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Nuclear magnetic resonance studies on yeast $tRNA^{Phe}$ I. Assignment of the iminoproton resonances of the acceptor and D stem by means of Nuclear Overhauser Effect experiments at 500 MHz

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ABSTRACT

Resonances of the water exchangeable iminoprotons of the acceptor and D stem of yeast $tRNA^{Phe}$ have been assigned by means of Nuclear Overhauser Effects (NOE's). Assignments were made for spectra recorded from tRNA dialysed against a buffer with 110 mM sodium and 5 mM magnesium ions and against a buffer with 430 mM sodium and no magnesium ions. Remarkable is the assignment of a resonance at 13.6 - 13.7 ppm to the iminoproton of C11G24. This assignment as well as those of G1C72, G3C70, U7A66, U12A23 and C13G22 are different from those made previously on the basis of less direct evidence. NOE experiments performed at 45°C support the view that the D stem together with the tertiary interaction U8A14 is one of the most stable parts of the molecule in the presence of magnesium ions. A comparison of the spectra recorded under the two different buffer conditions shows that an excess of 320 mM sodium ions is not capable to force the tRNA in the same conformation as 5 mM magnesium ions can do.

INTRODUCTION

NMR studies of the ^1H spectra of the hydrogen bonded imino protons of tRNA have contributed significantly to our understanding of the structure and dynamics of these molecules in solution. However, up till now it has not been possible to employ to information contained in these spectra to its full extent. The main reason for this is that in most cases the assignments of resonances to particular protons in the molecule could not be made unambiguously. Attempts to assign these resonances have been made using ring current calculations, specific chemical modifications, paramagnetic ions and tRNA fragments, but these approaches have met with only a limited amount of success.

A more satisfactory approach was pioneered by Redfield and coworkers. They successfully employed Nuclear Overhauser Effects (NOE's) to assign specific resonances in the iminoproton spectra of several tRNA's [1,2,3]. Briefly, the Overhauser Effect is the change in integrated intensity of the resonance signal(s) of one set of nuclei as a result of the saturation

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of another set of nuclei by a strong resonant r.f. field. It arises as a result of cross relaxation processes occurring in the spin system.

The employment of the Overhauser effect in the study of relaxational and structural properties of small molecules has a long history [4]. NOE's have also been successfully used in the study of proteins [5,6,7]. Its application to the iminoprotons in nucleic acids, such as tRNA, is more complicated, because the effects have to be observed in the presence of a huge waterpeak; moreover, under some circumstances these iminoprotons are subject to exchange with waterprotons. Most of the NOE's that could be recorded were of the order of 20% [1]. With the advent of a new generation of high resolution NMR spectrometers, operating at 500 MHz, resolution and sensitivity makes the observation of Nuclear Overhauser Effects as small as 2-5% possible on a routine basis. In this way the usefulness of NOE measurements is increased substantially, as it permits the observation of transfer of magnetization between iminoprotons of adjacent basepairs, thus leading to a spectral assignment on pure physical grounds.

We employed the method to assign iminoproton resonances in the 500 MHz ^1H NMR spectrum of $tRNA^{Phe}$ from yeast. The strategy used was to start preirradiating a solidly assigned resonance and then to look for the NOE's to nearby iminoprotons. Subsequently, the so assigned resonances of these iminoprotons were taken as the new starting points. Following this step by step procedure a complete and internally consistent assignment of iminoprotons participating in secondary and tertiary interactions could be obtained. In the first paper of this series the results for the acceptor and D stem of yeast $tRNA^{Phe}$ are presented.

MATERIALS AND METHODS

Preparation of samples

Yeast $tRNA^{Phe}$ was purchased from Boehringer Mannheim; it had an amino-acid acceptance of 1460 pmol/A260 as determined by the manufacturer. The NMR samples were prepared by dialysing small volumes of tRNA (6 mg. in about 280 µl buffer) in microdialysis cells for at least 24 hours against 50-100 ml of buffer at 4°C. The dialysis buffer was changed at least three times. The final dialysis buffer contained 5% D_2O to be used for the spectrometer field/frequency lock. Two different buffers were employed: buffer 1 containing 80 mM NaCl, 30 mM sodiumcacodylate, 5 mM $MgCl_2$, 0.1 mM EDTA, pH=7.0; buffer 2 containing 400 mM NaCl, 30 mM sodiumcacodylate, 0.2 mM EDTA, pH=7.0.

In the preparation of the samples in buffer 2 special care was taken to remove all magnesium ions. They were first dialysed against buffer 2 with 20 mM EDTA at pH=7.5. This treatment was repeated with a similar buffer containing 2 mM instead of 20 mM EDTA, after which the final dialysis was performed. From atomic absorption measurements it was found that the number of moles of Mg^{++} ions per mole of tRNA was less than 0.01. All glassware was acid-washed to avoid contamination with polyvalent ions; suprapur NaCl was used (Merck, Darmstadt).

NMR spectroscopy

^1H NMR spectra at 500 MHz were recorded on a Bruker WM 500 spectrometer operating in the Fourier Transform mode with quadrature detection. Chemical shifts are quoted relative to DSS (2,2-dimethyl-2-silapentane-5-sulfonate). Downfield shifts are defined as positive. Chemical shifts were measured relative to the solvent H_2O peak and converted to the DSS reference by correcting for the H_2O to DSS chemical shift using the appropriate temperature and salt calibration curves. When the tRNA was prepared in buffer 1, spectra were recorded at 28°C and in buffer 2 at 21°C except where stated otherwise.

To avoid excitation of the water resonances the spectra were recorded with a "Redfield 214 pulse" [8] with a total pulse length of 250 μs and a carrier offset 4000 Hz away from the water resonance (acquisition time 0.82 s, relaxation delay 0.3 s). Dynamic range problems posed by the limited computer word length were overcome by a so-called alternate delayed acquisition [9]. Before fourier transformation and phase correction spectra were resolution enhanced by a Lorentzian to Gaussian tranformation [10], except when needed for integration of resonances. No baseline corrections were made.

Nuclear Overhauser effects were measured by employing the pulse sequence $[(RD - Dec(t_1,\omega_A) - FID(+))_{32} - (RD - Dec(t_1,\omega_{off}) - FID(-))_{32}]_n$. The first cycle fo this sequence starts with a relaxation delay of 0.3 s after which a selective preirradiation pulse of duration t_1 is applied by the ^1H decoupler at the chosen resonance frequency ω_A. This is followed by a "Redfield 214 observation pulse" and the resultant FID is recorded in a 16 K channel. 32 FID's are accumulated this way. The second cycle is exactly equal to the first except that the presaturation pulse is applied at an off resonance position ω_{off}. The accumulated FID's from both cycles are stored separately. Subtraction of FID(-) from FID(+) yields the NOE difference FID. To perform proper experiments the preirradiation pulse has to be of

sufficiently low power (35 dB below 0.1 W) to achieve selective saturation of site A and short enough to avoid extensive spin diffusion effects outside the sphere of nearest neighbours to A. In our case a preirradiation time of 0.3 - 0.4 s was chosen. NOE difference spectra were given a linebroadening of 5 - 10 Hz for a better signal to noise ratio.

Calculation of expected NOE's from crystalstructures

The coordinates of carbon and nitrogen atoms from three X-ray studies on crystals of $tRNA^{Phe}$ from yeast [11,12,13] were obtained from The Protein Data bank (Brookhaven, U.S.A.) as deposited there by the authors. These were used to generate proton coordinates by taking standard bond lengths and angles. From the latter coordinates the distances of each proton to its nearest neighbour protons were calculated. Distances between methylgroups and individual protons were calculated by taking the carbon atom of the methyl-groups as the center of these groups. Relevant data are listed in table 1.

From standard theory [4,14] it follows that the magnitude of the NOE is proportional to $1/r^6$. It is of interest to establish which distances may be covered in practice by NOE experiments. With tRNA concentrations of 0.8 mM, about 3 hours of signal accumulation permitted the detection of NOE's as low as 1-2%. Then, using available spin lattice relaxation rate constants for iminoprotons in yeast $tRNA^{Phe}$ [15,16,17] and known rotation correlation times [18,19] it may be shown that first order effects between iminoprotons should be detectable up till a distance of 4.8 - 5.3 Å between these protons. From our calculations of distances it was found that the distances between nearest neighbour iminoprotons may vary from 3 to more than 5 Å. Most are below 5 Å. Again using standard theory it follows that NOE's of 1-10% between adjacent iminoprotons can be expected provided that the crystal

distances in secondary structures	
acceptor stem:	
G 1 – G71	3.8
G71 – G 3	4.1
G 3 – G 4	4.0
G 3 – U69	4.0 – 5.5
G 4 – U69	2.4
G 4 – U68	4.6
U69 – U68	2.5 – 3.9
U68 – U 6	3.5
U 6 – U 7	4.1
U 7 – G65	4.9
D stem:	
G22 – U12	4.7 – 5.8
U12 – G24	3.3
G24 – G10	3.5

distances in tertiary structures	
G15 – U 8	5.1
U 8 – G22	3.1
U 8 – m^7G46	6.8
m^7G46 – G22	5.7
m^7G46 – U12	>7.0
m^2_2G26 – m^2G10	4.5 – >7.0

distances of the m^2G10 methylgroup to nearest neighbour iminoprotons		
methylgroup:	iminoproton:	
m^2G10	m^2G10	4.1
m^2G10	G24	4.5 – 5.7

TABLE 1
Selected distances (Å) between iminoprotons of neighbouring base-pairs. Average values, derived from three different crystal structures (11, 12, 13) are tabulated. When the calculated distances differed by more than 0.5 Å from the average value the extreme values are given.

structure does not largely deviate from the solution structure. Most iminoproton resonances between 9 and 14.5 ppm give first order NOE's of about 5% on other iminoproton resonances, which is in accordance with our general expectation based on the crystal structure.

These relatively small, but distinct NOE's were used for the assignments described below.

RESULTS

To facilitate reading of the text the cloverleaf structure of yeast $tRNA^{Phe}$ is presented in Fig. 1.

Iminoprotons resonate between 15 and 9 ppm. This part of the 500 MHz ^{1}H NMR spectrum of yeast $tRNA^{Phe}$ prepared in buffer 1 and 2 is shown in Figure 2a and 2b respectively. The peaks are lettered A to Z. On the basis of other investigations three types of iminoprotons are expected to resonate in this region: a) Iminoprotons, hydrogen bonded to ring nitrogens have been found between 15 and 11.5 ppm [20]. Each classical Watson and Crick basepair has such a proton; several non classical basepairs, involved in tertiary interactions in tRNA's posses these protons as well. b) Iminoprotons

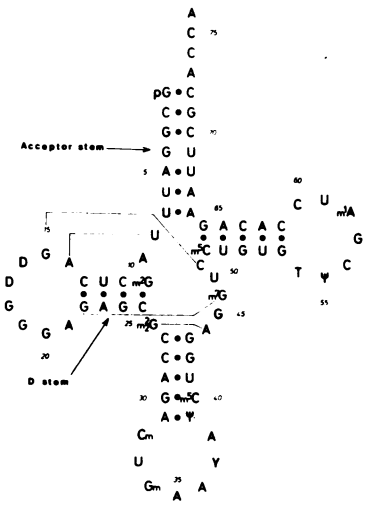

FIGURE 1
Cloverleaf structure of yeast $tRNA^{Phe}$ (according to RajBhandary, U.L. and Chang, S.H. (1968) J. Biol. Chem. 243, 598-608). Solid lines indicate tertiary interactions discussed in the text.

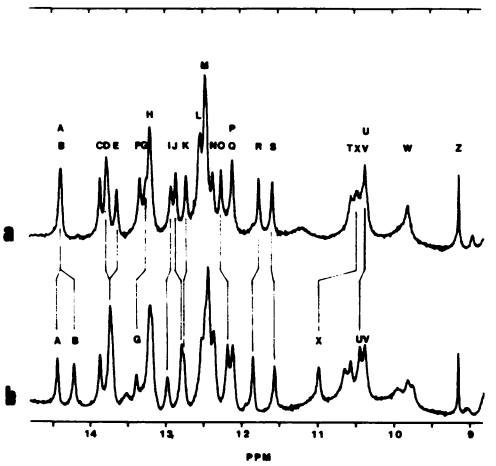

FIGURE 2
500 MHz ^{1}H NMR spectra (2240 accumulations) of the iminoproton "region" of yeast $tRNA^{Phe}$ recorded in the presence of magnesium ions (buffer 1) at 28°C (a) and in the absence of magnesium ions (buffer 2) at 21°C (b).

hydrogen bonded to carbonyl oxygens are found between 10 and 12.5 ppm [20]. GU basepairs contain two such protons. c) Non hydrogen bonded iminoprotons, protected from exchange with waterprotons have also been found in this region [21,22].

Assignment of iminoproton resonances from the acceptor stem

To identify the resonances in a molecule on the basis of NOE experiments it is convenient to have as a starting point a resonance which has been assigned unambiguously. In the case of the acceptor stem of yeast $tRNA^{Phe}$ the iminoprotons of the G4U69 basepair may serve as such. The distance between the iminoprotons of the GU basepair is the smallest of all distances between iminoprotons in the molecule (2.4 Å, as was calculated from crystal structures). Consequently, the NOE between the GU iminoproton resonances can be distinguished from NOE's between other iminoproton resonances by being more intense. This indeed is found and demonstrated in Fig. 3 in an experiment employing a sample dissolved in buffer 1. After preirradiating resonance R (see Fig. 3b), the NOE difference spectrum shown in Fig. 3c was obtained. A NOE of about 25% is measured on resonance U. Irradiation of resonance U gives the same result on resonance R. Because such large NOE's could not be found between other resonances of iminoprotons, the resonances R and U are assigned to the iminoprotons of the GU basepair. This assignment was previously established by Johnston and Redfield [16]. In an analogous experiment we also identified the iminoproton resonances of the GU basepair under the conditions of buffer 2 (see Fig. 4c). The individual assignment of resonance R to the N1 proton of U69 and resonance U to the N1 proton of G4 is based on ring current calculations by Geerdes and Hilbers [23].

In addition to these NOE's of about 25% measured after irradiation of R and U, two smaller NOE's (ca 5%) are observed on resonances C and O (see Figs. 3c and 4c). Because of their positions (see Fig. 3c) we conclude that resonance C must come from the hydrogen bonden ring N proton in an AU basepair and resonance O from such a proton in a GC pair. This conclusion also follows from the observation that we see a sharp NOE in the aromatic region after preirradiation of resonance C and not after preirradiation of resonance O (vide infra) [27]. Therefore, according to the cloverleaf structure (see Fig. 1) resonance C comes from A5U68 and resonance O from G3C70. The NOE connections were confirmed by performing the reverse experiments, i.e. preirradiation of resonances C and O gave rise to a NOE on resonance R as well as on resonance U, although the latter was very weak and in some cases not observable (an example is shown in Fig. 5d). This may

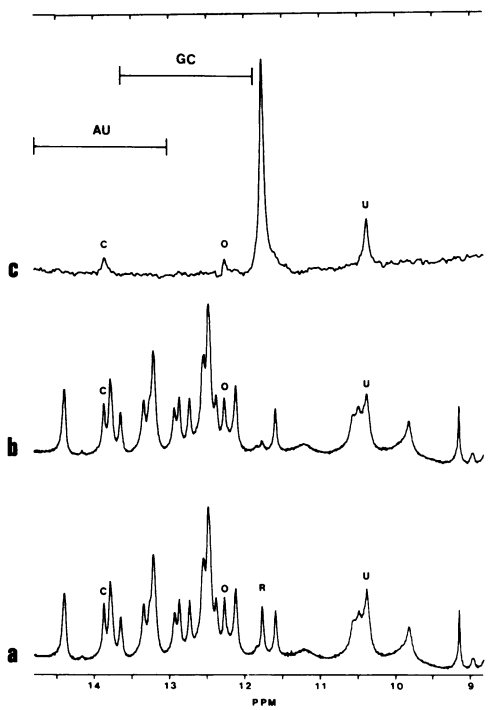

FIGURE 3
Nuclear Overhauser experiment performed with yeast $tRNA^{Phe}$ dissolved in buffer 1.
(a) Reference spectrum irradiated at an off resonance position.
(b) Spectrum showing the degree of saturation after irradiation of resonance R.
(c) NOE differences spectrum (b subtracted from a).
Observed NOE's are lettered corresponding to the signals from which they originate.
Bars indicate the spectral regions in which iminoprotons of GC and AU basepairs resonate.

reflect a difference in relaxation behaviour between the two iminoprotons of the GU basepair.

Preirradiation of resonance O not only affected resonance R and U, but also resonances M and J. Because the effect on peak M is much more pronounced than on J, one of the resonances under M is assigned to the iminoproton of C2G71. Here a limitation of the method is encountered. Since many resonances are overlapping in peak M one cannot proceed unambiguously from here to the proton resonance of G1C72. However, the following evidence indicates that the iminiproton of G1C72 most likely resonates under peak J. First, among the NOE's observed after irradiating peak M, we find one on resonance J and one on resonance O. Secondly, irradiating resonance J results in a NOE on peak M and a weaker NOE on peak O. An example of this result in buffer 2 is demonstrated in Fig. 6b. Under these conditions peak J coincides with peak K. In buffer 1, when peak J is resolved the same result was obtained. Thirdly, after completion of our assignment of all iminoprotons participating in secondary or tertiary interactions (to be published) only resonance J could not be assigned unless it belongs to the iminoproton of G1C72. The weak NOE

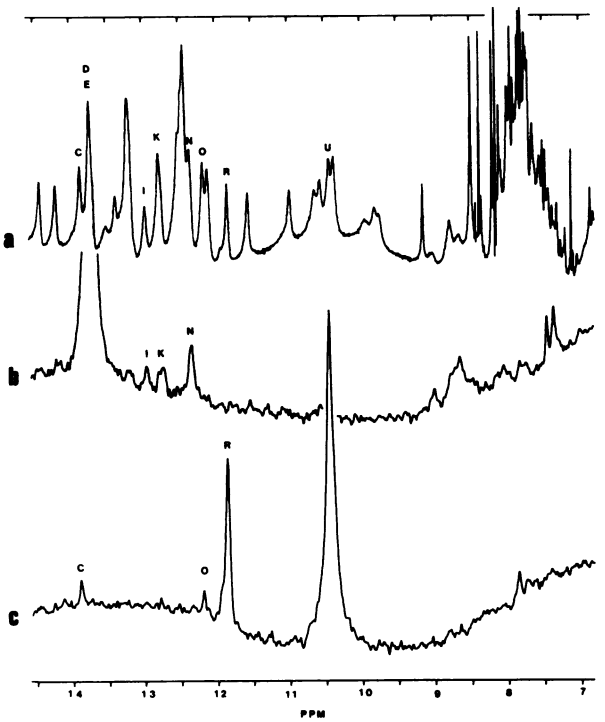

FIGURE 4
NOE experiments on yeast $tRNA^{Phe}$ dissolved in buffer 2. Reference spectrum (a); NOE difference spectra obtained after irradiation of the overlapping resonances D and E (b); idem after irradiation of resonance U (c).

connection between resonance J and O, described above, most likely represents transfer of saturation intermediated by imino and aminoprotons of C2G71.

We proceeded further down the acceptor stem by preirradiating resonance C (assigned above to A5U68), which, for the tRNA in buffer 1, results in an NOE on peak A/B. However, when the tRNA is dissolved in buffer 2 (i.e. absence of Mg^{++} ions) peak A/B is resolved into two resonances (Fig.2b). Performance of the analogous NOE experiments shows that there is a NOE connection between resonance C and A (see Fig. 5b). Thus, resonance A is assigned to the iminoproton of U6A67.

Preirradiation of peak A/B, for tRNA dissolved in buffer 1, not only gave the reverse effect on resonance C, but gave NOE's for resonances G and I as well (see Fig. 7b). Repetition of the experiment on tRNA dissolved in buffer 2 showed that resonance A is connected with resonances C and G and resonance B with resonance I (see Fig. 5c and 5b). The NOE's of resonance B will be dealt with in the next section. Since resonance A belongs to the iminoproton from basepair U6A67, resonance G must come from the iminoproton of basepair U7A66. It is noted that resonance G is not well resolved from resonance H under the conditions of buffer 1 (see Fig. 2a). The reverse experiment, i.e.

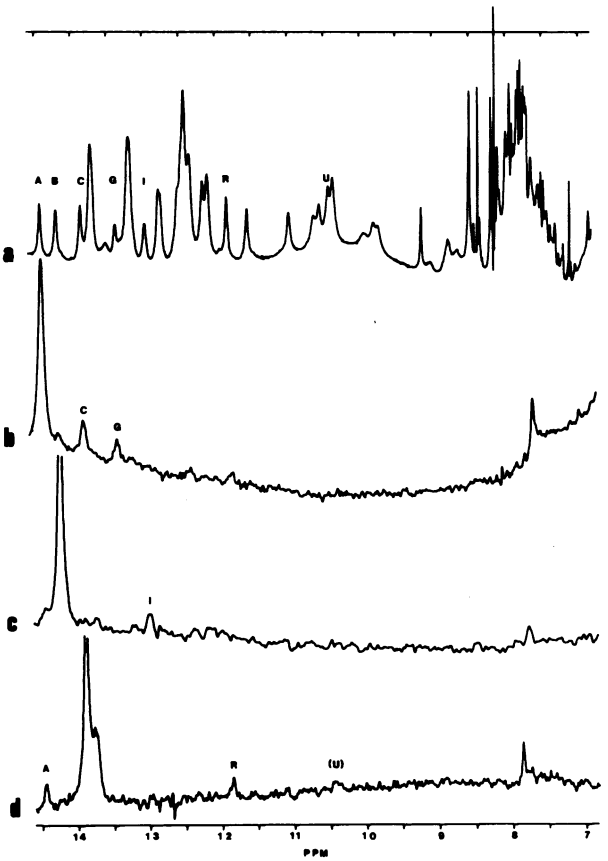

FIGURE 5
NOE experiments on yeast $tRNA^{Phe}$ dissolved in buffer 2. Reference spectrum (a); NOE difference spectra obtained after irradiation of resonance A (b), resonance B (c) and resonance C (d) respectively. The difference spectrum (b) was recorded at 15°C, all other spectra were recorded at 21°C.

irradiation of resonance G, is therefore expected to be ambiguous due to a possible spillover of saturation power to resonance H and to a lesser extent F. However, the NOE from resonance G to peak A/B was completely lost when the preirradiation pulse was set on resonances F or H, notwithstanding that the latter is only 20 Hz away from the top of resonance G. This confirmed our assignment of this resonance to the iminoproton of U7A66 and completes the assignment of the iminoproton resonances of the acceptor stem. From Table 1 it may be noted that the iminoproton of U7A66 is not too far away from the iminoproton of C49G65 opening a possibility to "jump" from the acceptor stem to the T stem. In a forthcoming publication we will demonstrate that a NOE on resonance M, observed after irradiating resonance G, represents such a "jump". Here we proceed with resonances from the D stem.

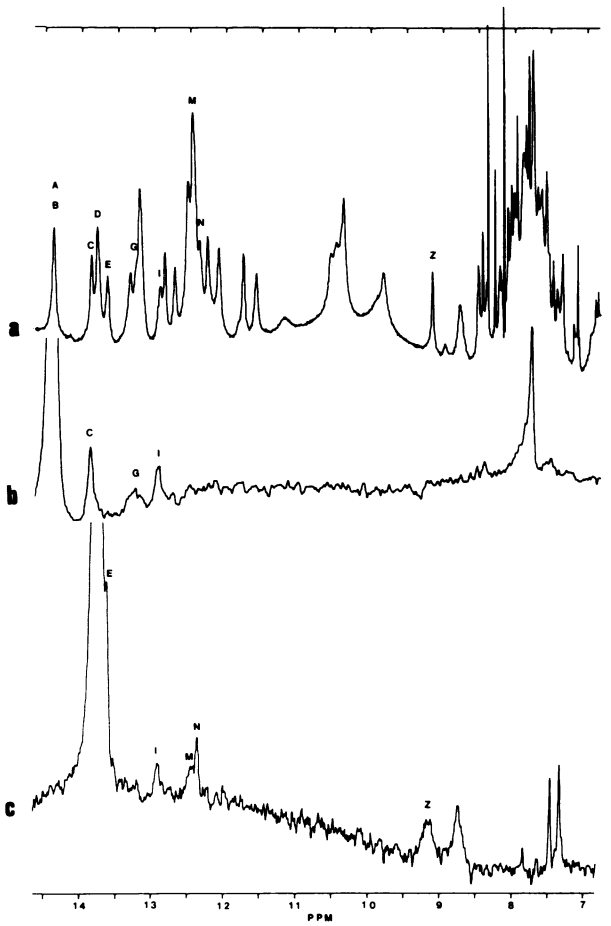

FIGURE 7
NOE experiments on yeast $tRNA^{Phe}$ dissolved in buffer 1. Reference spectrum recorded at 25°C (a); NOE difference spectra obtained after irradiation of the overlapping resonances A and B at 25°C (b) and of resonance D at 28°C (c).

Assignment of the iminoproton resonances from the D stem

Yeast $tRNA^{Phe}$ contains several methylgroups (see Fig. 1) whose protons resonate between 0 and 4 ppm. This region of the 500 MHz NMR spectrum at 30°C is shown in Fig. 8. Several of the resonances of methylgroups proved to be useful as starting points in our assignments.

The D stem contains a methylgroup on residue G10. Previously, at lower magnetic fields, its protons have been found to resonate at 2.8 ppm [24,25]. However, below 50°C this resonance was found to overlap with the resonance of the C5 methylene protons of D16 and 17 [25]. At 500 MHz two separate resonances are observed at 2.8 ppm marked 2 and 3 in Fig. 8. We followed these resonances up till 80°C and used the high temperature assignments by

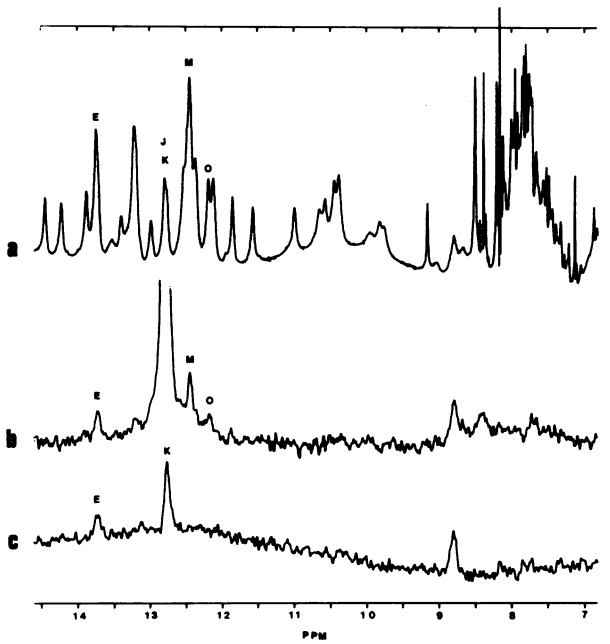

FIGURE 6
NOE experiments on yeast $tRNA^{Phe}$ dissolved in buffer 2. Reference spectrum (a); NOE difference spectra obtained after irradiation of the overlapping resonances J and K (b) and of the methylresonance of m^2G10 (c).

Kan et al. [24] to establish that resonance 2 belongs to the methylgroup of G10 and resonance 3 to the C5 methylene protons of the dihydrouridines. As low as 35°C resonance 3 could be observed as a triplet (see inset Fig. 8), which further confirms these low temperature assignments. Because resonance 1 has been assigned by Davanloo et al. [25] to the C6 methylene protons of D16 we expected to find a strong NOE on resonance 1 after irradiation of resonance 3 and vice versa. However, no effects could be observed from which we conclude that the assignment of resonance 1 should be revised.

Both resonance 2 and 3 were irradiated while observing the iminopart of the spectrum. Only irradiation of resonance 2 resulted in detectable NOE's. These were on resonance K and E (see Fig. 6c and 9c); the effect on K being much stronger than on E. The iminoproton of $m^2G10C25$ is closest to the methylgroup (Table 1) and therefore resonance K is assigned to this proton. The NOE on resonance K has been previously found by Johnston and Redfield at 270 MHz [1]. Because the iminoproton of C11G24 is the second closest to the methylgroup (Table 1) resonance E is attributed to this proton. In good agreement with this assignment we observed a NOE on resonance E after irradiating resonance K and vice versa (see Fig. 6b and 9b). It is noted that the position of resonance E (13.6 - 13.7 ppm) is quite far

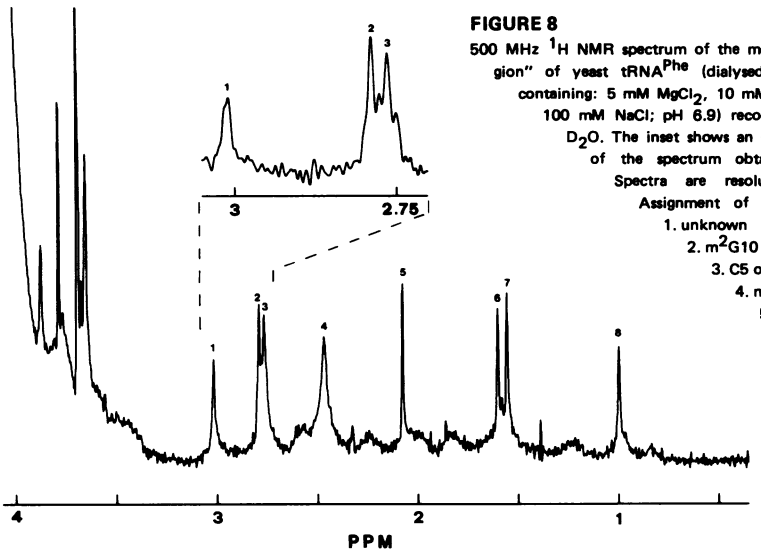

FIGURE 8

500 MHz ^1H NMR spectrum of the methylproton "region" of yeast $tRNA^{Phe}$ (dialysed into a buffer containing: 5 mM $MgCl_2$, 10 mM Na_2HPO_4 and 100 mM NaCl; pH 6.9) recorded at 30°C in D_2O. The inset shows an expanded region of the spectrum obtained at 35°C. Spectra are resolution enhanced. Assignment of the resonances:

1. unknown
2. m^2G10
3. C5 of D16,17
4. m^2_2G26
5. Y37(C11-CH3)
6. m^5C40
7. m^5C49
8. T54

downfield for an iminoproton in a GC basepair. In further confirmation of the nature of this resonance we did not find any sharp NOE from resonance E to the aromatic region between 6 and 9 ppm (see Fig. 9b). Such a NOE is typical for standard AU basepairs, which show NOE's for adenine C2 protons when the ring N3 H of the uridines are preirradiated [26]. In the present study all resonances assigned to standard AU basepairs display such sharp NOE's in the aromatic region.

Irradiating resonance E also resulted in an effect on peak D (see Fig. 9b). This may be caused by spillover of saturation power, but not necessarily so, because, as we will show below, a resonance under peak D comes from the neighbouring iminoproton of basepair U12A23. The latter proton as well as the other unassigned iminoproton of the D stem, i.e. C13G22, were assigned using the iminoproton of U8A14. There is general concurrence that this proton can be assigned to peak B. Evidence is provided by a comparison with several bacterial tRNA's where this proton in the U8A14 interaction was shown unequivocally to resonate in the region of peak B [26 and references therein]. This has been substantiated by Sanchez et al. [27], who found that C8 deuteration of yeast $tRNA^{Phe}$ results in the loss of a NOE from peak B to the aromatic region, which is only expected to occur for reverse Hoogsteen basepairs. Resonance B appeared to be connected by mutual NOE's to peak I (see Figs. 5c, 7b and 10b - the other NOE's from B to C and G have been dealt with above). In

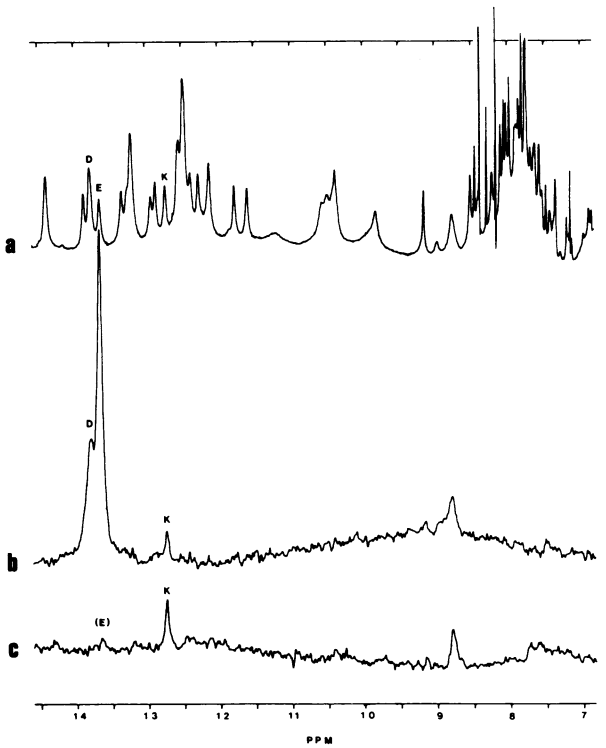

FIGURE 9
NOE experiments on yeast $tRNA^{Phe}$ dissolved in buffer 1. Reference spectrum (a); NOE difference spectra obtained after irradiation of resonance E (b) and the methylresonance of m^2G10 (c).

our distance calculations the iminoproton of G22 in the C13G22 basepair is the iminoproton nearest to the ring N proton of the U8A14 basepair (see Table 1). Therefore it is concluded that resonance I belongs to the iminoproton of C13G22. Interestingly, between 28°C and 40°C, when the tRNA was dissolved in buffer 1, we discovered that preirradiation of peak A/B gives rise to weak NOE's at 12.14 ppm (resonance Q) and at 10.42 ppm (just downfield from peak U/V) in addition to those described above. In a forthcoming publication we will present evidence that these result from preirradiation of B and that peak Q (see Fig. 11) can be assigned to the tertiary interaction G15C48.

Apart from an effect on peak B, preirradiation of resonance I also results in a NOE on peak D (see Fig. 10b). Because the reverse NOE was found as well (see Fig. 7c and 4b) one of the two resonances under D can be assigned to the iminoproton of U12A23. After irradiation of D additional effects could be observed on peaks M, N and E (see Fig. 7c). The latter effect was expected because resonance E has been assigned above to the

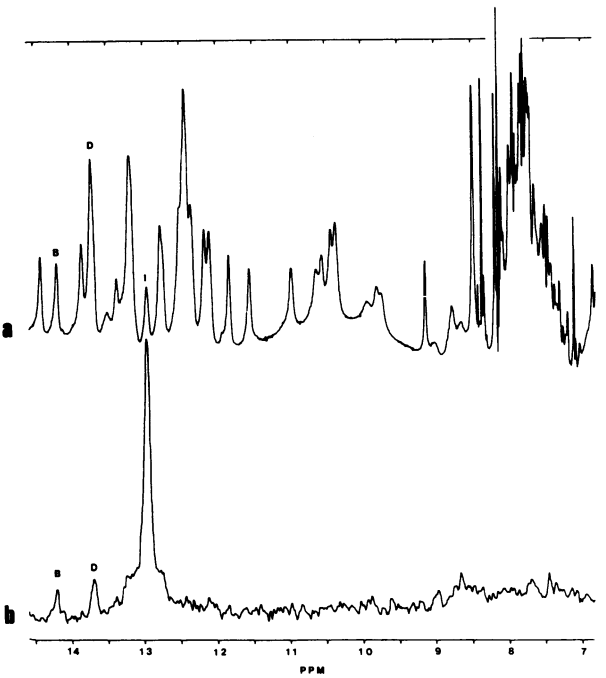

FIGURE 10
NOE experiment on yeast $tRNA^{Phe}$ dissolved in buffer 2. Reference spectrum (a). NOE difference spectrum after irradiation of resonance I (b).

neighbouring basepair C11G24. We may conclude that the NOE's observed on M and N come from basepairs neighbouring the basepair contributing the second proton resonance to D. It is noted that in the aminopart of the spectrum (from about 8 - 10 ppm) a NOE is observed on peak Z (9.1 ppm) after irradiation of D. This may seem rather peculiar because resonance Z has been assigned by Hurd and Reid [29] to the C8 proton of m^7G46, which is more than 7 Å away from the iminoproton of U12A23. Because the NOE at Z is broad in contrast to the appearance of this resonance it is likely that some aminoprotons resonate beneath Z as well.

Furthermore it is worth mentioning that at 45°C, when a number of NOE connections are no more detectable due to increased exchange kinetics, those within the D stem can still be found when the measurements are performed on tRNA dissolved in buffer 1. Two examples are presented in Fig. 11. At this temperature resonance A is resolved from B (see inset Fig. 11). In contrast to the situation without magnesium ions the U8A14 resonance (B) is now downfield from the U6A67 resonance (A). When resonance B is preirradiated we see a NOE on resonance I (iminoproton of C13G22) and when resonance E is irradiated we see a NOE on resonance K (iminoproton of $m^2G10C25$). Preirradiation of resonances A, C and R at this temperature did not result

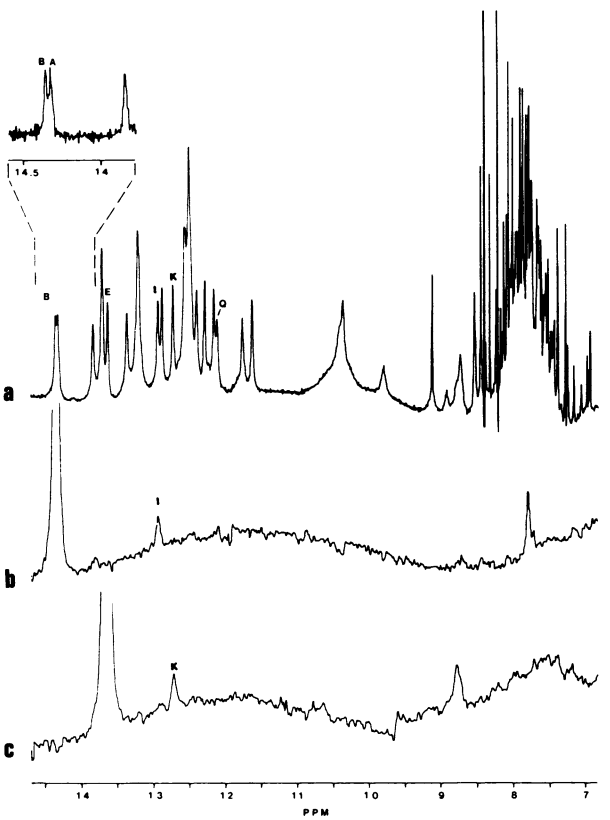

FIGURE 11
NOE experiments on yeast $tRNA^{Phe}$ dissolved in buffer 1 at 45°C. Reference spectrum (a); the inset shows a more resolution enhanced and expanded part of the spectrum. NOE difference spectra obtained after irradiation of resonance B (b) and after irradiation of resonance E (c).

in NOE's on iminoproton resonances, but NOE's could still be observed on M after irradiation of J or O.

DISCUSSION

In the present paper we have used the Overhauser effect to assign the resonances of exchangeable iminoprotons of the acceptor and the D stem of yeast $tRNA^{Phe}$. The use of tRNA solutions with and without magnesium ions permitted a step by step procedure which makes the assignments highly reliable (except for the resonance from G1C72). In hindsight it turns out that at these conditions the resonances of the stems are favourably distributed over the spectrum, thus allowing this approach. For instance in the presence of Mg^{++} the resonances E and K (see Fig. 2), which were assigned to basepairs C11G24 and m^2G10C25 respectively, are sufficiently isolated to allow the use of a step by step procedure, while in the absence

of Mg^{++} this is true for resonance A which was assigned to U6A67. Such a procedure was not always possible for the remaining part of the molecule (paper in preparation) where we had to take recourse to elimination procedures in some cases. In addition, the present assignments are self-consistent. For instance, the only NOE of about 25% is expected between the iminoprotons in the GU basepair. Indeed, we find only one combination of resonances, R and U, which gives rise to such an effect. When proceeding from these resonances we find only at one side AU basepairs. This is in accordance with the cloverleaf structure and reinforces the assignment of resonances R and U. On the other side of the GU pair we find only GC basepairs. In this direction the step by step procedure was not entirely applicable to the resonances originating from G1C72 and C2G71. Preirradiation of resonance O (G3C70) resulted in an NOE on resonance M and a smaller effect on resonance J. Since we cannot be completely sure that spillover of saturation power has some effect on resonance M in this case, the assignments of resonance J (G1C72) and one of resonance M (C2G71) are in principle interconvertible. However, this seems unlikely because under the conditions of buffer 1 irradiation of resonance J is possible without spillover to resonance M and this shows that the NOE on M is stronger than on O.

It has been mentioned already that the iminoproton resonance from C11G24 resonates at a rather low field position (13.65 ppm in the presence of Mg^{++}). Our assignment ultimately depends on the correctness of the assignment of the methylresonance attributed to the m^2G10 residue (2 in Fig. 8). At high temperatures when the tRNA is in the random coil form its resonance can be assigned by comparing its position with that of a free m^2G residue [24]. As the m^2G10 methyl resonance shifts continuously, its position in the native state can be established by following it to lower temperatures. In the present experiment at 500 MHz this resonance remains free from the resonance of the C5 methylene protons (recognizable from its triplet shape) contrary to older experiments [24,25]. Preirradiation of this resonance gives rise to a NOE on resonance K which can therefore be assigned unambiguously to the iminoproton of the m^2G10C24 basepair. A small effect is observed on resonance E. Subsequently preirradiation of resonance K gives the expected NOE on resonance E which is therefore attributed to C11G24. The assignment of resonance K to the m^2G10C24 basepair is bolstered by the fact that irradiation of the iminoproton of m^2_2G26 (marked V in Fig. 2), who is adjacent to the m^2G10C24 basepair, gives among other effects a NOE on

resonance K. Therefore we are confident of the assignment of K and as a result of that of E. The characterization of E was further substantiated by our finding that upon irradiation of this resonance sharp NOE's were absent in the 6 - 9 ppm region as is expected for GC resonances.

Several research groups [29,30,31,32] have attempted to assign resonances in the iminoproton spectra of tRNA's by means of ring current calculations. We have shown that if these calculations are based on different sets of crystalcoordinates the positions of some resonances may vary widely [29, 30] (variations up till 0.5 ppm) while others remain more or less at the same position (within 0.2 ppm). The latter category of predicted resonance positions is reasonable close (ca 0.25 ppm or less) to the measured position e.g. the iminoproton resonance of U5A68 is predicted at 13.9 ppm and observed at 13.87 ppm. Other positions may still be off by ca 0.4 ppm, so that a more detailed discussion seems not appropriate.

The most recent assignment of resonances in the iminoproton spectrum of yeast $tRNA^{Phe}$ has been given by Johnston and Redfield [1]. Their work was based partly on NOE experiments, but for the greater part on melting experiments and ring current calculations. Most assignments were therefore necessarily tentative. For comparison purposes we have collected their assignments with ours in Table II. It is of particular interest to compare the resonances from the D stem. As mentioned above, resonance K (our notation) is assigned to the iminoproton of m^2G10C25 by both groups. There is a difference in the assignment of G11C24, that we assign to resonance E but Johnston and Redfield to resonance H (again our notation). Instead,

basepair	spectral marking (Fig. 2)	POSITIONS (ppm from DSS) 1[a]	2[a]	J&R[b]	H&R[c]
G1C72	J	12.88	12.78	12.45 (M)	
C2G71	M	12.49	12.44	12.45 (M)	
G3C70	O	12.30	12.19	12.45 (M)	
G4U69	U	10.38	10.46	10.42 (U)	
	R	11.76	11.85	11.79 (R)	
A5U68	C	13.87	13.87	13.88 (C)	
U6A67	A	14.40	14.46	14.35 (A)	
U7A66	G	13.27	13.39	13.79 (D)	
m^2G10C25	K	12.74	12.77	12.77 (K)	12.60
C11G24	E	13.65	13.73	13.18 (H)	12.30
U12A23	D	13.80	13.73	13.79 (E)	13.84
C13G22	I	12.92	12.97	12.15 (O,P,Q)	13.05
U8A14	B	14.40	14.20	14.35 (B)	14.90

TABLE II

Assignment of iminoproton resonances from the acceptor stem and augmented D helix in yeast $tRNA^{Phe}$.

a) Numbers indicate the buffers used (see Materials and Methods).

b) Assignments by Johnston and Redfield [1] of the iminoprotons of the acceptor and D stem of yeast $tRNA^{Phe}$; the capitals indicate their assignment to resonances marked in our notation.
Conditions: 10mM EDTA, 10 mM sodium cacodylate (pH7), 0.1 M NaCl, 10 mM added $MgCl_2$, 20°C.

c) Assignments by Hare and Reid [34] of the iminoprotons of the D stem of E. coli $tRNA^{Val}$.

resonance E (our notation) was assigned to U12A23 by Johnston and Redfield while we find the corresponding iminoproton resonance 0.15 ppm downfield (under D). We assign resonance I to the iminoproton from C13G22 contrary to Johnston and Redfield who attributed it to G19C56. We note that the resonances, we have assigned to the iminoprotons of the D stem were observed to exchange very slowly with solvent at 15°C in the presence of Mg^{++} ions in real time solvent exchange studies [33]. In particularly this is true for resonance E which remains visible for hours. These observations are bolstered by NOE experiments at 45°C on resonances of the D stem. At this temperature a number of NOE's are lost due to the predominant exchange to water. Not however the NOE's between resonances of the D stem and also not between resonances I (C13G22) and B (U8A14). It is therefore justified indeed to speak of the augmented D helix.

At this point it is interesting to compare our results with those obtained for the D stem of E.coli $tRNA^{Val}$ in a recent paper by Hare and Reid [34] which appeared during the preparation of this manuscript. The D stem of E.coli $tRNA^{Val}$ has the same basepairs in the same sequence and polarity as those of the D stem of yeast $tRNA^{Phe}$ and therefore the positions of the iminoproton resonances are expected to be similar. This turns out to be true for the resonances of the basepairs m^2G10C25, U12A23 and C13G22 (see Table II) the positions of which differ by 0.2 ppm or less. However, the position of the iminoproton of C11G24 differs by more than 1.3 ppm. Provided that neither Hare and Reid nor we have made an error in the assignment procedure this could point to an interesting difference in the structure of the two tRNA's.

Finally it is worthwhile to point out some differences between the spectra from the samples with and without Mg^{++} (see Fig. 2). The integrated intensity (downfield from 11.5 ppm) remains the same under both conditions indicating that secondary as well as tertiary basepairs remain intact. Yet the spectra are clearly different. Almost all resonances assigned to the acceptor and D stem change their positions when proceeding to the Mg^{++} free solution. Furthermore resonance G (from U7A66) has an intensity smaller than unity (Fig. 2b). Up till now we could not trace its neighbour resonance F in the Mg^{++} free sample. Most likely this is the broad resonance at 13.5 ppm. Since the total intensity between 13.1 and 13.6 ppm remains the same this indicates different conformations of or around the corresponding basepairs. ^1H NMR studies of the "melting of basepairs" of yeast $tRNA^{Phe}$ have appeared in the past [17,35]. With the assignments presented here these can now be placed on a firm basis as far as the resolution of the published spectra goes. It is

observed that, in contrast to magnesium containing samples, in the absence of magnesium the stability of the D stem decreases. Its melting almost coincides with the early melting of the AU basepairs of the acceptor stem.

Both with and without Mg^{++} ions resonance J and probably resonance O (assigned to G1C72 and G3C70 respectively) appear to "melt out" at higher temperatures than the resonances of the AU and GU basepairs in the acceptor stem implicating that the top part of this stem is more stable than anticipated previously [17,35]. This is supported by our experiments at 45°C in which the NOE's between the resonances of the iminoprotons of the latter basepairs have disappeared, while those between resonances assigned to the GC basepairs in the acceptor stem could still be observed.

Mg^{++} free samples of $tRNA^{Phe}$ from yeast have mainly been studied in buffers with about 100 mM sodium ions. We have deliberately employed a buffer with 430 mM sodium ions to see whether it could mimic the effect of Mg^{++} ions. The spectra taken under this condition are essentially the same as spectra of Mg^{++} free samples at about 100 mM sodium ions [17,35]. The above discussion and Figure 2 clearly shows that an excess of 320 mM sodium ions cannot replace 5 mM Mg^{++} ions in their effect on the structure of yeast $tRNA^{Phe}$. We have extended this result by other experiments and arrived at the conclusion that certain, possibly important, conformations of the tRNA are Mg^{++} dependent (to be published).

After submission of this manuscript we learned that S. Roy and A.G. Redfield have independently assigned the iminoproton spectrum of yeast $tRNA^{Phe}$. It is gratifying to note that most assignments made by us and by Roy and Redfield agree (A.G. Redfield, personal communication).

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