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Chemical Synthesis and Biological Investigation of a 77-mer Oligoribonucleotide with a Sequence Corresponding to *E. coli* tRNA^{Asp}

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Abstract—A 77-mer RNA with the sequence of *Escherichia coli* tRNA^{Asp} has been chemically synthesised using standard automated phosphoramidite chemistry with the coupling reagent 4,5-dicyanoimidazole (DCI). The synthesis was carried out on a 1000 Å CPG-column and, after deprotection and gel purification, a yield of about 7 nmol with a purity of >95% was reproducibly obtained. By comparing automated synthesis of the 77-mer RNA using 1H-tetrazole and DCI as activator, DCI is advantageous in producing longer RNAs. However, for shorter RNAs (<40 mer) no difference could be observed. In addition to the all-ribo tRNA^{Asp} carrying the wild-type sequence, two variants were synthesised, one with a single C to G48 mutation and the second with a 2′-deoxy modification at C48. The three tRNAs were tested for their aminoacylation efficiency and high affinity binding to *E. coli* RNase P RNA. The results demonstrate that chemically synthesised 77-mer oligoribonucleotides can be successfully used for structure–function studies.

Introduction

tRNA molecules can be produced by in vitro transcription using T7 RNA polymerase or by chemical synthesis of RNA fragments which are ligated to a full-length tRNA. Chemical synthesis of long RNAs is a powerful method for site-specific labelling and it is therefore of interest to improve both yield and activity of these RNAs. The application of modified oligonucleotides has been reviewed recently by Verma and Eckstein.[1]

Synthesis of tRNA for functional studies by in vitro transcription is so far the most common strategy. However, limitations associated with T7 RNA polymerase are poor yields in the case of transcripts lacking G residues at their 5′-ends[2] and 3′-heterogeneity of transcripts.[3] It has also been shown that T7 RNA polymerase may produce errors at the 5′-end of transcripts.[4]

To date several studies have reported the chemical synthesis of full-length tRNAs or shorter oligoribonucleotides that were assembled to the full-length tRNA by enzymatic ligation. *Escherichia coli* tRNA$_f^{Met}$,[5,6] yeast tRNA^{Ala} [7] and *Ascaris suum* mitochondrial tRNA^{Met} [8] were prepared by enzymatic ligation of chemically synthesised oligoribonucleotides. Only *E. coli* tRNA$_f^{Met}$,[9] yeast tRNA^{Met},[10] *E. coli* tRNA^{Ala},[11] yeast tRNA^{Phe},[12] *E. coli* tRNA^{Gly} [12] and *Ascaris suum* mitochondrial tRNA^{Met} [13] were chemically synthesised as a single RNA chain. However, previous characterisations of chemically synthesised full-length tRNAs were restricted to aminoacylation as the sole functional assay,[9–12] did not include a comprehensive kinetic analysis of aminoacylation,[9–12] focused on significantly shorter mitochondrial tRNA species,[13] and/or used native modified tRNA instead of in vitro transcribed unmodified tRNA as the control for the chemically synthesised unmodified tRNA.[9–11] In addition, the synthesis of longer RNAs is still difficult in terms of reproducibility, yields and time consumption despite advances in automated solid-phase synthesis.

In a recent study a new activator for the coupling step of phosphoramidites to the 5′-hydroxyl group during the synthesis has been presented.[14] The new activator 4,5-dicyanoimidazole (DCI) is twice as fast as the traditionally used 1H-tetrazole. Here we report the use of DCI for the synthesis of an RNA sequence corresponding to the

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E. coli $tRNA^{Asp}$. Our synthesis protocol improves synthesis efficiency and reproducibility. We also demonstrate that the chemically sythesised full-length tRNAs are of a quality highly suitable for structure–function studies, as inferred from the kinetic parameters of aminoacylation and specific binding to bacterial RNase P RNA.

Results

Chemical synthesis

We have chemically synthesised a 77-mer oligoribonucleotide with the sequence of *E. coli* $tRNA^{Asp}$ (Fig. 1), and two variants thereof. No difference in yield was observed with either benzoyl- or *tert*-butylphenoxyacetyl-protection of the exocyclic amino function of adenosine. Standard RNA synthesis conditions were used except that the traditionally used activator 1H-tetrazole was replaced with a 0.5 M solution of DCI in acetonitrile. The coupling time was 8 min for the ribonucleotides and 30 s for the deoxynucleotide. After work up and purification a yield of 5–7 nmol corresponding to about 3–4 A_{260} units of the oligoribonucleotide was obtained with a purity of >95%, as analysed by anion exchange chromatography (data not shown). Synthesised tRNAs were further analysed with respect to their length by analytical 12% denaturing PAGE in comparison with a transcribed *E. coli* $tRNA^{Asp}$ (Fig. 2). The chemically synthesised tRNAs showed the same mobility as the transcribed tRNA.

Deprotection and degradation study of $tRNA^{Asp}$ exposed to TEA-3HF

In order to optimise the deprotection conditions we 5′-^{32}P-end-labelled the chemically synthesised all-ribo tRNA and exposed it to neat TEA-3HF. The crude product was analysed by 12% denaturing PAGE, showing that exposure of the tRNA to TEA-3HF for 20 and 80 h resulted in a 10–15% and 30–40% degradation, respectively (Fig. 3). Despite some degradation a reaction time of 25 h was chosen in order to favour complete deprotection of the 2′-hydroxyl groups.

Kinetic results

The aminoacylation capacity of chemically synthesised all-ribo tRNA, either ligated from two fragments or synthesised as a single 77-nt RNA chain, was compared with that of transcribed $tRNA^{Asp}$. The activity of the first two tRNAs was the same (Table 1), but compared with the transcribed tRNA their k_{cat}/K_m was 2-fold reduced. The K_m value of the transcribed $tRNA^{Asp}$ was 0.8 μM and that of the synthesised tRNAs ranged between 1.3 and 1.5 μM. No differences in k_{cat} values were observed. Different syntheses of the all-ribo $tRNA^{Asp}$ reproducibly showed equal aminoacylation efficiencies. This result encouraged us to synthesise two additional tRNAs with modifications in the variable loop.

Exchanging C48 to G48 in the $tRNA^{Asp}$ system resulted in a 1.7-fold loss of specificity compared with the non-

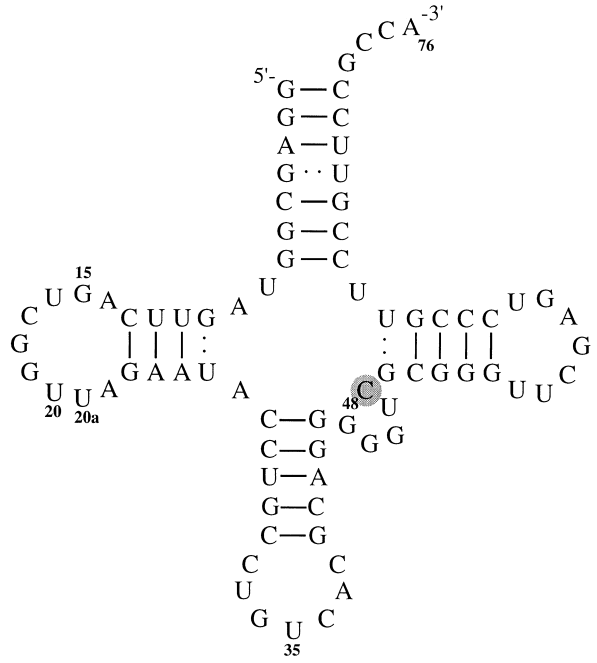

Figure 1. Secondary structure of the *E. coli* $tRNA^{Asp}$ showing position C48 in the variable loop.

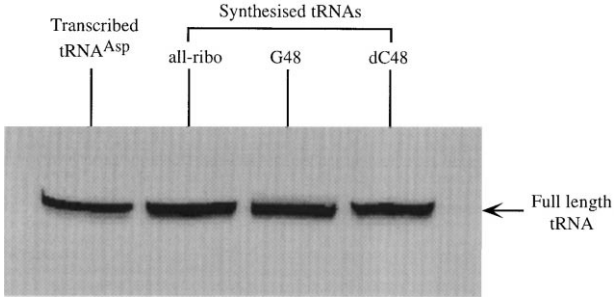

Figure 2. High resolution 12% denaturing PAGE analysis of chemically synthesised $tRNA^{Asp}$ variants in comparison with transcribed $tRNA^{Asp}$. At the end of electrophoresis, tRNAs had migrated about 25 cm into the gel matrix.

mutated counterpart. The K_m was increased about 5-fold and k_{cat} about 3-fold. Exchanging the 2′-hydroxyl group at the C48 position to a 2′-deoxy group resulted in a 1.3-fold loss of specificity compared with the synthesised all-ribo tRNA. The K_m was increased about 2-fold and k_{cat} 1.5-fold.

Gel retardation

Gel-resolvable binding of tRNAs to *E. coli* RNase P RNA has been shown to be very sensitive to structural changes in tRNA molecules,[15,16] particularly to disruptions of its tertiary fold.[17] Therefore, we chose the gel retardation assay to further examine the structural integrity of chemically synthesised $tRNA^{Asp}$ variants. For this purpose, trace amounts (<1 nm) of ^{32}P-end-labelled $tRNA^{Asp}$ were preincubated with increasing amounts of *E. coli* RNase P RNA and analysed by gel electrophoresis on a native 7.5% PAA gel. The transcribed $tRNA^{Asp}$ and the chemically synthesised

Table 1. Kinetic parameters of aminoacylation of transcribed $tRNA^{Asp}$, $tRNA^{Asp}$ prepared by ligation of shorter chemically synthesised RNA fragments to full-length $tRNA^{Asp}$ and chemically synthesised full-length $tRNA^{Asp}$ variants[a]

	K_m (mM)	K_m relative	k_{cat} (s^{-1})	k_{cat} relative	k_{cat}/K_m ($mM^{-1}s^{-1}$)	k_{cat}/K_m relative
Transcribed tRNA	0.8	—	12	—	15	—
Ligated all-ribo tRNA	1.3	—	11	—	8.5	—
Synthesised all-ribo tRNA	1.5	1	10	1	6.7	1
dC48	2.9	1.9	15	1.5	5.2	0.78
G48	8	5.3	32	3.2	4.0	0.60

[a]K_m and k_{cat} determined from Lineweaver–Burk plots. Each experiment was repeated at least three times. Error $\leq$30%.

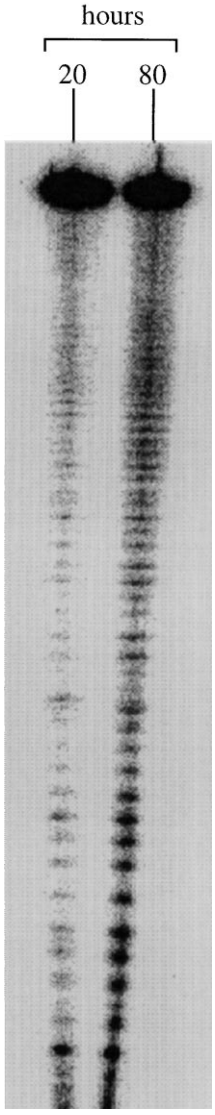

Figure 3. Degradation of a synthesised all-ribo tRNA exposed to neat TEA-3HF for 20 and 80 h, respectively. For further details, see Experimental.

tRNAs, i.e., the all-ribo and the dC48 modified ones, had apparent (app) K_d values between 140 and 200 nm (Table 2). Differences observed between the three tRNA species were statistically insignificant, although a tendency towards slightly higher app K_d values was evident for the chemically synthesised tRNAs. In conclusion, all tRNAs analysed here bound with very similar affinity to RNase P RNA, indicating that the chemically synthesised variants

Table 2. App K_d values for complex formation of transcribed and synthesised $tRNA^{Asp}$ variants with *E. coli* RNase P RNA

tRNA	Transcribed all-ribo	Synthesised all-ribo	Synthesised dC48
App K_d (nM)[a]	140±30	200±40	180±40

[a]App K_d values represent mean values of at least three independent experiments. Deviation between individual experiments are indicated. For further details, see Experimental.

essentially adopt the correct L-folded tertiary conformation. Further, the 2′-hydroxy group at the C48 position is not crucial to the binding of $tRNA^{Asp}$ to *E. coli* RNase P RNA.

Discussion

Chemical synthesis

The versatility of chemical nucleic acid synthesis is due to the easy incorporation of modified nucleotides, including modifications of the phosphodiester backbone, the purine and pyrimidine heterocyclic bases, and the sugar moiety. Also, oligoribonucleotides with site-specific photoactive groups are important tools for structural and conformational studies.[18,19] In a recent study, where we screened the importance of 2′-hydroxyl groups in *E. coli* $tRNA^{Asp}$ for efficient aminoacylation,[20] tRNA variants were assembled by a combination of chemical synthesis and ligation of shorter RNAs to produce the full-length tRNAs. This procedure turned out to be very time-consuming, not always reproducible and its efficiency was largely dependent on the site of ligation. A chemical synthesis without the ligation step was therefore an attractive alternative for the production of full-length tRNAs.

Despite advances in automated solid-phase synthesis it is still difficult to synthesise longer RNAs. Progress has been achieved by changing the protecting groups of the phosphoramidites or by changing the activator for the coupling step.[21,22] We were interested in using commercially available phosphoramidites such as β-cyanoethyl diisopropylphosphoramidites. Here we changed the activator in the coupling step from 1H-tetrazole to DCI, which increased the rate of the coupling step such that a 77-nt oligoribonucleotide could be produced in a significantly shorter time. The purified 77-mer was obtained in highly reproducible yields of 5–7 nmol corresponding to 3–4 A_{260} units within about 25 h. The relatively low

yield can be attributed to some extent to the fact that full-length tRNAs were excised as very narrow bands from preparative gels to avoid any contamination with shorter species. Goodwin et al.[12] reported the chemical synthesis of 12 A_{260} units of $tRNA^{Phe}$, whereas only 4 A_{260} units were obtained for a $tRNA^{Gly}$ when applying the same methodology. This was explained by the presence of a G-rich region in the middle of the $tRNA^{Gly}$, which is also present in the $tRNA^{Asp}$. Thus, it is evident that synthesis yields of longer RNAs are sequence-dependent.

Deprotection and degradation study of tRNA exposed to TEA-3HF

A commonly used reagent to remove the t-butyldimethylsilyl groups (TBDMS) from the 2′-OH groups is tetrabutyl ammonium fluoride (TBAF). The removal is achieved by using 1 M TBAF in THF. However, in our hands it was not possible to obtain complete deprotection of the oligoribonucleotide even though more reagent and longer reaction times were used. A similar observation has been made by Gasparutto et al.[11] In addition, TBAF is very hydroscopic.[23] Lately, neat triethylamine trihydrofluoride (TEA-3HF) has been employed, which turned out to be a more efficient desilylating agent that is less sensitive to water contamination than TBAF.[11,24] Even though it has been reported that exposing RNA to neat TEA-3HF does not result in any significant degradation,[11] we decided to treat a gel-purified 5′-^{32}P-end-labelled tRNA, devoid of any detectable degradation products (not shown), with neat TEA-3HF for 20 versus 80 h (Fig. 3). Exposing the tRNA for 20 h resulted in a 10–15% degradation and this proportion increased to 30–40% after 80 h. Similar degradation problems have been noticed by Wincott et al.[21] We decided to choose a reaction time of 25 h to favour complete deprotection while accepting some degradation.

Functionality of chemically synthesised tRNA

Early attempts to fully or partly chemically synthesise tRNAs resulted in products with low aminoacylating capacity. However, two groups, Gasparutto et al.[11] and Ohtsuki et al.,[13] have reported relatively efficient aminoacylation of chemically synthesised *E. coli* $tRNA^{Ala}$ and *Ascaris suum* mitochondrial $tRNA^{Met}$, respectively. The k_{cat}/K_m for aminoacylation of the $tRNA^{Ala}$, which also carried three naturally occurring modified nucleosides, was 2-fold lower in comparison with bulk *E. coli* tRNA.[11] The 64-nt-long mitochondrial $tRNA^{Met}$ was reported to have methionine-accepting activity 5-fold lower than that of native $tRNA^{Met}$, but equivalent to that of a corresponding $tRNA^{Met}$ transcript.[13] Although the data for the different systems are not directly comparable, our results (Table 1) suggest that the functionality of the chemically synthesised $tRNA^{Asp}$ reported here is at least as good as that of the previously characterised tRNAs obtained by chemical RNA synthesis. The 2-fold reduced aminoacylation efficiency of the chemically synthesised tRNA suggests that exisiting protocols still allow some RNA damage and/or incomplete deprotection.

Gel-resolvable binding to *E. coli* RNase P RNA was employed as a second method to study the function of $tRNA^{Asp}$ variants. The gel retardation analysis did not reveal statistically significant differences for the binding affinity of the chemically synthesised tRNAs versus transcribed tRNA, although a slight tendency of chemical tRNA species towards higher app K_d values may be inferred from the values shown in Table 2. It is well known that the elbow of the L-shaped tRNA, particularly the T-arm, are part of the contact surface between tRNA and bacterial RNase P RNA, and the D-arm is important for a correct positioning of the T-arm.[17,25] Structural perturbations in these regions of the chemically synthesised tRNAs would have been expected to cause significant losses in binding energy, which, however, were not observed. We thus conclude that the majority of chemically synthesised $tRNA^{Asp}$ molecules essentially adopted the same authentic tRNA tertiary fold as the transcribed $tRNA^{Asp}$.

We further introduced structural changes at nucleotide 48 in the variable loop of the $tRNA^{Asp}$, illustrating the potential of chemical synthesis to provide RNA species with single base exchanges or single-site modifications. C48 is expected to form a reversed Watson–Crick base-pair with G15 as in yeast $tRNA^{Phe}$,[26] and the 2′-hydroxy group at this position was shown to be important for efficient charging.[20] Exchanging C48 to dC48 of the same *E. coli* $tRNA^{Asp}$ resulted in a 1.3-fold loss of aminoacylation efficiency. The result is in good agreement with our previous study, where usually a 1.1- to 1.8-fold loss of aminoacylation efficiency was observed when exchanging a functionally important single ribonucleotide for a deoxynucleotide.[20] Exchanging C48 to G48 resulted in a 1.7-fold loss of specificity. The effect is relatively small if compared with other tRNAs, for example the $tRNA^{Pro}$ system, where exchanging C48 to G48 results in a 20-fold loss of efficiency.[27] We conclude that the base identity at position 48 contributes relatively little to synthetase recognition in the *E. coli* $tRNA^{Asp}$ system.

Conclusion

We have been able to chemically synthesise a 77-nt-long RNA with the sequence of *E. coli* $tRNA^{Asp}$ in good and reproducible yields using DCI as an activator in the coupling step. The kinetics of aminoacylation and the affinity to *E. coli* RNase P RNA demonstrate that the tRNAs synthesised according to the methodology presented here are well-suited for structural and functional studies.

Experimental

Materials

The tRNAs were synthesised on an Applied Biosystems 394A DNA synthesiser. Ribonucleoside phosphoramidites were purchased from Perseptive (Germany). CPG-Columns for the tRNA synthesis were either a

0.84 μmol 1000 Å column bearing t-butylphenoxyacetyl protection of the exocyclic amino function of adenosine (rAtBPA) or a 1 μmol 1000 Å CPG-column with a benzoyl protecting group of the exocyclic amino function (rAbz). 4,5-dicyanoimidazole (DCI) and triethylamine hydrofluoride (TEA-3HF) were purchased from Aldrich. [γ-^{32}P]ATP (specific activity approx. 5000 Ci/mmol), [^{32}P]pCp (specific activity approx. 3000 Ci/mmol) and L-[U-^{14}C]aspartic acid (specific activity approx. 216 mCi/mmol) were from Amersham Buchler. T4 polynucleotide kinase (10 U/μL) and T4 RNA ligase (20 U/μL) were purchased from New England Biolabs. RNase Inhibitor (25 U/μL) and calf intestine alkaline phosphatase (1 U/μL) were obtained from MBI Fermentas. The *E. coli* aspartyl-tRNA synthetase (specific activity 10 000 units/mL) was a kind gift of D. Moras (Strasbourg). Anion-exchange HPLC columns (NucleoPac Pa-100 column, 250×4) were obtained from Dionex corporation.

Radiolabelled tRNAs were quantified with a Bio-Imaging Analyzer BAS-1000 (Fujifilm).

Solid phase synthesis and work up

Syntheses were carried out at a 1 μmol or 0.84 μmol scale using either a 1000 Å rAbz column or a 1000 Å rAtBPA column and a 0.5 M filtered solution of DCI in acetonitrile as activator. The coupling times were 8 min for ribonucleotides and 30 s for the deoxynucleotide. The polymer-bound oligoribonucleotide was transferred from the synthesiser column to a 4 mL vial and suspended in a mixture of 3:1 (v/v) (32%) NH_3: EtOH at 55 °C for 6 h for the rAtBPA column and 12 h for the rAbz columns. After cooling on ice for 10 min the supernatant was removed and the solid support was washed twice with double-distilled water. The two combined supernatants containing the oligoribonucleotide were concentrated to dryness in a speed vac, whereupon 500 μL EtOH was added and the oligoribonucleotide was again dried in a speed vac in order to remove any water left from the preceding step. The base-deprotected oligribonucleotide was resuspended in 3.6 mL anhydrous triethylamine trihydrofluoride (TEA-3HF) and reacted for 24 h at rt, whereupon 3.6 mL double-distilled water was added to a final volume of 7.2 mL. The solution was divided into ten 720 μL fractions after rigorous mixing. To each fraction 12 mL 1-butanol was added and stored at −80 °C overnight. The tubes were then centrifuged at 3000 rpm for 15 min at 4 °C. The butanol phase was carefully removed and the resulting pellets were dissolved in 600 μL double-distilled water and concentrated in a speed vac to about 200 μL. The oligoribonucleotides were purified by 10% denaturing PAGE and the full-length band visualised by UV, excised from the gel and eluted using a Bio-Trap elution chamber (Schleicher-Schüll) at 100 V in sterile TBE buffer. Desalting of the oligoribonucleotide was accomplished by using a Sep-Pak C18 cartridge (Waters). The average yield of tRNA was 5–7 nmol. Oligoribonucleotides were analysed by anion-exchange HPLC before and after purification by denaturing PAGE. About 0.3 A_{260} units were analysed and the 77-meric RNA was eluted using a gradient from 20 to 200 mM $NaClO_4$ at 60 °C. The synthesised full-length tRNA and RNase T1 fragments were analysed by mass spectrometry on a Voyager DE Biospectrometer. RNase T1 fragments had the correct molecular weights.

Deprotection and degradation analysis of *E. coli* tRNA^{Asp}

0.06 nmol of chemically synthesised all-ribo tRNA^{Asp} was 5′-end-labelled using T4 polynucleotide kinase and [γ-^{32}P]ATP according to the manufacturer's instructions. The 5′-^{32}P-end-labelled tRNA was purified by 12% denaturing PAGE, excised from the gel, eluted in 1 M NaOAc, pH 4.6, and concentrated by ethanol precipitation. The purified 5′-[^{32}P]tRNA was exposed to 500 μL neat TEA-3HF for 20 or 80 h at rt. Reaction mixtures were precipitated by ethanol and analysed by 12% denaturing PAGE.

In vitro transcription of *E. coli* tRNA^{Asp}

E. coli tRNA^{Asp} transcripts were prepared by using the plasmid AspUC encoding *E. coli* tRNA^{Asp}.[28] Plasmid DNA was linearised with BstNI to obtain runoff transcripts with genuine CCA 3′-termini. Transcription was carried out in 120 mM HEPES–KOH, pH 7.5, 3 mM of each rNTP, 1 mM spermidine, 60 mM $MgCl_2$, 5 mM DTT for 2 h at 37 °C using 50 U/μL of T7 RNA polymerase in a total volume of 300 μL. The tRNAs with authentic 3′-CCA ends were purified by a 12% denaturing PAGE.

5′-Dephosphorylation and end labelling of *E. coli* tRNA^{Asp} transcripts

Transcribed tRNA was dephosphorylated at the 5′-end using calf intestine alkaline phosphatase. Transcripts (400 pmol) were heated to 90 °C for 1 min in 7% aqueous DMF and immediately put on ice before addition of 10 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$ and enzyme (1 U/μL) in a total volume of 100 μL at 37 °C for 1.5 h. Dephosphorylated transcripts were extracted with phenol–chloroform and concentrated by ethanol precipitation. Chemically synthesised tRNAs as well as transcribed tRNA^{Asp} were 3′-end-labelled using T4 RNA ligase and [^{32}P]pCp and 5′-end-labelled using T4 polynucleotide kinase and [γ-^{32}P]ATP according to the manufacturer's instructions.

Enzymatic ligation of tRNA halves to full-length *E. coli* tRNA^{Asp}

The ligation was carried out as described.[20] The ligation site was placed in the anticodon loop between positions 33 and 34.

Aminoacylation kinetics

Aminoacylation reactions were carried out in 50 mM HEPES–KOH, pH 7.5, 30 mM KCl, 20 mM $MgCl_2$, 2.5 mM ATP, and 77 μM L[U-^{14}C] aspartic acid at 37 °C. The concentration range used for transcribed,

ligated and chemically synthesised full-length tRNAs was between 0.3 and 5 μM using a synthetase concentration of 0.158 nM in a total volume of 60 μL as described by Sampson and Uhlenbeck.[29] The kinetic parameters are averages of at least three independent experiments and were derived from Lineweaver–Burk plots using the KaleidaGraph software. Deviations between individual experiments did not exceed ±30%.

Preparation of *E. coli* RNase P RNA and gel retardation analysis

E. coli RNase P RNA was obtained by in vitro transcription essentially as described[30] and using plasmid pJA2′ as the template.[31] Gel retardation experiments and determination of apparent K_d values were performed essentially as described,[15,30] except that the tRNA was preincubated at 55 °C and 37 °C for 10 min and 20 min, respectively, and the RNase P RNA was preincubated at 37 °C for 60 min. Finally, both tRNAs were incubated together at 37 °C for 30 min before gel loading.

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