Nuclear magnetic resonance studies on yeast tRNA\textsuperscript{Phe}.II. Assignment of the iminoproton resonances of the anticodon and T stem by means of nuclear Overhauser effect experiments at 500 MHz

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ABSTRACT

Resonances of the water exchangeable iminoprotons of the T and anticodon stem of yeast tRNA\textsuperscript{Phe} were assigned by means of Nuclear Overhauser Effects (NOE's). Together with our previous assignments of iminoproton resonances from the acceptor and D stem (A. Heerschap, C.A.G. Haasnoot and C.W. Hilbers (1982) Nucleic Acids Res. 10, 6981-7000) the present results constitute a complete assignment of all resonances of iminoprotons involved in the secondary structure of yeast tRNA\textsuperscript{Phe} with a reliability and spectral resolution not reached heretofore. Separate identification of the methyl-protons in m\textsuperscript{5}C\textsuperscript{40} and m\textsuperscript{5}C\textsuperscript{49} was also possible due to specific NOE patterns in the lowfield part of the spectrum. Our experiments indicate that in solution the Y39 residue in the anticodon stem is orientated in a syn conformation in contrast to the normally observed anti orientation of the uracil base in AU basepairs. Evidence is presented that in solution the acceptor stem is stacked upon the T stem. Furthermore, it turns out that in a similar way the anticodon stem forms a continuous stack with the D stem, but here the m\textsuperscript{2}G26 residue is located between the latter two stems (as is found in the X-ray crystal structure). The stacking of these stems is not strictly dependent on the presence of magnesium ions. NOE experiments show that these structural features are preserved when proceeding from a buffer with magnesium ions to a buffer without magnesium ions although differences in chemical shifts and NOE intensities indicate changes in the conformation of the tRNA.

INTRODUCTION

The discovery by Kearns, Patel and Shulman [1] that the hydrogen bonded iminoprotons in transfer RNA's can be observed by means of NMR has provided a spectroscopic window through which the most important junctions in these macromolecules can be observed. The resonances originating from these protons are found between 15 - 9 ppm downfield from 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) and are well resolved from the bulk of the proton resonances arising from the rest of the molecule. In this part of the spectrum each Watson-Crick basepair is represented by one resonance coming from its hydrogen bonded iminoproton. GU basepairs, because they have two iminoprotons, contribute two resonances. In principle these spectra
contain detailed information on the molecular structure and its dynamics. However, disclosure of this information greatly depends on the reliability by which the resonances can be assigned to particular protons in the molecule. Recently, substantial progress has been made in this area by studying Nuclear Overhauser Effects (NOE's). The application of this method to transfer RNA's was pioneered by Redfield and his coworkers, who for the first time observed NOE's between the two hydrogen bonded iminoprotons in the GU basepair present in the acceptor stem of yeast tRNA$^{\text{Phe}}$ [2]. Subsequently it turned out that -particularly at 500 MHz- NOE's can be observed between the iminoprotons of adjacent basepairs in the double helical regions of tRNA [3,4,5]. Using these internucleotide NOE's highly reliable assignments of the resonances can be achieved in a relatively short time.

Briefly the procedure for these assignments is as follows. After selective presaturation of a proton resonance during a short time (usually about 0.3 s) the NOE manifests itself in the $^1$H NMR spectra of macro-molecules as a reduction in the signal intensity of nearby proton spins. In our experimental set-up, NOE's from protons up to 4 Å away from the pre-irradiated proton can be observed routinely and in suitable cases effects on protons at a distance of about 5 Å are found. The assignment procedure then follows the algorithm described below: a) Find a resonance of a proton that has been reliably assigned earlier or establish such an assignment through a unique NOE relationship. b) Take this resonance as a starting point and generate NOE's from it on other resonances by which these can be assigned to nearby protons. In the low field part of the tRNA spectrum these may be iminoprotons of adjacent basepairs. c) Take the so assigned resonances as new starting points and repeat operation b). In this way it is possible to "walk" through the molecule up to sites where distances between protons are more than ca. 5 Å or insufficient resolution prohibits unambiguous assignments in which case a new starting point is needed.

Recently we have employed 500 MHz NOE experiments in this manner to assign the resonances of the iminoprotons of the acceptor and the D stem of yeast tRNA$^{\text{Phe}}$ [5]. The present paper is a continuation of this work: here we describe the assignments of the iminoprotons of the anticodon and T stem. The results permit a discussion of some interesting structural features observed for yeast tRNA$^{\text{Phe}}$ in solution. All experimental evidence available at present indicates that the Y39 residue in the anticodon stem adopts a syn conformation. Moreover, it turns out that the acceptor stem is costacked.
with the T stem. It is also found that the D stem is stacked upon the anticodon stem with the G26 residue in between.

**MATERIALS AND METHODS**

Yeast tRNA was purchased from Boehringer Mannheim; it had an amino acid acceptance of 1460 pmol/A260 as determined by the manufacturer. The tRNA samples were prepared in two different buffers (for a detailed description see ref.5): buffer 1 containing 80 mM NaCl, 30 mM sodium cacodylate, 5 mM MgCl₂, 0.1 mM EDTA, pH 7.0; buffer 2 containing 400 mM NaCl, 30 mM sodium cacodylate, 0.2 mM EDTA, pH 7.0. The 500 MHz ¹H NMR spectra and the Nuclear Overhauser experiments were performed on a Bruker WM 500 spectrometer, equipped with an Aspect 2000 computer. The water signal was suppressed using a Redfield 214 observation pulse [6] in conjunction with an Alternate Delay Acquisition method [5,7]. The semi-selective Redfield pulse had a total length of 250 µsec and a carrier offset 4000 Hz away from the water resonance. The data were accumulated with an acquisition time of 0.82 s and a relaxation delay of 0.3 s.

The Nuclear Overhauser effects are presented in NOE difference spectra which were obtained by subtraction of a spectrum in which a particular resonance is selectively saturated during 0.3 - 0.4 s from a spectrum in which the saturation power is at an off resonance position [5]. The magnitude of the recorded NOE drops with the inverse sixth power of the distance between two proton spins [8]. Calculations reveal that with a saturation pulse of 0.4 s direct effects between nearest neighbour proton spins in tRNA's (i.e. first order NOE's) may be detectable up to a distance of ca. 5 Å at sufficient signal-to-noise ratio's [5]. Sometimes unexpectedly large NOE's are observed (see results) which we think are second order NOE's. These are defined as a transfer of magnetization between spins mediated by a third spin. Chemical shifts are quoted relative to DSS (2,2-dimethyl-2-silapentane-5-sulfonate) with downfield shifts defined positive. Relevant distances between nearest neighbour iminoprotons and between methylgroups and individual protons were calculated from the proton coordinates derived from three sets of crystal coordinates [9,10,11] (see reference [5]).

**RESULTS**

The sequence of basepairs in the T and anticodon stem of yeast tRNA is schematized in Fig. 1. Relevant distances between different protons, as
FIGURE 1
Schematic representation of the stacking pattern of the basepairs in the T and anticodon stem of yeast tRNA^{Phe}. Iminoprotons are indicated by boldface capitals H's. Other protons discussed in the text are also indicated. Relevant distances (Å) between protons, derived from three different crystal structure coordinates [9,10,11] are given as average values. When the calculated distances differed by more than 0.5 Å from the average value the extreme values are given.

FIGURE 1a
T stem together with the flanking basepair U7A66 from the acceptor stem and the tertiary basepair T54m1A58.

FIGURE 1b
Anticodon stem together with the tertiary basepair m2G26A44 and the first basepair of the D stem m2G10C25. Residue Ψ39 is depicted in a syn conformation (see Fig. 7 and discussion).

calculated from the crystal structure coordinates, are indicated.

For the assignment of the resonances from the acceptor and the D stem in our previous paper [5] the iminoproton resonances of the G4U69 and the tertiary U8A14 basepairs served as starting points. For the anticodon and T stem such unambiguously assigned iminoproton resonances are not available. We therefore turn to the well assigned resonances of the methyl groups which are observed between 0 and 4 ppm [12,13] (see Fig. 2).

Assignment of the iminoproton resonances of the T stem
Both the T and the anticodon stem contain a $^5$C-methyl group in basepairs
FIGURE 2
500 MHz $^1$H NMR spectrum of the methyl "region" of yeast tRNA$^{\text{Phe}}$ (dialysed into a buffer containing 5 mM MgCl$_2$, 100 mM NaCl and 10 mM Na$_2$HPO$_4$; pH 6.9) recorded at 30°C in D$_2$O.

Assignment of the resonances:
1. unknown; 2. m$^2$G10;
3. D16,17(C5H); 4. m$^2$G26;
5. Y37(C11-CH$_3$); 6. m$^5$C40;
7. m$^5$C49; 8. T54.

G30$^m$C40 and m$^5$C49G65 (see Fig. 1), which may be utilized as starting points for the assignment in these two stems. The resonances of the protons of both m$^5$C methyl groups are found at 1.6 ppm [12,13] (resonances 6 and 7 in Fig. 2), separate identification of these resonances has so far, not been possible [13]. Distance calculations (see Fig. 1) show that NOE's can be expected from these methyl groups to the iminoprotons of the adjacent basepairs U7A66 and A31V39. Moreover, NOE's may be observable for the iminoprotons of the basepairs to which these methyl groups are attached, i.e. basepairs G30$^m$C40 and m$^5$C49G65. At 23°C, with the tRNA prepared in buffer 1, resonance 7 was preirradiated and the "iminopart" of the spectrum recorded. In the NOE difference spectrum (Fig. 3b) NOE's are observed at the peaks marked M, H and G. As resonance G has already been assigned to the iminoproton of basepair U7A66 from the acceptor stem [5] and presaturation of resonance 6 does not lead to a NOE on resonance G (U7A66) (see Fig. 3c), it is concluded that the methylprotons of residue m$^5$C49 give rise to resonance 7 (Fig. 2). From chemical shift considerations resonance M is due to GC basepairs. The observation of a NOE at this position thus implies that the iminoproton of m$^5$C49G65 resonates under M. It is noted that this NOE is much larger than the effect observed at peak G (U7A66), which does not seem to be in correspondence with our distance calculations (see Fig. 1a). We think that in addition to first order, second order NOE's contribute to the intensity decrease of peak M. Saturation of magnetization may be transferred efficiently via the two aminoprotons of m$^5$C49 which are situated halfway the methyl group and the iminoproton of m$^5$C49G65 (see Fig. 1a). The remaining NOE observed at peak H is attributed to the other neighbour of basepair m$^5$C49G65, i.e. U50A64. This effect was not expected; according to the crystal structure the distance between the methyl group of
FIGURE 3

NOE experiment at 23°C on yeast tRNA^Phe dissolved in buffer 1. "Iminopart" of the 500 MHz ^1H NMR spectrum as reference spectrum (a); NOE difference spectrum obtained after preirradiation of the methyl resonance marked 7 (b) and of the methyl resonance marked 6 in Fig. 2 (c); NOE difference spectrum obtained after irradiation of peak T (d).
m^5C49 and the iminoproton of U50A64 is more than 7.0 Å (see discussion). In the absence of Mg^{++} ions the methyl resonances marked 6 and 7 are no longer resolved below 35°C (not shown). Preirradiation of these overlapping resonances again results in NOE's at peaks G, H and M (see Fig. 4b) in accordance with the results obtained above. The other NOE's, observed in this experiment, on peaks L and T arise from methyl resonance 6 (see below). Inspection of the iminoproton spectrum in Fig. 3a shows that the resonances so far assigned to the basepair in the T stem severely overlap with other resonances. Therefore we could not proceed unambiguously from these assigned resonances to the non-assigned resonances of the basepairs G51C63 and U52A62. A new starting point was provided by the iminoproton resonance of G53C61, which in the absence of Mg^{++} ions is part of resonance N (indicated in Fig. 4a). This assignment was obtained after preirradiation of the methyl resonance of base T54 which is adjacent to G51C63 (see following paper). Previously we showed that a NOE connection exists between peaks N and D/E (indicated in Fig. 4a; see reference [5]). Peak D/E comprises three resonances of which two were assigned to the iminoprotons of U12A23 and C11G24 [5]. Furthermore it was shown that the NOE observed on resonance N after preirradiating of peak D/E did not arise from these two protons [5]. We therefore conclude that the iminoproton of U52A62 which is adjacent to G53C61 is the third proton that resonates under peak D/E. However, the intensity of the NOE at peak N after preirradiation of D/E and vice versa is appreciably larger (12-16%) than expected from the distance between the iminoprotons of G53C61 and U52A62 (Fig. 1a). This indicates that between these peaks an additional NOE connection exists. Because at the conditions of buffer 2 (absence of Mg^{++} ions) there is no other NOE from U52A62 than to N we conclude that the iminoproton from G51C63, which is the other neighbour of U52A62 also resonates under N. In summary, with the tRNA prepared in buffer 2, we find two resonances under peak N, one belonging to the iminoproton of G53C61 and the other to the iminoproton of G51C63. These assignments were obtained from experiments performed in the absence of Mg^{++} ions (buffer 2). With the tRNA prepared in buffer 1 (with Mg^{++} ions) the situation is somewhat different. After preirradiation of peak D (U52A62), which at these conditions is separated from peak E, we find NOE's under N as well as under M (see reference [5]). In the presence of Mg^{++} ions peak N consists of only one individual iminoproton resonance. This means that in going from a Mg^{++} free to a Mg^{++} containing solution either the resonance of basepair G53C61 or from basepair G51C63 shifts downfield. Preirradiation
FIGURE 4
NOE experiment at 20°C on yeast tRNA^Phè dissolved in buffer 2. Reference spectrum (a); NOE difference spectrum obtained after preirradiation of the overlapping methyl resonances of m^C40 and m^C49 (b) and after preirradiation of resonance G (U7A66) (c).

of the T54 methyl resonance in the presence of Mg^{++} ions demonstrates that it is the resonance from basepair G53C61 that shifts downfield under M (see accompanying paper). Further evidence for this conclusion may be inferred from Fig. 1a: the iminoproton of U52 is further away from the iminoproton of G53 than from the iminoproton of G51 and therefore we expect a difference in NOE intensity between the resonances of G53 and G51. This indeed was found (see reference [5]): the NOE on M (G53C61) is about half as intense as on N (G51C63). Finally both with and without Mg^{++} we find a NOE from peak N to peak H and vice versa (see Fig. 5d) which agrees with the assignment -made above- for the iminoproton of U50A64 to one of the resonances under peak H.

Assignment of the iminoproton resonances of the anticodon stem

In the preceding section resonance 7 (see Fig. 2) was assigned to the methylprotons of m^5C40. Consequently resonance 6 belongs to the methylprotons of m^5C49. Irradiating this resonance results in NOE's on peaks H, L
FIGURE 5
NOE experiment at 28°C on yeast tRNA\textsuperscript{Phe} dissolved in buffer 1. Reference spectrum (a); NOE difference spectra obtained after irradiation of peak U/V (b), peak L (c) and peak H (d).
The vertically expanded part of the latter spectrum around 7 ppm was obtained after irradiating peak H at 38°C.

and T (see Fig. 3c). Following the same line of reasoning, employed for the explanation of the NOE's observed after preirradiation of the m\textsuperscript{5}C\textsuperscript{49} methyl resonance, we ascribe the effect observed at resonance H to the iminoproton participating in the basepairing of A31 and U39 (because the distance of the m\textsuperscript{5}C\textsuperscript{40} methyl group to the iminoproton of U41 is more than 6 Å basepair A29U41 is excluded as a possibility). The iminoproton of basepair G30m\textsuperscript{5}C\textsuperscript{40}
is attributed to resonance L. Again we find a larger effect on the resonance of the basepair to which the methyl group belongs (G30mC49) than on the resonance of the adjacent basepair. As we did above for basepair mC49G65 we attribute this effect to second order contributions to the NOE. On resonance T a NOE is found as well, it is situated in a spectral region where we expect resonances from iminoprotons hydrogen bonded to oxygen atoms [14] or from non-hydrogen bonded iminoprotons sufficiently shielded from water [15]. Only the non-hydrogen bonded iminoproton attached to Y39, either at the N1 or N3 position, is close enough (see Fig. 1b) to explain the NOE observed on peak T. We will return to this problem in the next section.

The next step in the assignment of iminoproton resonances of the anti-codon stem makes use of the ring N proton of mG26. As described in the accompanying paper irradiation of the methylproton resonance of this residue results in the assignment of peak V (see Fig. 5a) to the iminoproton of mG26 (see also Johnston and Redfield [16]). In the presence of Mg ions this peak coincides with resonance U (N1 proton of G4) (see Fig. 5a).

Preirradiation of these combined peaks (see Fig. 5b) gives rise to NOE's at peaks C, K, L, O and R. Previously we have assigned [5]: C(A5U68), O(G3C70), K(mG10C25) and R(N3H of U69). Therefore, taking into account the distances in Fig. 1b, the NOE observed at L must be attributed to the iminoproton of basepair C27G43.

At this point in the assignment procedure it is clarifying to bring out those resonances downfield from 12 ppm that have not yet been characterized. These are (see Fig. 5a): F, one resonance under peak H, two under peak M and two under peak P/Q. In the accompanying paper resonance F is ascribed to m7G46G22, resonance Q to G15C48 and one of the resonances under M to T54m1A58. The iminoprotons from the anticodon stem that have not yet been assigned are those from C28G43 and A29U41. The only reasonable candidate for A29U41 is peak H; resonance M or P are the only reasonable candidates for C28G43. If the iminoproton of A29U41 belongs to peak H we expect a NOE at this position after irradiating the iminoproton resonance of its flanking basepair G30m5C40 (peak L). This indeed was found: in both buffers (with and without Mg ions) preirradiating peak L (G30m5C40) resulted in a NOE on peak H (see Fig. 5c). Furthermore we see an effect on peak P/Q. Because the iminoproton of C27G43 was also found to resonate under peak L and resonance Q is assigned to G15C48 (see next paper) resonance P must be attributed to C28G42. Preirradiation of peak H (U50A64, A29U41 and A31Y39) results in NOE's on peaks L, M, N and P (see Fig. 5d). These are all consistent with
FIGURE 6

NOE experiments on yeast tRNA\textsuperscript{Phe} dissolved in buffer 1. Reference spectrum obtained at 38\textdegree C (a); partial NOE difference spectrum obtained after irradiating peak P/Q at 28\textdegree C (b); NOE difference spectrum after irradiation of peak P at 38\textdegree C. At this temperature the NOE on peak L is less obscured from a "spillover NOE" from peak O to peak M (c).

the assignments established above. The assignment of P to C28G42 was further substantiated by irradiating this resonance. As expected this yielded a NOE on peak H (see Fig. 6b), but also on peak L (see Fig. 6c). The latter effect was best revealed in an experiment performed at 38\textdegree C. The NOE on peak L is in nice agreement with the fact that the iminoproton of the neighbouring basepair C27G43 resonates under peak L.

The resonances from \textsuperscript{43}P

Above we have demonstrated that the iminoprotons of \textsuperscript{43}P give rise to resonances that contribute to peak H and peak T. We have still to establish whether resonance T (or resonance H) originates from the N1H or the N3H proton. If T belongs to the \textsuperscript{43}P N1 proton we would expect a distinct NOE from this resonance to the resonance of the adjacent \textsuperscript{43}P N6 proton (see Fig. 7) in the aromatic part of the spectrum. Such a NOE was found at 7.25 ppm in the case of the \textsuperscript{55}N1 proton (see accompanying paper). Preirradiation of resonance T (see Fig. 3d) did not reveal any NOE in this spectral region. Thus it is quite unlikely that this resonance belongs to the \textsuperscript{43}P N1 proton. Instead it is justified to ascribe it to the \textsuperscript{43}P N3 proton which has no
"aromatic" protons nearby (see Fig. 7). Hence it is the $\Psi^{39}$ N3 proton which is not hydrogen bonded and consequently it must be the $\Psi^{39}$ N1H, resonating under peak H, which is involved in the hydrogen bond with A31. Peak H integrates to three resonances now assigned to basepairs A29U41, U50A64 and A31$\Psi^{39}$. Therefore, upon preirradiation of peak H at least 4 NOE's are expected in the aromatic region between 6.5 and 8 ppm: the iminoprotion resonances of the first two AU basepairs should give NOE's due to their H2 protons, while the $\Psi^{39}$ N1 proton resonance should give a NOE to the $\Psi^{39}$ H6 and A31H2 proton resonance (see Fig. 7). These expectations are indeed borne out by experiment: in the absence of Mg$^{++}$ ions a very pronounced NOE of ca. 70% was found at 6.85 ppm. In the presence of Mg$^{++}$ ions this NOE resolves into four peaks (see Fig. 5d) and in addition we find a NOE at 7.20 ppm.

The assignment of all iminoprotons in the T and anticodon stem are collected in Table I.

DISCUSSION

In this paper the assignment of the resonances of the iminoprotons of the anticodon and the T stem of yeast tRNA$^\text{Phe}$ is established. A similar study has been performed independently by Roy and Redfield (A.G. Redfield, personal communication). Moreover, we have been able to assign resonances 6 and 7 at 1.60 and 1.55 ppm (see Fig. 2) to the methyl groups of $^5$C40 and $^5$C49 respectively. In the present set of assignments we could make only limited use of the step by step procedure by which the iminoprotons of basepairs adjacent to a basepair with a known iminoprotion position can be assigned; a procedure that worked so well previously for the D stem and the acceptor stem [5].
Assignment of iminoproton resonances from the T and anticodon stem in yeast tRNA\textsubscript{Phe}.

<table>
<thead>
<tr>
<th>basepair</th>
<th>spectral marking (Fig. 5a and 4a)</th>
<th>positions (ppm from DSS)</th>
<th>basepair</th>
<th>spectral marking (Fig. 5a and 4a)</th>
<th>positions (ppm from DSS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>m$^5$C49G65</td>
<td>M</td>
<td>12.49 12.44</td>
<td>C27G43</td>
<td>L</td>
<td>12.54 12.52</td>
</tr>
<tr>
<td>U50A64</td>
<td>H</td>
<td>13.21 13.21</td>
<td>C28G42</td>
<td>P</td>
<td>12.14 12.14</td>
</tr>
<tr>
<td>G51C63</td>
<td>N</td>
<td>12.38 12.38</td>
<td>A29U41</td>
<td>H</td>
<td>13.21 13.21</td>
</tr>
<tr>
<td>US2A62</td>
<td>D</td>
<td>13.80 13.73</td>
<td>G30m$^5$C40</td>
<td>L</td>
<td>12.54 12.52</td>
</tr>
<tr>
<td>G53C61</td>
<td>M/N</td>
<td>12.49 12.38</td>
<td>A31Y39(H1)</td>
<td>H</td>
<td>13.21 13.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(H3)</td>
<td>T</td>
<td>10.58 10.58</td>
</tr>
</tbody>
</table>

Numbers indicate the buffers used (see materials and methods).

This limitation is mainly a result of the fact that the resonances of the T stem and the anticodon stem are part of overlapping peaks. That the assignments could still be performed successfully is due to the large abundance of methyl groups in yeast tRNA\textsubscript{Phe}. These methyl groups provided a sufficient number of known resonances from which the assignments of the iminoprotions could be started.

From the present study some important structural features of the tRNA molecule in solution can be derived. In the first paper of this series [5] we noted that it was possible to observe a NOE on peak M after irradiating resonance G (U7A66), indicating that the iminoproton of a GC basepair is close to the iminoproton of U7A66. In the present paper we have identified this GC pair as m$^5$C49C65. This was possible after preirradiation of the methyl resonance of the m$^5$C49 residue, which led to NOE's at resonance G (U7A66), at resonance M (m$^5$C49G65) and at resonance H (U50A64). These effects can only be rationalized if basepair m$^5$C49G65 is sufficiently stacked upon basepair U7A66 and we may therefore conclude that in solution the acceptor stem is stacked upon the T stem as is observed in the crystal structure [9,10,11]. It should be realized that this statement has only qualitative significance. From the present experiments we cannot tell whether the extent of basepair overlap and/or the exact distance between the basepairs is exactly the same as in the crystal structure. In this context it is interesting to note that, after preirradiation of the methyl-group of m$^5$C49, we observed a NOE for basepair U50A64 that we did not expect.
on the basis of the distance between the \( m^5C49 \) methylgroup and the N3 proton of U50 (> 7.0 Å as derived from crystal structures). This may be a higher order NOE arising from cross relaxation via the amino- and iminoprotons of \( m^5C49G65 \) to the iminoproton of U50A64. On the other hand it cannot be excluded that the overlap between this basepair and \( m^5C49G65 \) differs in solution from that in the crystal structure.

The results obtained in the absence of Mg\( ^{++} \) ions yield further insight into the properties of yeast tRNA\(^{Phe}\). Again we find a NOE on resonance G (U7A66) after irradiation of the (at these conditions coinciding) methyl group resonances of \( m^5C49 \) and \( m^5C40 \). Furthermore, preirradiation of resonance G results in a NOE on peak M (\( m^5C49G65 \)) (see Fig. 4c). These observations are of interest because it demonstrates that the stacking of the acceptor and T stem is maintained in the absence of Mg\( ^{++} \) ions (at least at 0.4 M Na\(^+ \) ions).

This conclusion was substantiated by prolonged irradiation (0.8 s) of resonance A (U6A67, which is one basepair further away from the acceptor and T stem junction than U7A66). This prolonged irradiation not only gives rise to a NOE on resonance G (U7A66) but also on peak M which can be ascribed to second order NOE's on the iminoproton resonance of \( m^5C49G65 \). Recently, Hare and Reid [17] found that for E.coli tRNA\(^{Ile}\) in solution the T stem is also stacked upon the acceptor stem.

From the present results it is also concluded that the D stem stacks upon the anticodon stem with the \( m^2G26 \) residue sandwiched in between. This conclusion follows directly from an experiment in which the iminoproton resonance V (\( m^2G26 \)) is preirradiated giving rise to NOE's on the iminoproton resonances of \( m^2G10C25 \) and C27G43 (see Fig. 5b). Similar results were obtained when the methyl resonance of \( m^2G26 \) was presaturated (see accompanying paper). Interestingly, the first result could only be obtained in the presence of Mg\( ^{++} \) ions and the second one in the absence of Mg\( ^{++} \) ions. This may indicate some difference in stacking dependent on the presence or absence of Mg\( ^{++} \) ions. It is noted that with and without Mg\( ^{++} \) ions we do not find a NOE from peak L (C27G43) nor from peak K (\( m^2G10C25 \)) to peak V (N1 proton of \( m^2G26 \)). This finding may be understood in terms of an enhanced relaxation of the \( m^2G26 \) iminoproton due to the freely rotating methyl groups nearby (see Fig. 1b), which may impair the building up of the NOE.

From the A31\( ^39 \) basepair in the anticodon stem we assigned peak T and one resonance under peak H to the \( ^39 \) N3 proton and the \( ^39 \) N1 proton resp. This indicates that the \( ^39 \) N1 proton is involved in basepairing with residue A31. Hence \( ^39 \) is in a \textit{syn} orientation which is in contrast to the normally \textit{anti}
conformation observed for uracil bases in AU basepairs (see Fig. 7). It should be realized that the conclusions are based upon the fact that we did not see a NOE from resonance T to the spectral region where the non-exchangeable aromatic ring proton resonances are found, i.e. we did not see a NOE to the V39 H6 which we would have expected if resonance T belonged to the V39 N1 proton (see Figs. 3 and 7). On the other hand we do see four NOE's in this spectral region if resonance H is presaturated (see Fig. 5d). This is the number of NOE's expected if V39 N1H contributes to this resonance. Although conclusions based on not seeing effects must be met with due caution, all evidence presently available points to the fact that V39 adopts a syn orientation (see also Roy et al. [18]). There are a number of examples of tRNA's in which the basepair that closes the anticodon loop is an AΨ basepair. On the basis of the estimated resonance positions of the iminoproton of these basepairs in a number of E.coli tRNA's and on the basis of model compound studies Hurd and Reid [19] proposed that the V39 residue might be in a syn orientation. The present experiments provide a more solid support for this view. For certain tRNA's the presence of the Ψ residue in the anticodon stem appears to be an essential element for the control of the operon which is involved in the regulation of the biosynthesis of the cognate amino acid [20]. For example histidyl tRNA's having a pseudouridine in the anticodon stem are able of repressing the histidine operon [21]. Naturally the replacement of a U residue by a Ψ residue at position number 39 will have chemical consequences, but it might only be functionally significant if this Ψ is orientated in a syn conformation. In that case the anticodon helix has a ring NH donor and carbonyl acceptor available in the major groove contrary to the situation in which the residue is in the anti position where only the ring NH donor is available. If the present results can be extrapolated to the regulating tRNA's this might provide the structural basis for their controlling function.

To conclude the discussion it is interesting and legitimate to ask whether we could have performed the assignment of iminoprotons in all four stems and reached the above conclusions about the stacking of the arms of yeast tRNA Phe without a knowledge of the crystal structure. This question can be answered affirmative with a few reservations. To get a starting point at one end of the D stem it was necessary to know that basepair U8A14 is stacked upon the D stem [5]. Furthermore, it seems difficult to obtain a starting point at the loop side of the T stem without knowledge of the crystal structure at this location. To arrive at the conclusion that the acceptor stem is stacked upon the T stem it is only necessary (in the case...
with Mg$^{++}$ ions) that we assume it is the methyl group of $\text{m}^{5}\text{C}49$ which is close to the acceptor stem and not the methyl group of $\text{m}^{5}\text{C}40$. All evidence so far available from NOE experiments make it very unlikely that this is not so. The conclusion that the $\text{m}^2\text{G}26$ base is stacked upon the D stem follows directly from NMR, independently from the X-ray results. The NOE experiments also demonstrate that the other neighbour of the $\text{m}^2\text{G}26$ base must be a GC basepair. That this is the C27G40 basepair follows from an educated guess based on the cloverleaf structure. Subsequently, NOE experiments then lead to a selfconsistent set of assignments. The conclusion that the $\Psi39$ residue in the anticodon stem adopts a syn conformation was achieved independently from the X-ray results. In fact the crystal structures [9,10,11] were presented with the $\Psi39$ base in an anti orientation although the available X-ray data may be explained in terms of a syn orientation (G.J. Quigley, private communication).

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