Pulsed FT-NMR double resonance studies of yeast $tRNA^{phe}$: specific nuclear Overhauser effects and reinterpretation of low temperature relaxation data

Paul D.Johnston* and Alfred G.Redfield†

*Department of Biochemistry, Brandeis University, Waltham, MA 02154, and †Departments of Biochemistry and Physics and Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, MA 02154, USA

Received 10 July 1978

ABSTRACT

Cross-relaxation effects are demonstrated between the imino protons and other protons in yeast $tRNA^{phe}$ and H_2O. A detailed examination has been made of the observed relaxation rate of the proton resonance at 11.8 ppm from DSS as a function of the D_2O content in the solvent. This result, as well as the size and number of observed nuclear Overhauser effects, suggests that dipolar magnetization transfer between solvent H_2O, amino, imino, and other tRNA protons may dominate the relaxation processes of the imino protons at low temperature. At higher temperatures the observed relaxation rate is dominated by chemical exchange.

The selective nuclear Overhauser effects are shown to be an important aid in resonance assignments. By these means we were able to identify two protons from the wobble base pair GU4 at 11.8 ppm and 10.4 ppm.

INTRODUCTION

Transfer of saturation between H_2O and the low field protons in yeast $tRNA^{phe}$ indicated that the relaxation of these protons is dominated by a mechanism involving water protons.[1] There exists an ambiguity in a transfer of saturation experiment because it cannot distinguish between a dipolar cross-relaxation mechanism and a chemical exchange mechanism.[2,3] We have examined the observed relaxation rates ($R_{1_{obs}}$) for the imino protons in $tRNA^{phe}$ as a function of the $^1H/^2H$ ratio in the solvent. In principle, this should allow one to distinguish between the two mechanisms[3-5], and our results suggest that magnetic cross-relaxation dominates the observed relaxation rates of the imino protons at low temperatures. Cross-relaxation is further demonstrated by direct transfer of saturation between peaks in the region 6.2 - 9.0 ppm and 11.5 - 15 ppm.

We previously reported that we had observed no nuclear Overhauser effects (NOEs) between the imino and other protons in yeast $tRNA^{phe}$.[1] However, in a more sensitive re-examination we have found definite NOEs between these protons. The NOEs are a manifestation of the cross-relaxation phenomena and can be directly used in conformational analysis and peak assignments. A specific NOE allowed us to identify the low field proton resonances of the GU4 wobble base pair.

MATERIALS AND METHODS

Yeast $tRNA^{phe}$ was purchased from Boehringer Mannheim and used without further purification (lot #1077326) or was purified as previously described.[1] All $tRNA^{phe}$ accepted at least 1.5 nmoles phe per A258 units tRNA.

The NMR samples were prepared by dissolving 5 mgs of tRNA in 190 µl of a buffer solution containing 10 mM EDTA, 0.1 M NaCl, 10 mM cacodylate (pH 7.0) and either 5% or 60% D_2O. A micro NMR tube with a cylindrical cavity was used to obtain the spectra (Wilmad no. 508-CP). Chemical shifts were measured relative to solvent H_2O and converted to DSS reference by correcting for the H_2O to DSS chemical shift using the appropriate calibration curve.

All data were obtained in the same way as described previously;[1] a long (0.1 - 0.3 sec) weak monochromatic preirradiation pulse of frequency (F_2) was followed by a special observation pulse which flips the imino and/or aromatic and amino protons without flipping solvent protons. For rate measurements a delay τ was programmed between preirradiation and observation pulses, and F_2 was placed at the resonance in question. After Fourier transformation, the peaks were integrated and fit to an exponential recovery versus τ with a first order recovery rate R_{1obs}. For NOE measurements, τ was set to be a few milliseconds and the preirradiation pulse was 0.1 to 0.5 secs. A spectrum obtained with F_2 set on a resolved or partly resolved line was subtracted from one with F_2 set on a blank part of the spectrum. In the broad-

band saturation transfer experiment, F_2 was frequency-modulated between 6.2 and 9.0 ppm about 25 times in 0.5 sec.

RESULTS

A. Relaxation and effects of solvent deuteration, broad-band saturation and solvent saturation.

An examination was made of the effect of varying the isotopic composition of solvent on the observed relaxation rates for the imino proton at 11.8 ppm in yeast $tRNA^{phe}$. The data for the rate measurements and the broad-band saturation were taken on the samples whose spectra are shown in Figure 1 (a and b), respectively.

The effect of varying the $^1H/^2H$ ratio in the solvent upon $R_{1_{obs}}$ for the proton at 11.8 ppm is presented in Figure 2. Similar results were obtained for the resonance at 14.35 ppm. The rate is sensitive to D_2O content only at low temperatures. At temperatures below about 21°C, it appears proportional to the D_2O content. At high temperatures $R_{1_{obs}}$ is the same, within experimental error, for both high and low D_2O levels.

We believe that the high temperature rate data represent chemical exchange as we proposed previously.[1] Chemical exchange would be consistent with the strong temperature dependence and isotopic independence of the observed rate.

At low temperatures the data are consistent with a model in which one or more protons cross-relax with the 11.8 proton. Since the efficiency of cross-relaxation between two nuclei depends upon their respective spin and magnetic moment, protium-deuterium cross-relaxation will be exceedingly small; therefore, only nearby protons will contribute to relaxation while deuterons will not. Thus, if relaxation involves exchangeable protons, it will proceed more slowly in a more deuterated solvent.

This picture can be enlarged by the broad-band saturation experiment of Figure 3. Here we investigate the effect of partially saturating the

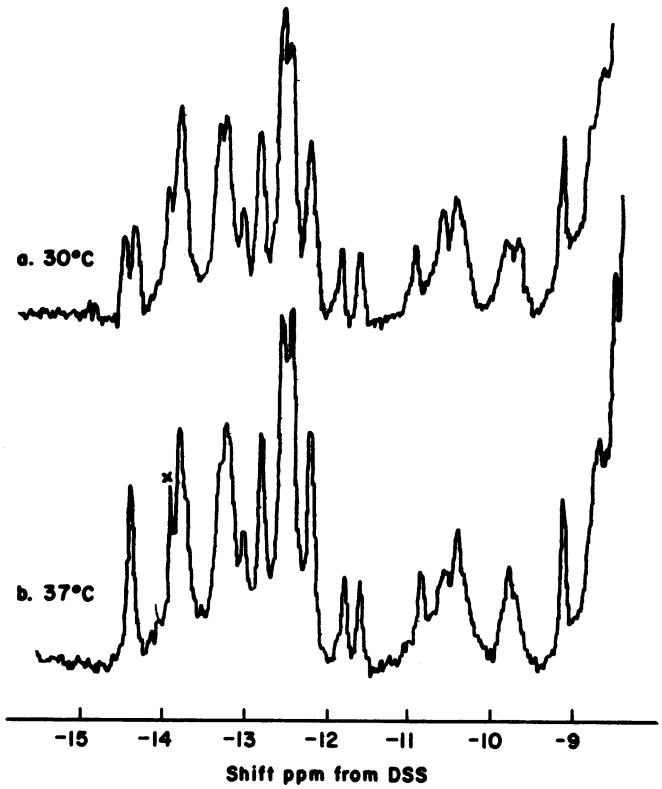

Figure 1. 270 MHz downfield spectra for the yeast $tRNA^{phe}$ used in this study: (a) 10 mM EDTA, 5% D_2O; (b) 10 mM EDTA, 10 mM $MgCl_2$, 5% D_2O. Each spectrum was the accumulation of 4000 FIDs and required 10 minutes. In all figures, center band gliches are marked by x.

region between 6.2 and 9.0 ppm. This region was directly saturated by about 50%; however, the presumably aromatic-rich region around 8 ppm, Figure 3 (c), was saturated to a larger extent than the amino-rich region (~6.8 ppm). This is expected because amino protons could have relatively shorter relaxation times. There is about a 56% magnetic transfer of saturation to the region below 11.5 ppm. Thus, there is nearly complete transfer of saturation between these two regions.

The absence of saturation transfer to the 9.0 - 11.0 ppm region (~15%) indicates that the direct broad-band saturation was confined to the region

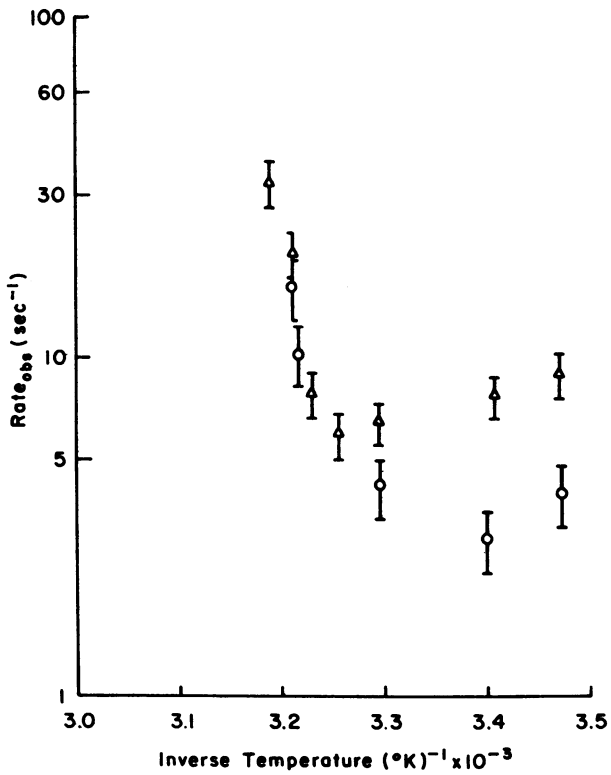

Figure 2. Recovery rate R_{1obs}, for the resonance at 11.8 ppm, versus reciprocal temperature for samples containing identical buffer components except for D_2O content which was 5% (triangles) and 60% (circles).

applied (6.2 - 9.0 ppm). In a separate run, we also verified that H_2O was saturated by about 15%.

Another observation suggested the importance of magnetic processes in the low temperature relaxation: in tRNA (unpublished results) and other macromolecules[2], saturating the solvent water protons produces a large intensity decrease for carbon protons as well as for exchangeable protons.

B. Selective nuclear Overhauser effects.

When a proton is saturated by preirradiation, its saturation can be transferred to its nearest neighbor protons by magnetic dipole coupling; this is the NOE.[6] It is strongly distance-dependent, being proportional to

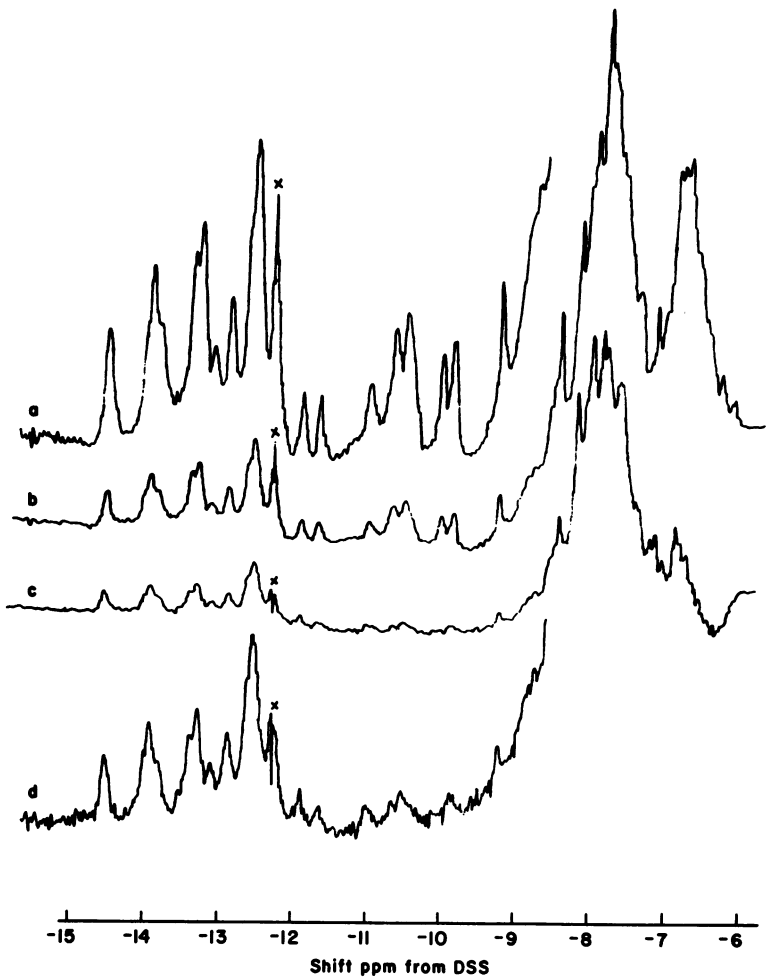

Figure 3. Broadband saturation for $tRNA^{phe}$ in 10 mM $MgCl_2$, 10 mM EDTA, 5% D_2O at 20°C: (a) normal spectrum, no preirradiation; (b) same spectrum as (a) except the gain was reduced to shown the aromatic - amino region; (c) difference spectrum of a control minus partial saturation of 6.2 to 9.0 ppm region, same gain as (b); (d) same difference spectrum as (c) except the gain was increased to that of (a) to distinctly show the transfer of saturation to the region below about 11.5 ppm. Each spectrum is the result of 4800 FIDs.

the inverse sixth power of the distance. Some selected difference spectra showing the NOE are given in Figure 4. In general, the percent of saturation transferred ranges between 20% and 30%.

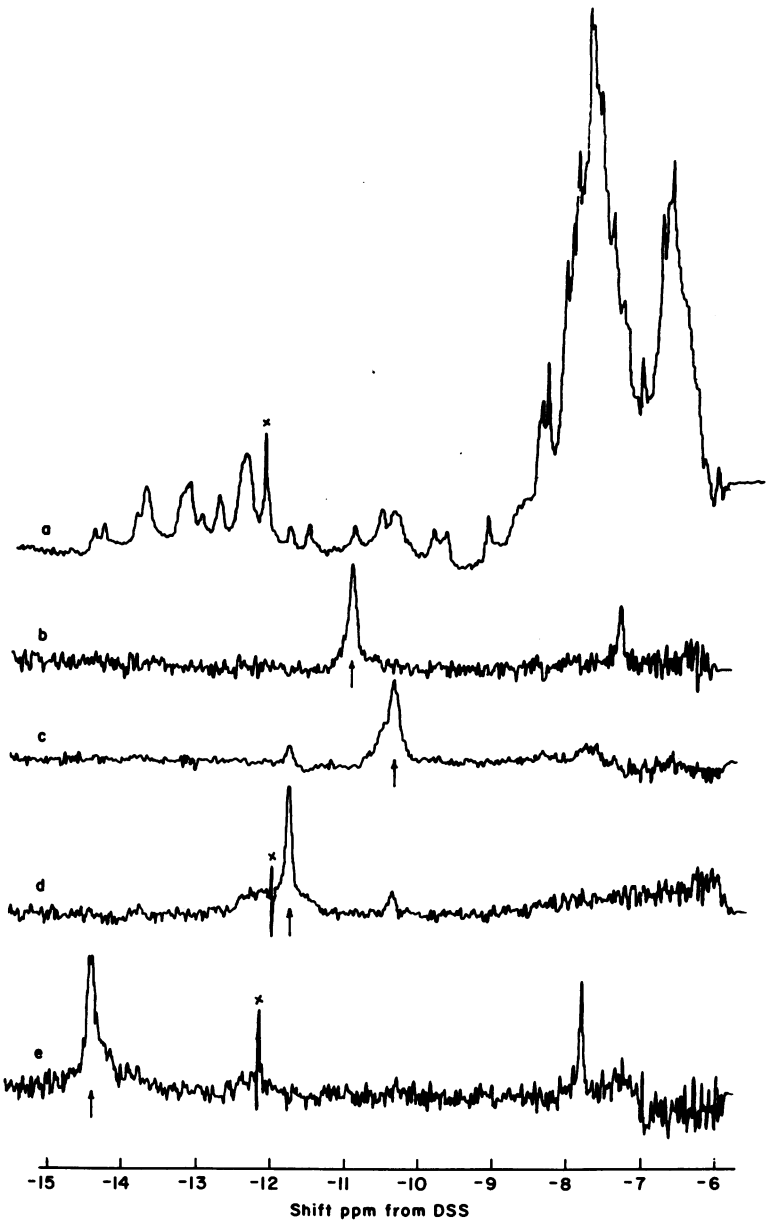

Figure 4. Selective NOE difference spectra for $tRNA^{phe}$ in 10 mM EDTA, 5% D_2O at 21°C. The arrows indicate the frequency of F_2: (a) normal spectrum, no F_2; (b) F_2 applied at 10.93 ppm for 0.4 sec; (c) F_2 applied at 10.4 ppm for 0.3 sec; (d) F_2 applied at 11.8 ppm for 0.3 sec; (e) F_2 applied 14.35 ppm for 0.4 sec. Each difference spectrum was plotted at a different gain. Four NOE runs of this quality require a total of 16 hours accumulation time. The control spectrum for the difference spectra has F_2 set at 11.2 ppm.

There have been predictions in the literature that selective NOEs would be unobservable in larger biopolymers because spin-diffusion would spread any selective excitation to many neighboring spins. The reverse seems to be true; selective proton-proton NOEs are easier to observe in larger molecules because of the dominance of mutual proton flips over other processes. The trick in observing these NOEs is to choose irradiation conditions correctly. The preirradiation should be applied for roughly the time required for a single mutual spin flip between typical near neighbors (about 0.3 sec for $tRNA^{phe}$), and the power should not completely saturate the directly irradiated resonance (about 80% is typical). These are the same conditions to which one is led by the desire to make NMR runs as short as possible and to optimize the selectivity of the preirradiation.

Of greatest interest is the observed 21% transfer from the proton at 11.8 ppm to a proton at 10.4 ppm, Figure 4 (d), which involves proton resonances of nearly equal linewidths. The reverse experiment, Figure 4 (c), (i.e., transfer from 10.4 ppm to 11.8 ppm) reinforces the observation. We have verified that there are no other proton pairs related by observable NOEs with both protons resonating in the 9-15 ppm region. A survey of base pair interactions, as seen in the crystal structure of yeast $tRNA^{phe}$ [7-9], suggests only two base pairs for which two exchangeable protons would be spatially close to each other (within about 3 Å) and would both resonate in the 10 to 12 ppm region. One possibility is the tertiary base pair G15-C48, where transfer of saturation would take place between G15 N_1H and G15 N_2H. The other possible interaction is the wobble base pair GU4; here transfer of saturation would take place between G4 N_1H and U69 N_3H protons (see Figure 5).

When the proton at 14.35 ppm is preirradiated, Figure 4 (e), there is a 30% NOE observable at 7.73 ppm. The linewidth of the proton at 7.73 ppm is half that of the proton at 14.35 ppm. The difference in linewidth can be explained if one assumes that the proton at 7.73 ppm is a carbon proton.

The linewidth of the 14.35 ppm proton is presumably due to ^{14}N relaxation broadening. This assertion is further substantiated by the fact that all the NOEs observed from lines in the region 14.5 to 13.2 ppm to protons resonating in the aromatic-amino region are as sharp as in Figure 4 (e). On the other hand, protons resonating between 13.2 and 11.5 ppm show relatively small and broad NOEs to this region (unpublished results). The former are likely to be AU base pairs and their narrow NOE presumably originates from the adenine C_2 proton. The latter are GC base pairs; the amino protons of such pairs are likely to have short T_1 values because they relax each other. Therefore, they should be less affected by the imino proton saturation even though they are slightly closer to the imino proton than is the C_2 adenine proton of an AU base pair.

The NOE shown in Figure 4 (b) is a 26% transfer of saturation from the proton resonating at 10.93 ppm to a proton resonating at 7.35 ppm. The linewidth of the proton at 7.35 ppm is about the same as the linewidth of the proton at 10.93 ppm, suggesting both are nitrogen protons. There are several possible interactions[7-9] that could generate resonances in this region. The two most likely candidates are the tertiary base pair G15-C48 or the G18-Ψ55-OP58 interaction.

DISCUSSION

This paper has addressed the mechanism of relaxation for the observed T_1 values of the low field imino protons in yeast $tRNA^{phe}$. In our earlier paper[1] we reported two general classes of rates observed: those showing strong temperature dependence and those showing little or no temperature dependence. All rates were thought to be exchange dominated because of the nearly complete transfer of saturation from H_2O protons to the imino protons. As discussed above, we still believe this to be true for the strongly temperature-dependent rates.

The absence of temperature dependence for the low temperature rates suggested a low-energy exchange mechanism. However, present data indicate that chemical exchange does not contribute significantly to these rates. In fact, within experimental error, the rates decreased by an amount equal to the percent H_2O replaced by D_2O, which is expected for a magnetically controlled mechanism. In addition, such a process should show the small temperature dependence observed.

The exact mechanism of relaxation is likely to be quite complicated and cannot be rigorously defined by the data presented here. The nearly complete transfer of saturation from H_2O to the imino region and the aromatic-amino region indicates the importance of H_2O protons in the relaxation of tRNA protons. The equally efficient transfer of saturation from the predominately aromatic protons to imino protons shows the extent to which saturation can be transferred through the macromolecule. In addition, the size of the NOEs and the isotopic sensitivity of the rates at low temperatures also points to the importance of a coupled dipolar relaxation mechanism. Thus, the data taken as a whole suggest that general spin magnetization diffusion[10] makes an important contribution to the observed rates, and this may explain why those protons showing temperature-independent rates have similar values (4-10 sec^{-1}).[1]

The protons in the region between 9 to 11 ppm show a somewhat different behavior. The lack of saturation transfer from the aromatic-amino region to, particularly, the resonances centered around 9.7 ppm and 10.5 ppm and the lack of specific NOEs from this region may indicate that exchange is important for most of these protons even at 20°C. Further experiments should elucidate this point.

An isotope effect, such as we observe at low temperature (Figure 2) could be due to the mass difference rather than the spin difference between ^1H and ^2H. However, the rate we observe is the off-rate for a proton independent of $^1H/^2H$ ratio. Thus, it would not show a primary mass isotope effect. We can-

not rule out the possibility that the effect shown in Figure 2 is non-magnetic (e.g., viscosity change, small change in pH, or intramolecular exchange), but the weight of evidence favors the explanation outlined above.

The selective NOEs can be useful in conformational analysis and in the assignment of the observed resonances. The effects, Figure 3 (c,d), relating a line at 11.8 ppm with one at 10.4 ppm show the usefulness of these observations. As we mentioned in Results, only two base pairs are capable of producing two resonances simultaneously in the region between 10.4 and 12.0 ppm arising from spatially neighboring protons. These two interactions are the tertiary reverse Watson-Crick G15-C48 and the secondary wobble base pair GU4. The wobble pairing would produce two resonances, both of which would be an H-bonded imino proton between the NH of one base (GN_1H or UN_3H) and the carbonyl group of the opposite base (see Figure 5). The G15-C48 interaction is a less likely assignment. The 11.8 ppm resonance could come from an imino proton (GN_1H) H-bonded to the carbonyl group of C48. The other proton from the H-bond between the amino group of G15 (GN_2H) and the ring nitrogen of C48 (N_3) would have to resonate at 10.4 ppm. This amino proton resonates at about 6.3 ppm in water[11] and should be shifted upfield by its neighboring bases, U59 and U8-A14. Thus, a downfield shift of more than 4 ppm due to its H-bonded interaction with C48 (see Figure 5) would be required to move it to 10.4 ppm. A shift of this magnitude seems unlikely. Therefore, the most reasonable assignment is the wobble base pair GU4.

This assignment is further substantiated by the observation of an analogous NOE in E. coli $tRNA_1^{fmet}$ (unpublished observation in collaboration with B.R. Reid). In that molecule we observe a definite NOE connecting a resonance at 12.44 ppm with one at 11.59 ppm, clearly too far downfield to expect an amino proton. Therefore, for E. coli $tRNA_1^{fmet}$ this NOE is very likely due to GU51. Using the ring current shift parameters of Arter and Schmidt[12], and assuming a normal RNA-11 helix in the vicinity of the GU base

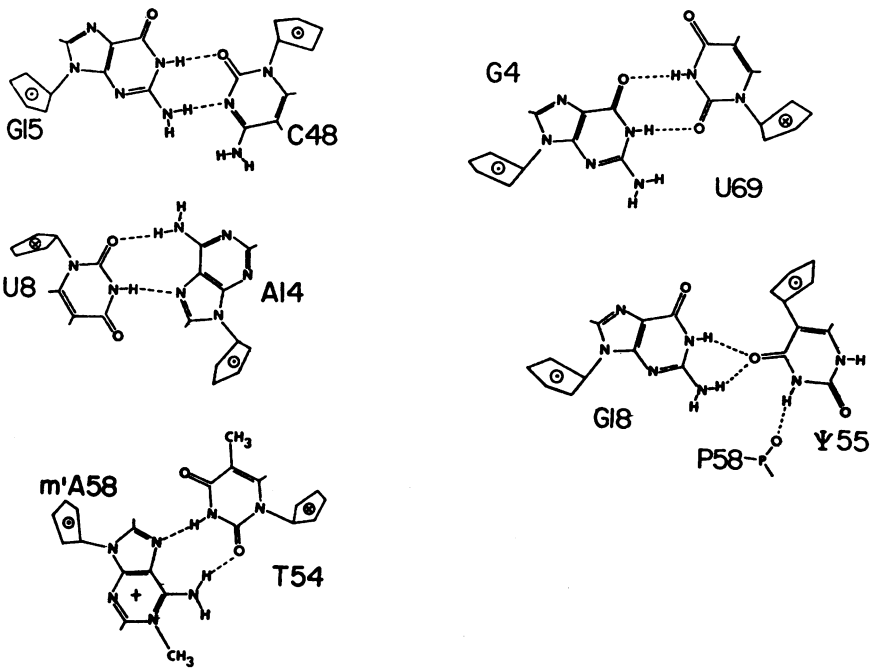

Figure 5. Base pairs discussed in the text, from reference 17.

pairs, we have calculated the chemical shift difference of the GN_1H in E. coli $tRNA_1^{fmet}$ and yeast $tRNA^{phe}$ to be 0.72 ppm, whereas we observe 1.19 ppm. In the same way, we calculated the difference in the UN_3H protons to be 0.46 ppm and observe 0.64 ppm.

Thus, for the first time there is direct experimental evidence to support the existence of the wobble GU base pair in solution. It is interesting to note that both resonances assigned to this base pair disappear with the bulk of the tertiary resonances between 36° and 42°C. The recovery rate of the proton resonance at 11.8 ppm was one of the six that showed strong temperature dependence between 36° and 42°C.[1] It was also one of the two proton resonances which rate appeared to be sensitive to temperature at intermediate levels of Mg^{++}.[1] These observations indicate the GU pair as a site of conformational flexibility in yeast $tRNA^{phe}$. It is confirmed that GU base pairs can, in fact, generate two proton resonances between 10 and 12 ppm which has been suggested

before based on the number of GUs and the general intensity in this region.[13,14] Also, the fact that one of the protons may resonate as far downfield as 12.44 ppm (E. coli $tRNA_1^{fmet}$) may explain why the intensity in the 10 to 11 ppm region does not correlate with the number of GU base pairs in the cloverleaf for some tRNAs.[11] Furthermore, our results agree with the model studies of Kallenback et al.[15], who found the N-H···O=C H-bonded proton to resonate at 11.8 ppm.

In addition, given the measured NOE (21%) and relaxation rate (7.7 sec^{-1}, 21°C) a calculation was done for the distance between the two imino protons in the GU4 base pair. Using equation (3) of Kalk and Berendsen[10] for the cross-relaxation rate and a value of (3 x 10^{-8} sec) for the rotational correlation time[18], a distance of 3.2 Å was calculated between GN_1H and UN_3H. This value is in excellent agreement with the distance of 3.2 Å calculated between these two protons using the X-ray coordinates of Rich et al. (coordinates from Gary Quigley, private communication).

Accompanying the dethiolation of S^4U8-A14 in E. coli tRNAs there is loss of a resonance around 14.8 ppm with the appearance of a new resonance near 14.2-14.3 ppm.[13,16] Our own high temperature exchange rate measurements on yeast $tRNA^{phe}$ suggested that the resonance at 14.35 ppm is one of the two tertiary reversed Hoogsteen base pairs, either U8-A14 or T54-m^1A58.[1] The NOE mate to this line at 7.73 ppm is presumably, on the basis of its linewidth as discussed above, the C8 proton of adenine (14 or 58, see Figure 5). However, this could conceivably be one of the adenine amino protons; its behavior would have to be studied at lower H_2O/D_2O ratios in order to completely rule out this possibility.

Using the dimension of a Watson-Crick AU base pair[19], where the AdC_2H and UN_3H proton-proton distance is about 3 Å (this is also about the distance between AdC_8H and UN_3H or TN_3H protons in the reversed Hoogsteen base pair), we have calculated a probable NOE value between these protons. For this cal-

culation we used the relaxation rate (8 sec^{-1}) for the non-exchangeable proton resonance at 9.1 ppm with the assumption that all aromatic carbon protons have nearly the same relaxation rates. We calculated a probable NOE (29%) which is in excellent agreement with our observed NOE (30%).

Indirect observation of identified carbon proton resonances, via NOE, should be interesting because these protons are much less sensitive than imino protons to electrostatic effects. They are shifted almost entirely by ring current shifts and, therefore, they may be more reliable conformational indicators than the imino protons. Thus, they will be useful in comparisons of different tRNAs, and for studies of buffer and cofactor influences on structure.

Finally, the relatively broad NOE-coupled peaks at 10.93 ppm and 7.35 ppm are presumably both nitrogen protons, and they could be either the H-bonded amino-imino protons of the G15-C48 base pair or the H-bonded amino proton of G18 and the imino proton of Ψ55 or G18 derived from the G-18-Ψ55-OP58 interaction. At this time we cannot distinguish between these two possibilities.

We have observed several other specific NOEs in yeast $tRNA^{phe}$ and in *E. coli* $tRNA_1^{fmet}$ (in collaboration with B.R. Reid). Most of these are either less interesting or less interpretable than those we report here, but it is clear that such observations are feasible on any tRNA. The conclusions presented here are only a preliminary sample of the potential of such observations.

ACKNOWLEDGEMENTS

The authors wish to thank Professor B.R. Reid for useful discussions and his generous gift of *E. coli* $tRNA_1^{fmet}$. We would also like to thank Dr. J.S. Stoesz whose ideas stimulated the proper re-examination of the NOEs and cross-saturation effects. We wish to express our appreciation to Ms. S.F. Waelder for her technical help in the preparation of the yeast $tRNA^{phe}$. This work was supported by U.S. Public Health Service Grant GM20168, by the National

Institutes of Health Grant 5 T01-GM00212-19, and by the Research Corporation. This is publication no. 1207 of the Biochemistry Department, Brandeis University.

ABBREVIATIONS

NOE, nuclear Overhauser effect; A, adenosine; C, cytidine; G, guanine; U, uracil; Ψ, pseudouridine; T, ribo thymine; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; EDTA, ethylenediamine tetra acetic acid; FT-NMR, Fourier Transform NMR; OP, double bonded phosphate oxygen; FID, free induction decay.

REFERENCES

1. Johnston, P.D. and Redfield, A.G. (1977) Nucleic Acids Res. 4: 3599-3615.
2. Stoesz, J.S. and Redfield, A.G. (1978) FEBS Letters 91: 320-324.
3. Campbell, I.D., Dobson, C.M. and Ratcliffe, R.G. (1977) J. Magn. Reson. 27: 455-463.
4. Waelder, S., Lee, L. and Redfield, A.G. (1975) J. Amer. Chem. Soc. 97: 2927-2928.
5. Waelder, S.F. and Redfield, A.G. (1977) Biopolymers 16: 623-629.
6. Noggle, J.H. and Schirmer, R.E. (1971) The Nuclear Overhauser Effect, Academic Press, New York.
7. Rich, A. (1977) Acc. Chem. Res. 10: 388-396.
8. Quigley, G.J. and Rich, A. (1976) Science 194: 796-806.
9. Jack, A., Ladner, J.E. and Klug, A. (1976) J. Mol. Biol. 108: 619-649.
10. Kalk, A. and Berendsen, H.J.C. (1976) J. Magn. Reson. 24: 343-366.
11. Kearns, D.R. (1976) Prog. Nucleic Acids Res. Mol. Biol. 18: 91-149.
12. Arter, D.B. and Schmidt, P.G. (1976) Nucleic Acids Res. 3: 1437-1447.
13. Reid, B.R., Ribeiro, N.S., Gould, G., Robillard, G., Hilbers, C.W. and Shulman, R.G. (1975) Proc. Natl. Acad. Sci. USA 72: 2049-2053.
14. Robillard, G.T., Hilbers, C.W., Reid, B.R., Gangloff, J., Dirheimer, G. and Shulman, R.G. (1976) Biochemistry 15: 1883-1888.
15. Kallenbach, N.R., Daniel, W.E., Jr., and Kaminker, M.A. (1976) Biochemistry 15: 1218-1228.
16. Wong, K.L., Bolton, P.H. and Kearns, D.R. (1975) Biochim. Biophys. Acta. 383: 446-451.
17. Rich, A. and RajBhandary, U.L. (1976) Ann. Rev. Biochem. 45: 805-860.
18. Komoroski, R.A. and Allerhand, A. (1974) Biochemistry 13: 369-372.
19. Bloomfield, V.A., Crothers, D.M. and Tinoco, I., Jr. (1974) Physical Chemistry of Nucleic Acids, Harper and Row, New York.