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Nuclear Overhauser effect study and assignment of D stem and reverse-Hoogsteen base pair proton resonances in yeast $tRNA^{Asp}$

Siddhartha Roy and Alfred G.Redfield

Departments of Biochemistry and Physics, and Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, MA 02254, USA

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ABSTRACT

Nuclear Overhauser effects (NOEs) in yeast $tRNA^{Asp}$ were found for all four GU and Gψ base pairs. NOEs of both reverse-Hoogsteen pairs were identified by comparison with a purine C8 deuterated sample. Several NOEs involving these resonances were also found which are clearly between single protons on adjacent base pairs. These interbase NOEs, combined with the assumption of reasonable similarity between the structure of yeast $tRNA^{Asp}$ and that of yeast $tRNA^{Phe}$, lead to unambiguous assignment of many resonances including all the ring NH and C2 protons in the D stem. The stability of the stem at 28°C, as recently deduced by Moras et al (Nature 288 669-674), from x-ray diffraction is confirmed. Assignments of the ring NH resonances of T54-A58 and of a Gψ pair are made for the first time.

INTRODUCTION

Nuclear magnetic resonance has become a powerful tool for structural and conformational analysis. In recent years efforts have been made to extend its usefulness to biological macromolecules. Among several different nuclei that are potentially available for NMR observations, protons have the advantages of sensitivity and abundance. In transfer RNAs, hydrogen bonded imino protons resonate downfield of the aromatic region, forming a relatively uncluttered set of NMR lines. Each base pair contributes one resonance to this region, except GU pairs which contribute two.[1] The resonances coming from these exchangeable protons are susceptible to dynamic influences in solution.[2,3] Thus, the hydrogen bonded imino protons have emerged as favorite markers for studying structure and conformational dynamics of transfer RNAs.

The major barrier to utilization of the imino proton region in tRNA is lack of correct assignments of resonances. Early attempts were made to assign spectral lines to specific protons on the basis of ring current shifts,[4] but these assignments have been challenged.[5,6] There were scant examples of convincing experimental assignments in transfer RNAs. We have

approached the assignment problem by using nuclear Overhauser effect (NOE), which is transfer of NMR saturation between nearby protons (generally <4Å apart). A discussion of NOE and its practical aspects is given elsewhere.[6,7]

Work in this laboratory demonstrated the usefulness of NOE in tRNAs, by showing the existence of the GU wobble base pair and identifying its resonances.[8] Later this approach was extended to distinguish and identify standard and reverse Hoogsteen AU pairs in yeast $tRNA^{Phe}$, using selective isotopic substitutions[5] and ring current shifts of carbon protons as a guide.[6] Most of the NOEs previously reported have been within single base pairs and therefore are relatively large. We have extended this approach in yeast aspartic acid tRNA and analyzed less intense NOEs, between protons on neighboring base pairs, as well as on the same base pair, using also isotopic substitution, to identify several resonances solely on the basis of structural criteria. The work described below shows that the imino proton resonances in this tRNA are amenable to reliable experimental assignment and may be useful in studying its structural and conformational properties in solution.

Yeast $tRNA^{Asp}$ is unusual in several respects: both reverse Hoogsteen base pair resonances could be located by comparison with a purine C8 deuterated sample; the D stem has an unusual sequence (Figure 1); and an unusual number of NOEs between base pairs are observable.

MATERIALS AND METHODS

Samples of tRNA prepared at the Institute de Biologie Moleculaire et Cellulaire, Strasbourg, were the kind gifts of Dr. R. Giege, and were prepared as described elsewhere.[9] Samples were prepared in our laboratory by use of benzoylated DEAE cellulose (BDC) chromatography at pH5, followed by chromotography on the same resin at pH4, and finally chromatography on sepharose 4B using a reverse ammonium sulphate salt gradient. Specific activities were at least 1.35 nmole/A_{258} unit. The purine C8 deuterated sample was prepared from a side fraction of the BDC step described by Sanchez et al.[5]

NMR samples were prepared by dissolving 5 mgs of tRNA in 190 µl of a buffer solution containing 20 mM EDTA, 10 mM phosphate (Na^+), 100 mM NaCl at pH 7.0. This was dialyzed in a microcell against several changes of buffer containing 1 mM EDTA, 100 mM NaCl, 10 mM phosphate (Na^+) at pH 7.0; EDTA was not included in the final change, for samples used in magnesium

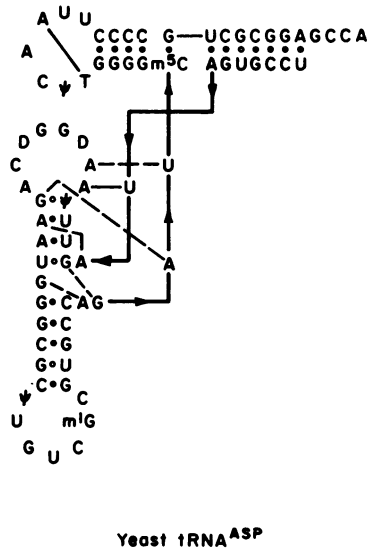

Figure 1 Nucleotide sequence of yeast $tRNA^{ASP}$. The sequence is shown in the form introduced by Kim, to emulate the tertiary strucutre. Several tertiary interactions expected from preliminary x-ray studies are shown as dotted lines between bases. The two expected reverse-Hoogsteen pairs are shown as lighter solid lines.

titrations. Final dialysis buffer contained 5% D_2O for NMR lock. Concentrated $MgCl_2$ was added to the tRNA samples containing no EDTA, as required. All spectra were recorded at 28°C in a homemade 270 MHz instrument. NMR methods have been described previously.[6,7]

RESULTS

The lowfield NMR spectrum of yeast $tRNA^{Asp}$ is shown in Figure 2. Almost all our studies to date have been carried out at 28°C because the spectrum appeared cleaner at this temperature than at room temperature. Conceivably there is either a conformational change, or association, for this tRNA at low temperature.

We concluded that peak A (Figure 2) was a single peak, as follows: Integration of peak A and the combination of peak B and C showed that the intensity ratio of peak A to peak B plus C is 1:5 ± 1. If peak A is assumed to arise from two protons, then peak B plus C would contain at least 10 ± 2 proton resonances. Presumably, the region 13.7-13.9 ppm (peaks B

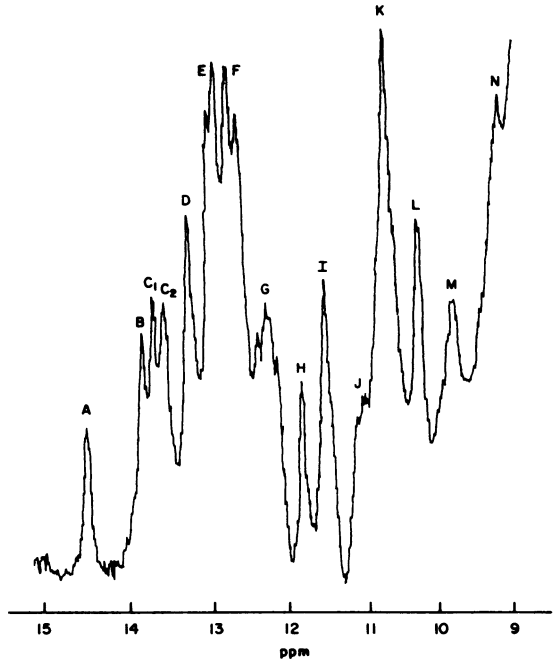

Figure 2 270 MHz downfield spectra for yeast $tRNA^{ASP}$. The sample contains 4 mM $MgCl_2$, 5% D_2O, 10 mM phosphate at pH 7.0. Spectra were recorded at 28°C. Letters A,B, and C etc. are arbitrary labels applied to the peaks and used in the text.

and C) contains only AU imino proton resonances. Since there are only six other AU imino protons in yeast^{Asp} (excluding at least one for peak A), it seems likely that peak A contains only one proton.

In secondary AU base pairs, the uracil imino proton is close to the C2 proton of adenine and gives rise to NOEs to the aromatic region characteristic of AU base pairs. In reverse Hoogsteen AU base pairs the adenine C8 proton is also close to the uracil imino proton, and also gives rise to similar aromatic NOEs. These classes of NOEs can be distinguished from each other by deuteration of the purine C8 position.[5] The most downfield peak A gives one NOE as shown in Table I, and Figure 3, at 7.93 ppm. The linewidth of the NOE is characteristic of a carbon proton. Peaks B, C_1 and C_2 give several NOEs, linewidths of which suggest them as carbon protons. Closeness of peaks B, C_1 and C_2 makes it difficult to nullify the spillover effects. By irradiating in small steps and observing the magnitudes of NOE, we have come to the conclusion that peak B gives a NOE at

Table I.

Intensities and Identifications of Selected NOEs in Yeast $tRNA^{ASP}$ at 28°C, 4mM Mg^{++}

Irradiated Resonances (PPM)		A 14.63	B 13.9	C_1 13.76	C_2 13.6	H 11.86	K 10.73	I 11.44	L 10.33
Assignments		U11(UA11)	U8(U8-A14)	T54(T54-A58)	U12(UA12)	G10-U25		ψ13-G22	
NOEs/Assignments		NOE Intensities							
14.63	U11(UA11)				5		5		
13.6	U12(UA12)	5							
11.85	G10/U25	5					20		
11.44	ψ13/G22								20
10.73	G10/U25					20			
10.33	ψ13/G22		5					20	
8.79	A(C2)				20				
8.4	A58(C8)			20*					
7.93	A24(C2)	30			10				
7.8	A14(C8)		20*					7*	
7.51	A23(C2)				20				

NOE intensities are listed, where found, as the percentage of a single proton resonance intensity. All protons are inter-base imino protons, except as marked. An asterisk denotes the NOE of a purine C8 proton.

7.8 ppm, peak C_1 has a major (~ 20%) NOE at 8.4 ppm, and peak C_2 has NOEs at 7.51, 7.93 (~ 10%) and 8.79 ppm. Also, peak A gives an NOE (~ 5%) at peak C_2.

In order to distinguish Watson Crick base pairs from reverse Hoogsteen base pairs, we prepared purine C8 deuterated tRNA as described in Materials and Methods. The far-downfield NMR spectrum for the deuterated sample was identical to that of unsubstituted yeast $tRNA^{Asp}$. Figure 3 shows the effect of C8 deuteration on NOEs from peaks A, B, C_1, and C2, to the aromatic region. Peaks I and II found in the undeuterated sample disappear in the deuterated sample. Other apparent differences between the two samples shown in Figure 3 are the result of spillover of saturation between imino peaks, and are well established NOEs for imino resonances

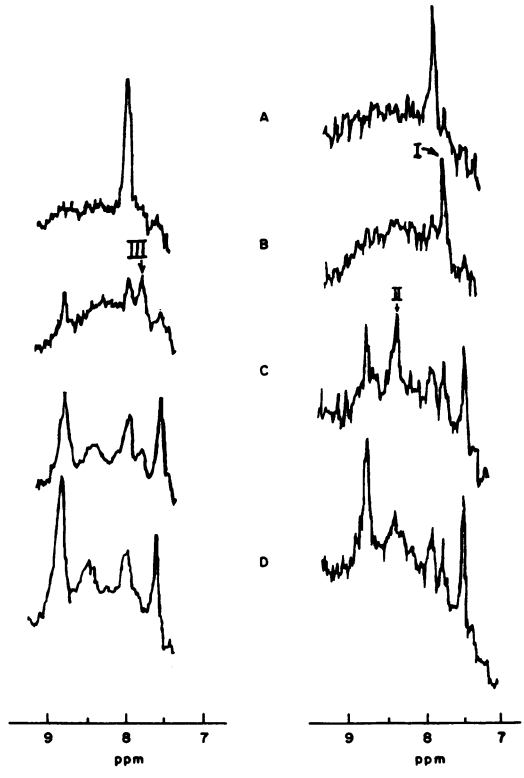

Figure 3 Downfield-to-aromatic NOEs. The spectra on the left are from the purine C8 deuterated sample, while those on the right are from a native sample. (A) Irradiation at peak A; (B) Irradiation at peak B; (C) Irradiation at peak C_1; (D) Irradiation at peak C_2. These are difference spectra which show only saturation-transfer to the aromatic region. Other spectral regions, and the irradiation point, are not shown.

whose frequencies are near those of the peaks of interest. Peak III, for example, is in a different position (7.69 ppm) from peak I (7.8 ppm); at lower temperatures these two NOEs split into two separate peaks of similar magnitude in the nondeuterated sample. Thus in this particular case two aromatic NOEs disappear, from peaks B and C_1, to 7.8 ppm and 8.4 ppm respectively. Therefore these resonances must come from the reverse Hoogsteen pairs U8-A14 and T54-A58. In addition, one smaller aromatic NOE, from peak I to 7.8 ppm, disappears on deuteration.

GU base pairs in several tRNA's have been identified to resonate in the region 10-12.5 ppm.[8] The characteristic NOE pattern for GU pairs is

often ~ 20% transfer of saturation from imino proton of guanine to imino proton of uracil and vice versa (both imino protons usually resonate in the region 10-12.5 ppm). In yeast $tRNA^{Asp}$ there are three possible GU pairs and one Gψ, which may be expected to be similar. We have observed all four possible GU or Gψ pairs, and we will explain below how we have identified two of them (peaks H and K; peaks I and L) as shown in Table I and Figure 4. The magnitude of the NOEs are of the order of 20-25% and the line widths are comparable to those of imino proton resonances (Figure 4).

The NOE observed between peaks A, B, and C and H, I, K, and L are of only 5-10% magnitude. For this reason, the NOEs have been verified on several samples prepared by two different methods, at Strasbourg, and at Brandeis. In addition, these NOE runs were run several times longer (60-100,000 accumulations) than other NOE runs. From the amplitudes and

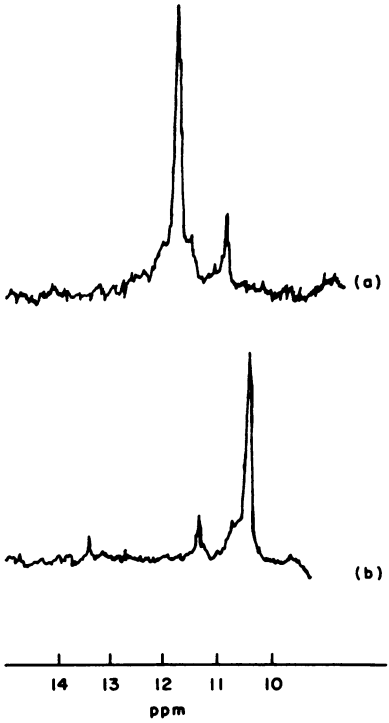

Figure 4 NOEs between GU or Gψ imino protons. (a) Irradiation at peak H; (b) Irradiation at peak L.

chemical shifts of these weak NOEs we conclude that they must be between protons on different, adjacent, base pairs. This is the first report of such NOEs other than several involving methyl groups,[12] and a single tentative report in reference 6. Their observation is the key to the identifications listed in Table I, as we now explain.

DISCUSSION OF ASSIGNMENTS

GU pairs

The GU or Gψ base pairs have two imino protons in near proximity and should show a major nuclear Overhauser effect (~ 25%). As described in the results section, two such pairs of protons resonating at peaks HK and IL are immediately evident (Figure 3). Identification of these resonances are discussed below. The other two GU NOEs which we have found will not be discussed here.

AU pairs

The yeast $tRNA^{Asp}$ sequence (Figure 1) contains 7 possible AU base pairs A1-U72, A7-U65, T54-A58, A15-U47, U8-A14, U12-A23, U11-A24. If the conformation in solution is similar to that of yeast $tRNA^{Phe}$ in the crystal,[10] the two reverse-Hoogsteen base pairs T54-A58 and U8-A14 will also form. As mentioned above, these base pairs should have an NOE from the uracil or thymidine imino proton to the C8 proton of adenine. Hence, they should disappear in tRNAs having C8 deuterated purines. Since yeast $tRNA^{Asp}$ has A46 (which does not have imino proton) instead of m^7G46, it cannot have an NOE from the m^7G46 (or equivalent) imino proton to the C8 proton of G22. Thus, it may be expected that only two major NOEs should disappear on C8 deuteration.

Indeed, as described in the results section two major NOEs from generally recognized AU regions to aromatic region disappear. These are the NOEs from peak B to 7.8 ppm and from peak C_1 to 8.4 ppm. By the argument presented above, they should be U8-A14 and T54-A58.

We assign the U8-A14 imino proton to peak B and the C8 proton of A14 to the NOE at 7.8 ppm by the following arguments. Peak B has an NOE of ~ 5% to peak L and vice versa. Peak I has an NOE to the aromatic region at 7.8 ppm which vanishes on C8 deuteration. Peaks I and L are connected by a NOE and therefore form one of the GU or Gψ pairs (see above). Since U8-A14 is the only reverse Hoogsteen AU base pair next to a GU or Gψ pair, we conclude that peak B belongs to the U8 imino proton and that the Gψ13 imino protons resonate at I and L.

It is possible, though, that the NOEs from peak B to peak L and from peak B to the C8 proton resonance at 7.8 ppm arise from different imino protons resonating at peak B. We think this is unlikely for two reasons. There is no major NOE from peak B to the aromatic region other than the one at 7.8 ppm, as there could be if resonance B arose from two AU protons. Secondly, in some samples peak B is well resolved from peak C and the intensity of B and A is similar. Since peak A has been concluded to contain one proton, we assume that peak B represents one proton only.

These assignments are further bolstered by the fact that at high magnesium concentration (16 Mg^{++}/tRNA), one peak moves upfield from peak I and gives rise to a resolved resonance; this resonance has a major NOE to peak L and a minor NOE to 7.8 ppm, indicating that this imino proton is the source of both NOEs. Hence, the resonance at 7.8 ppm must arise from a base adjacent to a GU or Gψ imino proton.

The peak at A has one NOE of ~ 30% at 7.93 ppm which does not disappear on C8 deuteration, indicating that it is a C2 proton resonance and that peak A belongs to a Watson-Crick type AU base pair. Two more NOEs of smaller size are also observed from peak A to 11.86 and 10.73 ppm (peak H and K), respectively.

The interpretation of this set of NOEs is that the imino proton at peak A has an NOE to a pair of GU protons (it was determined that a pair imino proton at peak A has an NOE to a pair of GU proton (it was determined that a pair of GU or GΨ protons resonate at H and K; see above). There are only two places where there is a Watson-Crick AU pair adjacent to a GU or GΨ pair. One is A24-U11 and G10-U25 and the other is A23-U12 and G22-Ψ13. Since we have already assigned G22-Ψ13 to peak I and L, peak A must belong to the imino proton of U11 and the G10-U25 protons resonate at peak H and K.

Base pair U12-A23 is assigned to peak C_2 by the following arguments: Peak A has an NOE at peak C_2 and vice versa. Since both peaks A and C_2 are presumably Watson-Crick type AU protons, they must represent the only adjacent secondary AU pairs in the molecule, i.e., U12-A23 and U11-A24. Peak A has already been assigned U11-A24, hence, U12-A23 must resonate at C_2. This assignment is also supported by the fact that peak C_2 has two major (7.51 and 8.79 ppm; ~ 20%) and one less intense (7.93 ppm; ~ 10%) NOE to the aromatic region. The less intense NOE at 7.93 ppm is assigned to the C_2 proton of A24 and the strong NOE at 7.51 ppm to the C_2 proton of A23. This assignment is supported by the fact that an imino proton reson-

ance moves from 13.76 ppm to 13.4 ppm when magnesium is added (0 Mg^{++}/tRNA to 16 Mg^{++}/tRNA). This imino proton resonance, when irradiated, gives two similar NOEs at 7.51 and 7.93 ppm. This shows that one imino proton is the origin of both of these peaks. The NOE from C2 to 8.79 PPM is apparently due to another Mg-insensitive base pair.

Distances can be estimated theoretically from the sizes of NOEs.[6,12] For a 5% NOE we estimate a distance of around 3.45A, based on an assumed rotational correlation time of 20 nanosec and an individual relaxation time of 0.1 sec. The latter time was not measured by us but 0.1 sec is typical of protons in other tRNA's. The inferred distance is propotional to the sixth root of the assumed correlation time and of the relaxation time. More detailed discussions of NOE distance estimates will be found elsewhere. We believe that error in the estimate is less than ± 10%.

DISCUSSION

Selective isotopic substitution and NOE study of tRNAs have proven to be fruitful approaches of assigning resonances in yeast phenylalanine transfer RNA and others. We have extended these approaches to yeast aspartic acid tRNA, and have identified several resonances without recourse to chemical shift theories. For the first time we have identified resonance of the internal ring NH of T54-A58, and a secondary Gψ resonance. Work is now in progress along these lines to identify more resonances in yeast $tRNA^{Asp}$.

The estimate of distance, between protons whose resonances show weak NOEs (3.45 ± 10% Å) is reasonable for protons more or less facing each other on adjacent base pairs. We calculated distances for some of the proton pairs in question, based on different sets of x-ray coordinates for yeast $tRNA^{Phe}$, and find that in some cases they range from about 3 to 4 Å, even for a single pair of protons.

Our spectra are similar to those of an earlier NMR study of yeast $tRNA^{Asp}$ but our conclusions are completely different from the previous work.[11] The interpretation of the earlier study was based largely on ring current shift theory, whereas we have not utilized such theory in the present paper. Barring some major blunder, the many assignments given in Table I may be regarded as certain. This is in marked contrast to most previous papers on tRNA NMR, which generally give many assignments, but few solid ones.

We have observed several other assignable NOEs in this tRNA, and

these will be presented in detail, together with other observations now in progress, in the future.

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