Demonstration of a tertiary interaction in solution between the extra arm and the D-stem in two different transfer RNA's by NMR

P. J. M. Salemink, T. Yamane* and C. W. Hilbers

Department of Biophysical Chemistry, University of Nijmegen, Nijmegen, Netherlands

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ABSTRACT

According to the X-ray structure of yeast tRNAPhe at 2.5 Å resolution, a hydrogen bond is formed between m^7G_46 and G_22. By removal of this m^7G_46-residue we demonstrate that this interaction is present in solution as well. Comparison of the 1H 360 MHz NMR spectra of intact yeast tRNAPhe and its m^7G-excised derivative locates the position of this tertiary H-bond at 12.5 ppm downfield from DSS. Additional evidence for the presence of this interaction in solution comes from a comparison of 1H NMR spectra of E. coli tRNA^Met and E. coli tRNA^Met, which differ only in a single position in the extra arm. In tRNA^Met residue 47 is a m^7G-residue, whereas in tRNA^Met it is A, resulting in the absence of the m^7G_47-G_23-C_13 triple interaction, characteristic of tRNA^Met. The resonance position of this tertiary interaction in tRNA^Met is located around -13.6 ppm, a chemical shift difference of 1.1 ppm with respect to the position observed for tRNAPhe. The origin of this chemical shift difference is discussed in relation to the structure of their respective augmented D-helices.

INTRODUCTION

Recent X-ray diffraction studies have provided a detailed picture of the 3-dimensional structure of yeast tRNAPhe. In addition to confirming the cloverleaf model as the secondary structure of tRNA, for which there is ample support from a variety of techniques, conclusive evidence for the existence of H-bonded interactions outside the cloverleaf framework became available. It was shown, that the 3-dimensional structure is stabilized by base pairing interactions between the DHU loop and the T\_C loop, between the extra arm and the DHU stem and by other interactions as well. With one exception these H-bond
interactions are not of the classical Watson-Crick type. Since these interactions involve a number of invariant bases, it is reasonable to assume their presence in other tRNA's.

It is crucial to the understanding of the molecular basis of tRNA function in protein synthesis and in other processes to know whether these structural elements are also present in solution. Detection of protons involved in hydrogen bonding is possible using nuclear magnetic resonance techniques by which it has been shown that imino protons involved in hydrogen bonding resonate at low field values well resolved from the resonances of other types of protons. Integration of these low field $^{1}H$ spectra, indicates that both secondary and tertiary H-bonds are present in solution. Additional evidence for the presence of tertiary base pairs had been obtained from earlier melting experiments. These results, in conjunction with spectra predicted from the crystal coordinates on the basis of ring current calculations, provide strong evidence for the existence in solution of the tertiary hydrogen bonds inferred from the crystal structure. Nevertheless independent experimental proof of the existence of particular (tertiary) hydrogen bonds in solution remains necessary to (i) test assignments made on the basis of ring current calculations (ii) to extend conclusions made for yeast tRNA-Phe-structure to other tRNA species and (iii) to provide a basis for the understanding of structure function relationships of tRNA.

Assignments of tertiary resonances have been made by chemical modification experiments and by spinlabeling of tRNA. For instance CNBr-modification of the $s^4U_8$-residue in crude E. coli tRNA and in E. coli tRNA$_{Val}$ led to the assignment of the resonance found around $-14.8$ ppm in E. coli tRNA's to the $A_{14}N_7 - s^4U_8 N_3H$ bond.

The electron density maps of both the orthorhombic and the monoclinic crystal forms of yeast tRNA$_{Phe}$ at 2.5 Å reveal an interaction between $m^7G_{46}$ and $G_{22}$, involving the ring $N_1H$ bond of the former to the ring $N_7$-atom of the latter. In this paper it will be shown that this interaction is present in solution in tRNA$_{Phe}$ as well as in E. coli tRNA$_{Met}$. The chemical shift dif-
ference between the two resonances from these species, is explained on the basis of structural differences in the two tRNA's.

MATERIALS AND METHODS

trNA^{fMet}_1 and trNA^{fMet}_3

tRNA^{fMet}_1 and trNA^{fMet}_3 were purified to homogeneity from unfractionated E. coli B tRNA: the following 4 preparative column chromatographical steps were used in the order indicated: 1. benzoylated DEAE cellulose, pH = 5.0 (Bio-Rad) 2. sephadex DEAE A-50, pH = 7.5 (Pharmacia, Sweden) 3. sepharose 4B, pH = 4.5 (Pharmacia, Sweden) 4. high pressure RPC-5 chromatography, pH = 4.5 (Adogen 464: Ashland Chem. Co.; Plaskon CTFB, 2300 grade: Allied Chem. Corp.).

The activity of these tRNA's was tested by aminoacylation with E. coli methionyl tRNA synthetase, partially purified by sephadex DEAE A-25 chromatography. In addition trNA^{fMet}_1 and trNA^{fMet}_3 were tested in a formylation assay, including the following treatments: after charging the formylmethionine specific tRNA with \(^{35}S\)-labeled methionine and subsequent formylation of the aminoacyl tRNA formed, the product was degraded chemically by incubation at high pH in a triethylamine solution at 37°C resulting in formation of oligonucleotides and free \(^{35}S\)-formylmethionine. Electrophoresis of the radioactive material on Whatman 3 MM paper and subsequent autoradiography yielded the percentage of formylated trNA^{fMet}. trNA^{fMet}_1 was formylatable to approximately 70% and trNA^{fMet}_3 to 90%. Note that these numbers are defined with respect to fully charged Met-trNA^{fMet}.

\(^7\text{G}\)-modification of yeast trNA^{Phe}

Yeast trNA^{Phe} was purchased from Boehringer Mannheim (batch no. 1236526) and used without further purification. Excision of \(^7\text{G}\) was accomplished using a modification of the procedure of Wintemeyer and Zachau. The tRNA was dialyzed.
against a solution containing 0.1 mM EDTA, pH = 7.0, followed by dialysis against distilled water. It was subsequently incubated in a 0.1 M Tris buffer at pH = 9.5 for 4 hours at 37°C. Afterwards the tRNA solution was titrated to pH 4.5 with 5N acetic acid and incubated at 37°C for 3 hours. Aniline HCl, which is usually employed in this second incubation step, was not added to the reaction mixture in order to prevent scission of the phosphate sugar backbone in the extra arm of the tRNA. The resulting product was analyzed in two ways: i) by electrophoresis on a 9% polyacrylamide gel containing formamide to establish the possible presence of 1/4- and 3/4-tRNA fragments resulting from cleavage at position 46. ii) by UV-difference spectroscopy, because the m^7G-residue exhibits an UV absorption band at 292 nm, which is resolved from the chromophore absorption at 260 nm and may serve as a marker for the m^7G removal.\(^{12,15A,15B}\).

In fig. 1A a densitometer scan of a polyacrylamide gel is shown for tRNA\(^{\text{Phe}}\), in which at position 46 the N\(_9\)-C\(_1\) glycosidic bond between the ribose moiety and the guanine base has been cleaved, according to the procedure outlined above, resulting in the loss of m^7G. That only one band is present in the gel,

![Figure 1A](image1.png)

**Figure 1A**
Densitometer scan of a 9% polyacrylamide gel of yeast tRNA\(^{\text{Phe}}\), modified at the m^7G\(_{46}\)-position.

![Figure 1B](image2.png)

**Figure 1B**
UV-difference spectrum between m^7G-modified and unmodified yeast tRNA\(^{\text{Phe}}\) in the spectral range between 250 and 350 nm.
is a strong indication, that after removal of the \( m^7G \) base the phosphate backbone remains intact. This was corroborated in a control experiment, where we observed that 1/4-tRNA fragments and 3/4-tRNA fragments, resulting from undesired backbone scission, are well resolved from the tRNA in the polyacrylamide gel. Thus, the densitogram in fig. 1A clearly establishes the full homogeneity of the macromolecular material and the absence of fragments. Moreover, the position of the \( m^7G \) modified tRNA in the gel is identical to that of unmodified tRNA, indicating a polynucleotide chain of correct size.

Fig. 1B presents an UV-difference spectrum between modified and unmodified tRNA in the spectral range between 250 nm and 350 nm. The chemically treated tRNA served as the reference substance in the spectrometer. This difference spectrum definitely establishes the disappearance of \( m^7G \) upon modification.

Yeast tRNA\(^{\text{Phe}}\) and its \( m^7G \) modified derivative were aminoacylated in a heterologous incorporation assay using partially purified E. coli phenylalanyl tRNA synthetase. The \( m^7G \) modified material displayed aminoacylation properties, very similar to those of intact yeast tRNA\(^{\text{Phe}}\), as shown in fig. 8B.

**NMR-spectra**

High resolution \(^1H\) NMR spectra of yeast tRNA\(^{\text{Phe}}\) were obtained using a Bruker 360 MHz spectrometer, operating in the correlation spectroscopy mode\(^{16,17}\). 2000 scans of 3 sec each were accumulated in a Nicolet BNC 12 computer. The tRNA was dissolved in a buffer, containing 10 mM cacodylate, 10 mM EDTA, 10 mM \( \text{Na}_2\text{S}_2\text{O}_3 \), \( \text{pH} = 7.0 \) and spectra were recorded at \( 35^\circ \text{C} \).

360 MHz \(^1H\) spectra of E. coli tRNA\(^{f\text{Met}}\) were recorded at \( 45^\circ \text{C} \), using the same instrumentation, by accumulating 300-400 scans of 6 sec – 12 sec each. These samples were dissolved in the same buffer, except for the \([\text{Na}_2\text{S}_2\text{O}_3]\), which was now 100 mM.

**RESULTS AND DISCUSSION**

According to the X-ray diffraction studies\(^1-4\) of yeast tRNA\(^{\text{Phe}}\), residue \( m^7G_{46} \) is base paired to \( G_{22} \). The latter also
participates in a normal Watson–Crick base pair with C13 as shown in fig. 2. A hydrogen bond is formed between the N1H-
moiety of m'G46 and the N7-atom of G22. This interaction is also indicated in the cloverleaf model of yeast tRNA Phe by a solid line in fig. 3A. From earlier experiments performed with oligo A-(oligo U)2 we expect this hydrogen bonded proton to resonate in the spectral region between 15 and 10 ppm downfield from DSS 7,18. In E. coli tRNA Met a m'G is present at position 47, which by analogy to yeast tRNA Phe is expected to hydrogen bond to G23 in the DHU-stem (see cloverleaf model in fig. 3B). Consequently a resonance from this triple interaction may be expected in the same spectral region. This was investigated by comparing the low field spectra of these tRNA's with the spectra from corresponding species modified at the m'G position.

Fig. 4 shows the 360 MHz proton spectra of intact tRNA Phe and tRNA Phe -m'G between 15 to 10 ppm downfield from DSS, together with their difference spectrum. The latter was obtained by subtracting spectra A and B after they were scaled. The scaling was performed by equating the low field resonances at -14.4 ppm in spectra 4A and 4B. From the difference spectrum it is clear that intensity is lost around -12.5 ppm after m'G excision. The integrated intensity of the peak in this spectrum corresponds to about 1.2 - 1.3 protons. Moreover, it is evident that removal of the m'G residue causes only small additional

Figure 2
Bonding scheme of m'G46, G22, and C13 in yeast tRNA Phe. The hydrogen bond between m'G46 and G22 discussed in the text is indicated by the large capital H.
Figure 3A
Primary structure of yeast tRNA\(^{\text{Phe}}\) as determined by RajBhandary and Chang\(^{25}\). Solid lines represent tertiary interactions expected to be visible in the NMR-spectrum.

Figure 3B
Primary structure of E. coli tRNA\(^{\text{Met}}\) as determined by Dube et al.\(^{29}\). Solid lines represent tertiary interactions expected to be visible in the NMR-spectrum on the basis of the crystal coordinates of yeast tRNA\(^{\text{Phe}}\). Bonding between G19 - G57 has been assumed. Note, however, that instead the neighboring residue G20 might be involved in this interaction.

Spectral changes. For instance around -13.3 and -13.7 ppm some slight shifts are observed, while around -10.5 and at -13.3 ppm some intensity is lost in the m\(^7\)G deficient material.

In fig. 5 the 360 MHz proton spectra of tRNA\(^{\text{fMet}}\) and tRNA\(^{\text{fMet}}\) are presented together with their difference spectrum. This spectrum is rather different from that in fig. 4. It shows that at -13.6 ppm a resonance with an integrated intensity of 1 proton, which we assign to m\(^7\)G NH, is lost when comparing the tRNA\(^{\text{fMet}}\) -spectrum to the tRNA\(^{\text{fMet}}\) -spectrum. Moreover, another resonance at -13.8 ppm with an integrated intensity of 1.1 protons is shifted 0.1 ppm upfield. In principle this interpretation can be reversed i.e. the peak at -13.8 ppm could
Figure 4. 360 MHz \(^1\)H NMR spectra of intact yeast \(\text{tRNA}^{\text{Phe}}\) (A) and yeast \(\text{tRNA}^{\text{Phe-m7G}}\) (B), together with their difference spectrum (A-B). Positions are given with respect to DSS. The difference spectrum is scaled with respect to spectra A and B.

Figure 5. 360 MHz \(^1\)H NMR spectra of E. coli \(\text{tRNA}^{\text{Met}}\) (1) and E. coli \(\text{tRNA}^{\text{3Met}}\) (2), together with their difference spectrum (1-2). Positions are given with respect to DSS. The difference spectrum is scaled with respect to spectra 1 and 2.

be assigned to the \(m^7G\text{ N}1\text{H}\). We prefer the first interpretation for reasons to be discussed below. Around -12.2 ppm effects of slight shifts due to the \(m^7G_{47} \rightarrow A_{47}\) transition are visible.

The difference spectra in fig. 4 and 5 in conjunction with
the X-ray data obtained for tRNA\textsuperscript{Phe} provide strong evidence that in solution the m\textsuperscript{7}G residues in the extra arms of tRNA\textsuperscript{Phe} and tRNA\textsubscript{fMet} are involved in hydrogen bonding to the DHU stem as indicated in fig. 2. The resonance positions of these hydrogen bonded protons are rather different, i.e. -12.5 ppm and -13.6 ppm and it is interesting to examine possible structural differences between the two tRNA's responsible for this shift. Both tRNA's are so called class 1 tRNA's, sometimes characterized as having a D\textsubscript{4}V\textsubscript{5} structure, i.e. a tRNA with four base pairs in the DHU stem and five bases in the extra arm. They have identical extra arms, consisting of the pentanucleotide sequence: A\textsubscript{24(45)} - G - m\textsuperscript{7}G - U - C\textsubscript{48(49)}. The numbers between the brackets refer to the numbering of the bases in tRNA\textsubscript{fMet} and those outside the brackets to tRNA\textsuperscript{Phe}. The relevant parts of the DHU domains of both tRNA's are given below:

<table>
<thead>
<tr>
<th>Yeast tRNA\textsuperscript{Phe}</th>
<th>E. coli tRNA\textsubscript{fMet}</th>
</tr>
</thead>
<tbody>
<tr>
<td>A\textsubscript{21} \cdots A\textsubscript{14} - U\textsubscript{8}</td>
<td>A\textsubscript{22} \cdots A\textsubscript{14} - s\textsuperscript{4}U\textsubscript{8}</td>
</tr>
<tr>
<td>m\textsuperscript{7}G\textsubscript{46} - G\textsubscript{22} - C\textsubscript{13}</td>
<td>m\textsuperscript{7}G\textsubscript{47} - G\textsubscript{23} - C\textsubscript{13}</td>
</tr>
<tr>
<td>A\textsubscript{9} \cdots A\textsubscript{23} - U\textsubscript{12}</td>
<td>G\textsubscript{9} \cdots C\textsubscript{24} - G\textsubscript{12}</td>
</tr>
<tr>
<td>G\textsubscript{24} - C\textsubscript{11}</td>
<td>U\textsubscript{25} - A\textsubscript{11}</td>
</tr>
<tr>
<td>C\textsubscript{25} - m\textsuperscript{2}G\textsubscript{10} \cdots G\textsubscript{45}</td>
<td>C\textsubscript{26} - G\textsubscript{10} \cdots G\textsubscript{46}</td>
</tr>
</tbody>
</table>

In tRNA\textsuperscript{Phe} the m\textsuperscript{7}G\textsubscript{46} - G\textsubscript{22} - C\textsubscript{13} triple is sandwiched between two triples: A\textsubscript{21} \cdots A\textsubscript{14} - U\textsubscript{8} at one side and A\textsubscript{9} \cdots A\textsubscript{23} - U\textsubscript{12} at the other side. A similar structure is to be expected in tRNA\textsubscript{fMet} i.e. A\textsubscript{22} \cdots A\textsubscript{14} - s\textsuperscript{4}U\textsubscript{8} at one side. However, in position 9 a G residue is found and a concomitant change in positions 24 and 12 to C\textsubscript{24} and G\textsubscript{12} has taken place. In analogy to the structure in tRNA\textsuperscript{Phe} (fig. 6A) Kim\textsuperscript{19}, and Brennan and Sundaralingam\textsuperscript{19} have proposed a hydrogen bonded scheme for this triple (compare fig. 6A and 6B).

It is now well established that the resonance positions of the hydrogen bonded imino protons in tRNA's are mainly determined by the ring currents of the neighboring bases. From our
earlier calculations, based on the Duke model, it follows that the ring current shift contributions to the $m^7G_{46} - N_1H - N_7G_{22}$ hydrogen bonded proton in tRNA$^\text{Phe}$ are comprised of 0.75 ppm from A$_{21}$, and 1.25 ppm from A$_{9}$. Other surrounding bases contribute positive and negative amounts of a few hundredths ppm. The total shift amounts to 2.0 ppm which leads to an intrinsic position of -14.5 ppm for the $m^7G N_1H$ proton in the hydrogen bond.

If the E. coli tRNA$^\text{fMet}$ structure is highly similar to that of yeast tRNA$^\text{Phe}$ we would expect the shift from A$_{22}$ in tRNA$^\text{fMet}$ to be equivalent to that of A$_{21}$ in tRNA$^\text{Phe}$. Instead of A$_{9}$, now G$_9$ would be expected to contribute an estimated shift of 0.5 ppm, when it is substituted for A$_{9}$ and hydrogen bonded as indicated in fig. 6B. This leads to a total shift of approximately 1.2 ppm and a resonance position of -13.3 ppm, i.e. 0.3 ppm to higher field than actually measured. This shift difference indicates the existence of slight structural differences between tRNA$^\text{fMet}$ and the Duke model of tRNA$^\text{Phe}$ around the $m^7G_{47} - G_{23} - C_{13}$ triple (apart of course from the substitutions discussed above). The shift difference may originate from a different overlap between G$_9$ and $m^7G_{47}$ compared to A$_{9}$ over $m^7G_{46}$ in yeast tRNA$^\text{Phe}$ or from a different orientation of A$_{21(22)}$ with respect to $m^7G_{46(47)}$ in the two tRNA's.

In fig. 7 residue A$_9$ is projected onto the plane determined by G$_{22}$, showing the orientation of A$_9$ with respect to the
$^{7}$GN$_1$ proton. Fig. 7A is based upon the crystal data of Sussman and Kim$^{20,21}$, while fig. 7B presents the orientation derived from the coordinates of Rich and coworkers$^{22,23}$. The ring current contribution of A$_9$ to the $^7$G$_{46}$ ring NH proton reduces from 1.2 to 0.9 ppm in the latter model. Given this difference between the two models of tRNAPhe the difference of 0.3 ppm obtained for the calculated and measured position in tRNA$^{fMet}_1$ on the basis of the Duke model leads us to conclude that the base overlap around $^7$G$_{47}$ in tRNA$^{fMet}_1$ is highly similar to the base overlap in the analogous region in yeast tRNA$^{Phe}$.

The difference in folding of the $^7$G-deficient tRNA's and the tRNA's from which they are derived seems to be negligible. Removal of the $^7$G$_{46}$ in tRNAPhe results in the disappearance of one resonance. According to our calculations this residue does not, via its ring current, contribute to the position of nearby hydrogen bonded protons. As expected no shifted resonances are observed in the difference spectrum (see fig. 4). In going from tRNA$^{fMet}_1$ to tRNA$^{fMet}_3$, the change $^7$G$_{47}$ to A$_{47}$ results, apart from the loss of one resonance at -13.6 ppm, in an

![Diagram](image)

**Figure 7**
Projection of A$_9$ onto the plane, determined by G$_{22}$ as computed from the model of Kim$^{20}$ (fig. 7A) and Rich$^{22}$ (fig. 7B). In space A$_9$ is located above the plane through G$_{22}$. 
upfield shift of 0.1 ppm of the resonance at -13.8 ppm. Originally this resonance was attributed to AU_{11}^{24}. If we assume that A_{47} in tRNA^{\text{Met}} occupies the cavity where in tRNA^{\text{Met}} the m^7G_{47} is situated an upfield shift of 0.1 ppm of AU_{11} under the influence of A_{47} is not unreasonable. This was the reason for the assignment of the resonance at -13.6 ppm to m^7G_{47} N_1 H. A reversal of this assignment, indicated above, will not affect the conclusion concerning the similarity of the tRNA^{\text{Phe}} and tRNA^{\text{Met}} structure around the m^7G_{47}...G_{23} - C_{13} triple. Furthermore we can say that structural differences between the isoacceptors tRNA^{\text{Met}} and tRNA^{\text{Met}} are negligibly small on the basis of the imino proton spectra.

Recently in a series of elegant experiments Daniel and Cohn made a NMR study of tRNA^{\text{Met}} and tRNA^{\text{Met}} in which they assigned the m^7G_{47} N_1 H resonance indirectly using spinlabeled material^{11,25}. This yielded a position of -13.3 ppm for this tertiary hydrogen bonded proton, i.e. 0.3 ppm upfield from the position obtained in the present experiments. Somewhat surprisingly, however, they find that four tertiary hydrogen bonded resonances from tRNA^{\text{Met}} are shifted 0.3 ppm upfield with respect to the positions obtained for tRNA^{\text{Met}}. This is particularly evident for the resonance at lowest field (-14.8 ppm in tRNA^{\text{Met}}) assigned to s^4U_{8} - A_{14}. We did not find such shifts and thus no substantial structural differences between the two isoacceptors. It is known, however, that the 3-dimensional folding of tRNA^{\text{Met}} is rather sensitive to solution conditions. In the absence of Mg^{++} ions, the optical melting temperature, corresponding to the disruption of tertiary structure, is 16°C lower for tRNA^{\text{Met}} than for tRNA^{\text{Met}}. Moreover, it is evident from the charging kinetics of the two species, that under our assay conditions the aminoacylation of tRNA^{\text{Met}} and tRNA^{\text{Met}} is rather different (fig. 8A; see also ref. 11). To assess the importance of m^7G in an independent way, the aminoacylation of yeast tRNA^{\text{Phe}} was compared with that of yeast tRNA^{\text{Phe}}-m^7G in a heterologous incorporation assay using E. coli phenylalanyl tRNA synthetase as shown in fig. 8B. The absence of m^7G has percentage wise significantly less influence in this instance. The relation between the chargeability and structure of tRNA's is the subject of further investigation.
Comparison of aminoacylation properties of E. coli tRNA^{Met} and E. coli tRNA^{3Met} using partially purified E. coli methionyl tRNA synthetase (fig. 8A). Comparison of aminoacylation properties of yeast tRNA and yeast tRNA-m7G using partially purified E. coli phenylalanyl tRNA synthetase (fig. 8B).

In conclusion we have found that a combination of specific chemical modifications and \(^1\)H NMR is able to reveal the existence of particular structural features of tRNA in solution, e.g. the presence of a tertiary m\(^7\)G\(46\) - G\(22\) hydrogen bond interaction between the extra arm and the D-stem. Moreover, it is shown that the structure of other tRNA's can be related to the structure of yeast tRNA\(^{Phe}\).

After this manuscript had been finished, a paper on the tertiary structure of yeast tRNA\(^{Phe}\) in solution appeared, in which the resonance position of the m\(^7\)G\(46\) - G\(22\) hydrogen bond was calculated near -12.5 ppm\(^{27}\). This is in excellent agreement with our assignment of this tertiary H-bond, based upon chemical excision of the m\(^7\)G\(46\)-residue.

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Present address: Bell Laboratories, Murray Hill, N.J., USA.

REFERENCES
