymmetric unit. A real-space Patterson-search method (Huber, 1995) was employed using data in the resolution range 15.0–4.0 Å and the unique axis along b . The self-rotation function was carried out by varying ψ and ϕ spherical polar angles in steps of 2° in the range $\psi = 0$ –180°, $\phi = 0$ –180° while restricting κ , the rotation angle around the putative non-crystallographic rotation axis, to 160°–180°. Similar rotation searches were done with κ set to the vicinity of 90° and 120°. The result of the self-rotation function analysis revealed two 2-fold axes, yielding a solvent content of 69% and four molecules in the asymmetric unit.

Acknowledgements

We are grateful to an anonymous reviewer for an important comment about the interpretation of Figure 3. We thank Dr R.J.Turner for technical assistance during cloning and for helpful discussions. We are also grateful to Drs L.Ribas de Pouplana, C.C.Wang and K.Wakasugi and all members of the Schimmel laboratory for helpful discussions and H.A.Lashuel for help with the analytical ultracentrifuge. We thank Dr Ronald V.Swanson and the Diversa Corporation for providing us with *A.aeolicus* genomic DNA. We appreciate X-ray data collection time provided by Drs Xiaoping Dai and Xiang Nguyen-Huu. This work was supported by grant numbers GM15539 and GM23562 from the National Institutes of Health.

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Received March 18, 1999; revised and accepted April 27, 1999