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Procedure for C2 deuteration of nucleic acids and determination of Aψ31 pseudouridine conformation by nuclear Overhauser effect in yeast $tRNA^{Phe}$

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ABSTRACT

Nuclear Overhauser effect (NOE) combined with semispecific deuteration provides a general strategy for identification of exchangeable protons in nucleic base pairs, and has been extended to NOEs involving purine C2 protons in tRNA. Deuterated tri-ethyl orthoformate was condensed with 5(4)-amino imidazole 4(5)-carboxamide to yield C2 deuterated hypoxanthine. C2 deuterated hypoxanthine was fed to a purine requiring mutant of yeast and C2 deuterated yeast $tRNA^{Phe}$ was isolated. This C2 deuterated $tRNA^{Phe}$ was used to identify AΨ31 and U8-A14. AΨ31 was found to be bonded through N1H. The utility of C2 deuteration in nucleic acid NMR is thus demonstrated.

INTRODUCTION

Proton NMR study of nucleic acids is a powerful method of determining structure, and thus eventually structure-function relationships.[1,2] Imino proton absorption lines provide favorite markers because of their relative isolation from the other proton resonances, and because of the dynamic information inherent in the rate of exchange of such protons with solvent. Assignment of imino proton resonances is obviously a prerequisite for correct interpretation of measurements based on these spectra.

It is well established that the imino proton spectra can be roughly divided into three regions; AU, GC and GU and non-internally bonded imino protons. AU imino protons resonate below about 13 ppm. When we selectively saturate a line in the AU region, we observe a relatively large transfer of saturation (15-30%) to a narrow line in the aromatic region by nuclear Overhauser effect (NOE). This indicates that the saturated imino proton is spatially close to an aromatic proton. In transfer RNA there are three possible AU or AΨ bonding modes that could give rise to such an NOE. In a standard Watson-Crick type AU pair this aromatic proton would be a C2 proton of adenosine. In a reverse-Hoogsteen base pair (such as U8-A14 and T54-m^1A58) the purine C8 proton is closest to the imino proton. In AΨ pairs, Ψ can hydrogen bond to adenosine through N1H or N3H (Figure 1). If it forms

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the hydrogen bond through N1H, then in addition to an NOE from the Ψ imino proton to C2H of adenosine there should be an NOE to C6H of Ψ. Thus, selective deuterium substitution in tRNA combined with NOE could be used to distinguish between these possibilities. Sánchez et al[3] performed biosynthetic purine C8 deuteration of tRNA for NMR spectroscopy. They observed that none of the AU NOEs then observed in yeast $tRNA^{Phe}$ vanish on purine C8 deuteration, and hence concluded that all of them belonged to other than reverse-Hoogsteen type AU pairs. Recently, in yeast $tRNA^{Asp}$ we have observed two NOEs from the AU region to the aromatic region that vanish on C8 deuteration.[4] This helped make a series of identification of NMR lines base pairs in the dihydoruridine stem and of two reverse-Hoogsteen base pairs.

In yeast $tRNA^{Phe}$ there is one AΨ base pair AΨ31. If ψ is bonded to adenosine through ΨN1H, there could be a single imino resonance showing two aromatic NOEs, one of which should survive both C8 and C2 deuteration. Thus C2 deuteration in conjunction with C8 deuteration could be used to assay for this type of base pair. C2 deuteration also removes most of the narrow AU-type NOEs, facilitating unambiguous observation of other weak overlapping NOEs. Here we describe a procedure for chemical synthesis of C2 deuterated hypoxanthine and isolation of C2 deuterated tRNAs from yeast. The usefulness of C2 deuterated tRNA was demonstrated by identifying AΨ31 and U8-A14 and by finding connecting NOEs to several other resonances. Elsewhere[5] we have used C2 deuterated yeast $tRNA^{Asp}$ to positively assign A15-U48. This method will certainly prove useful in other nucleic acids as well.

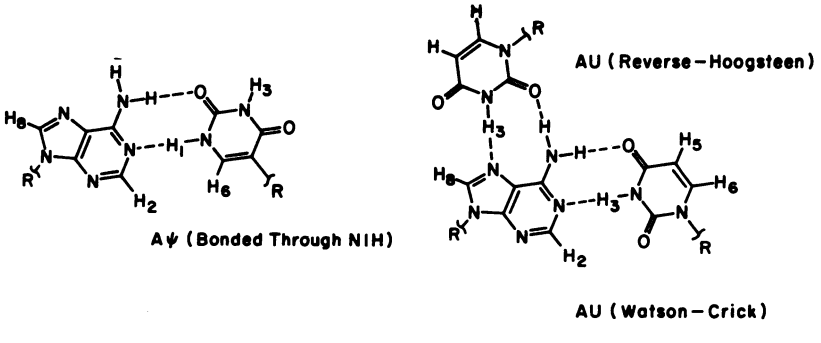

Figure 1. Three possible modes of hydrogen bonding in AU or AΨ pairs.

MATERIALS AND METHODS

5(4)-Amino imidoazole 4(5)-carboxamide hydrochloride was purchased from Sigma Chemical Company. Deuterated tri-ethyl orthoformate was purchased from Merck and was also prepared from deuterated chloroform and deuteroethanol (C_2H_5OD) according to Organic Synthesis.[6]

Chemical synthesis of C2 deuterated hypoxanthine was by a modified procedure of Richter et al.[7] 2.75 gms of 5(4)-amino imidazole 4(5)-carboxamide hydrochloride was dissolved in 50 ml of NN' dimethyl formamide. 5 ml of deuterated triethyl orthoformate was added and the mixture was refluxed for 5 minutes. It was allowed to cool, and filtered. The precipitate was washed several times with ethanol and dried. The dry precipitate was dissolved in 80 ml of 1M HCl. A total of 2.25 gms of this crude hypoxanthine in 1M HCl was diluted 3 fold in water and applied on a AG50-X8 column (1.5 x 30 cms) equilibrated with 0.33 M HCl. Elution was also performed with 0.33 M HCl. The first major peak (which contains 73% of total A_{250}) was pooled and evaporated to dryness. The product was found to be homogenous by TLC on a silica plate with n-butanol : acetic acid : water as solvent system. It also comigrates in the same solvent system with an authentic sample of hypoxanthine. NMR verified that the C2 position was fully deuterated and that the C8 position remained protonated. The ultraviolet absorption spectra was also identical with an authentic sample of hypoxanthine.

Purine requiring yeast cells were grown on the C2 deuterated hypoxanthine. The mutant strain used (A-27 MAta ade 5) lacks either glycinamide nucleoside or amino imidazole synthetase activity (or both) and was kindly provided by Dr. James Haber. Cells were grown to stationary phase in a 100 liter fermentor on 20 gms of dextrose per liter and 6.7 gms of yeast nitrogen base per liter. 1.7 gms of deuterated hypoxanthine was dissolved in 550 ml of 0.33 M HCl, titrated with NaOH back to pH 1.5, and millipore filtered. This millipore filtered solution was injected into the fermenter shortly after innoculation. The yield of cells was 525 gms. Isolation of yeast $tRNA^{Phe}$ and sample preparation was as described by Sánchez et al.[3] A small portion of the tRNA was degraded enzymatically to the nucleoside monomer as described.[8] The amount of C2 deuteration was checked by NMR in comparison with a non-deuterated crude tRNA degraded by the same procedure, and was nearly 90%. NMR spectra and NOEs were obtained as described previously,[9] mostly at 270 MHz. A few runs were also obtained at 500 MHz.

RESULTS AND DISCUSSION

Figure 2 shows the imino region spectrum of nondeuterated and C2 deuterated yeast $tRNA^{Phe}$. As expected, the spectra appear nearly identical. Figure 3 shows the methyl spectrum of non-deuterated yeast $tRNA^{Phe}$ (C2 deuterated $tRNA^{Phe}$ has an identical spectrum). Most of the methyl lines are well assigned.[9,10] Peak Z is the ribothymidine methyl and peak Y the methyls of m^5C 40 and 49. Peak X has been assigned to Wynosine 37 methyl and peaks W and V to m_2^2G26 and m^2GC10 methyls respectively.

The relevant NOEs are listed in Table I. When the composite methyl peak Y is saturated there are two NOEs (~10%) to the composite GC region peak IJ (see Figure 4). These two NOEs can be clearly resolved at 15 Mg^{++}/tRNA and at 500 MHz (data not shown). Since both m^5CG40 and 49 have only AU or AΨ as nearest neighbors (Figure 5), which should not have resonances upfield of 13 ppm, these NOEs must represent third order NOEs to the imino protons of m^5CG40 and m^5CG49 through the external amino group. The distances between m^5C methyl protons, amino protons and the guanosine N1 proton are relatively small (2-3Å), thus making the third order NOE possible. Similar NOEs have been observed[5] in yeast $tRNA^{Asp}$. In addition, there are two small NOES (~5%) to peaks F and O. These NOEs are most likely to be to the protons of adjacent base pairs. Peak O resonates in a region where non-internally hydrogen bonded imino protons resonate. The only such proton around either m^5C is the non-internally hydrogen bonded proton of Ψ39. In the yeast $tRNA^{Phe}$ crystal struc-

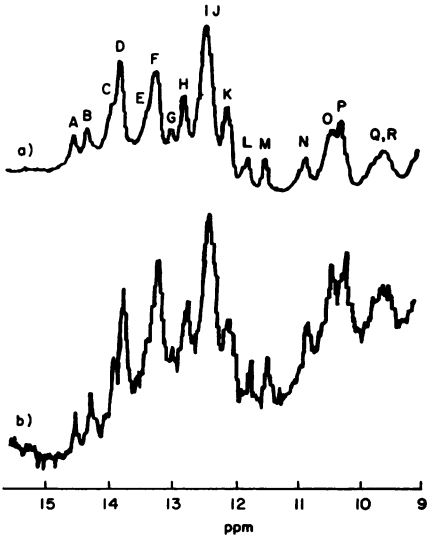

Figure 2. (a) NMR spectrum of yeast $tRNA^{Phe}$ obtained at 20°C, in 10 mM phosphate containing 0.1M NaCl, 1 mM EDTA, pH 7.0. (b) Spectrum of yeast $tRNA^{Phe}$ deuterated at C2 position. Buffer conditions are same as in (a).

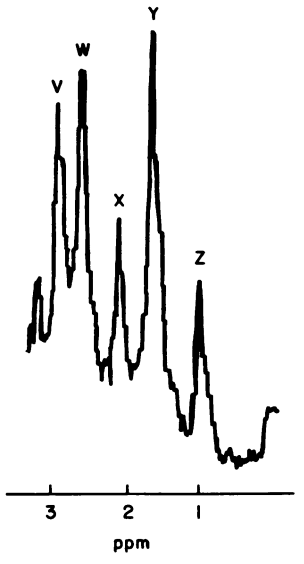

Figure 3. Methyl region spectrum of yeast $tRNA^{Phe}$ at zero magnesium concentration. The solution composition is same as in Figure 2(a).

TABLE I

Intensities and Identification of Selected NOEs in Yeast $tRNA^{Phe}$

Irradiated		Z	Y	B	F	G	O
Resonances (PPM)		0.95	1.5	14.2	13.2	13.0	10.6
NOE/Assignments							
						5	
B	U8-A14						
D	AU12					5	
F	A¥31		5				5
G	GC13			5			
IJ	GC53 GC40 GC49	8	15†				
O	¥39N3H		5				
7.75	A14(C8)			8			
6.85	¥39(C6H)				70*		

†Actually two NOEs; could be resolved in 500MHz spectrometer.
*Actually composed of two or more NOEs one of which is the ¥39 C6H.

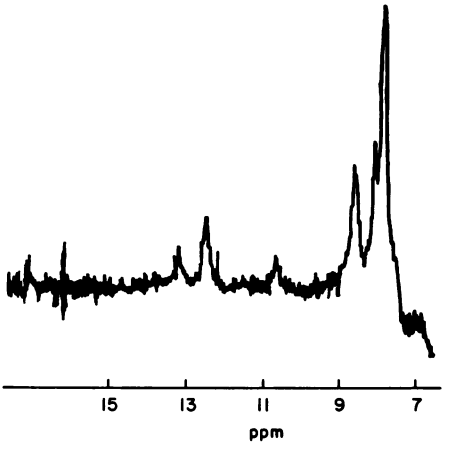

Figure 4. Downfield NOEs at zero magnesium concentration. The irradiated peak is peak Y in Figure 3.

ture, it is located within 4Å of the methyl group of m^5C40. Peak O could be either N1H or N3H depending on the mode of AΨ31 base pair formation (Figure 1). A way to distinguish between these possibilities is to saturate this proton and look for a large aromatic NOE to a pyrimidine proton. Such an NOE is possible for N1H but not for N3H. When peak O was irradiated no significant aromatic NOE was observed, thus indicating that Ψ39 N3H resonates at peak O. Absence of an expected NOE should never be taken as proof, but if there is another large NOE to a pyrimidine aromatic proton from the AU region it would become a convincing assignment of AΨ31.

When peak EF is saturated there is a large NOE to the aromatic region

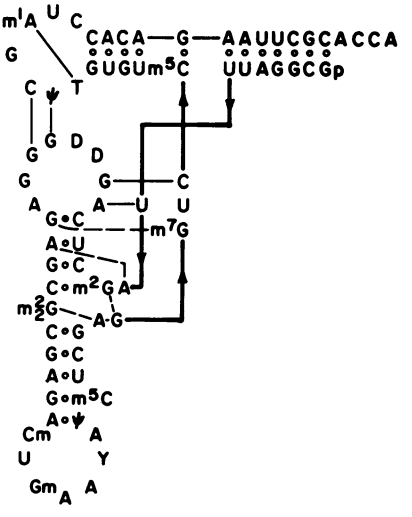

Figure 5. Nucleotide sequence of yeast $tRNA^{Phe}$. The sequence is shown in the form introduced by Kim, to emulate the tertiary structure.

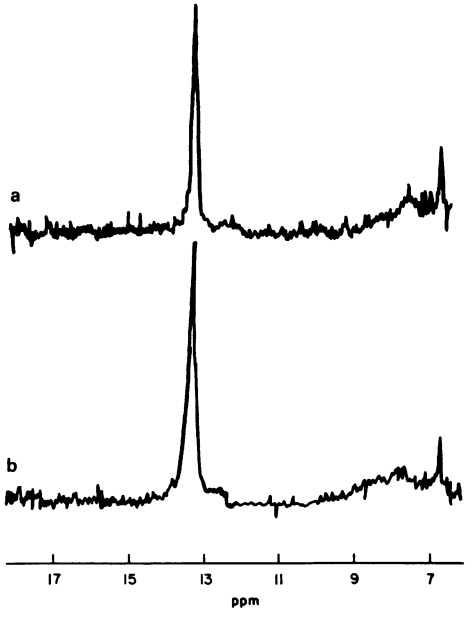

Figure 6. (a) NOE from peak F to aromatic region in yeast tRNA^{Phe}. Solution composition is same as in Figure 2(a). (b) Same NOE in C2 deuterated yeast tRNA^{Phe}. Solution composition is same as in Figure 2(a).

at 6.85 ppm (~70%) (Table I). Because of its size, it is probably a superposition of 2 or more NOEs. In the C8 deuterated sample the magnitude of the NOE remains the same.[3] In the C2 deuterated sample the magnitude is reduced to about one half. Therefore the NOE at 6.85 ppm is composed of two NOEs, one of which is to an adenosine C2 proton, and the other to a pyrimidine proton. The only imino proton that can have such a large NOE is AΨ31 N1H, and the NOE is to C6H of the same base. This observation is consistent with the fact that when the m^{5}C methyl protons (peak Y) are saturated there is a weak NOE to an imino proton at peak EF. Indeed, in the crystal structure both Ψ39 N protons are ~4Å away from the m^{5}C40 methyl. Thus we assign AΨ31 to peak EF and Ψ39 N3H to peak O and conclude that AΨ31 is base paired through N1H.

When peak Z, the thymine methyl, is saturated, in addition to a previously reported NOE to Ψ55 N1H[11], weak NOEs to peak M and IJ were observed. Peak M is assigned to Ψ55 N3H because it is the closest non internally bonded imino proton around T methyl that has not been assigned. Similar NOEs have been observed[5] in yeast tRNA^{Asp}, and *E. coli* tRNA^{Val} (Hare and Reid, private communication). GC53 is assigned to peak IJ. Again the argument is straightforward; there is no other GC base pair around the T methyl. The assignment of GC53 will be used later.

Peak B was tentatively assigned by Johnston and Redfield[9] to U8-A14.

They observed a possible NOE (~10%) to 7.74 ppm from peak B which seemed to vanish on C8 deuteration. Closeness of peak B to peak A, which has a large NOE at about the same position in the aromatic region, made it difficult to make a positive conclusion. On C2 deuteration the NOE from peak A at 7.75 ppm disappears and the NOE from peak B to 7.74 ppm (~10%) remains. From this we can conclude that peak B is a reverse-Hoogsteen base pair or m^7G46 tertiary interaction (the m^7G46 N1 proton is close to the C8H of G22). When peak B is saturated an NOE can be seen to peak G. When peak G is saturated we observe the back NOE to peak B and another NOE to peak D. Peak G is a single proton peak, which can be seen very convincingly in a 500 MHz spectrum (not shown). Thus the NOE pattern establishes a pattern for peaks B-G-D, as being AU (reverse-Hoogsteen) - GC - AU connection. We can then eliminate the possibility of B being the m^7G46 imino proton because m^7G46 is flanked by two AU pairs, U8-A14 and AU12. Even if peak G is assumed to be an AU pair, it has to be flanked by another AU pair to establish the connection B-G-D(AU pair). U8-A14 is flanked by two GC pairs and so is AU12. We can eliminate B being T54-m^1A58 because under this assumption the peak G has to be GC53; but GC53 has already been assigned above. Thus peak B is U8-A14, peak G is GC13 and peak D contains AU12. We note that GC13 and AU12 both resonate at similar positions in yeast $tRNA^{Phe}$ and[12] *E. coli* $tRNA^{Val}$, as expected from their sequence identity in the D stem and loop.

The role of post-transcriptional modifications in tRNA is not well understood. It was suggested[13,14] that modification of U to Ψ in position 39 in tRNA does not alter its function in protein synthesis, but alters possible regulatory function. Hurd and Reid[15] proposed that Ψ39 was bonded through N1H and this requires Ψ39 to be in the syn conformation. As a result the Ψ carbonyl projects into the major groove, making recognition of this minor modification easier to understand. Here we present conclusive evidence that Ψ39 is indeed bonded through N1H. This also raises the question whether all the Ψ in tRNAs are in a syn conformation. C2 and C8 deuteration in conjunction with NOE may be able to answer this question.

In conclusion, the utility of isotopic substitution in combination with NOE has again been demonstrated. C2 deuteration, C8 deuteration and NOE could elucidate such fine structural features as the bonding mode of AΨ pairs. The synthesis could also be used to introduce ^{13}C in the C2 position. This procedure could also be utilized in other nucleic acid NMR systems.

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