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Nuclear Overhauser effect in specifically deuterated macromolecules: NMR assay for unusual base pairing in transfer RNA

[imino proton/purine C(8)/reverse Hoogsteen/ring-current shift]

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ABSTRACT **We demonstrate a fairly general method for identification of NMR absorption lines of macromolecules extracted from microorganisms, based on nuclear Overhauser effects (NOE). Several NOE in tRNA are observable between resolved imino proton resonances and ring carbon resonances that are either C(2) protons of adenine or C(8) protons of adenine or guanine. Yeast tRNA^{Phe} was deuterated at the purine C(8) positions by heating in ^2H_2O and also biosynthetically. NOE between imino protons and adenine C(2) protons of standard A·U base pairs would not be affected by such a label, but some other NOE that might be otherwise similar, such as those of reverse Hoogsteen base pairs, should disappear. Six NOE were shown to be from standard A·U pairs by their nondisappearance. Four NOE from methyl resonances to aromatic proton resonances did disappear. The results disagree with previous assignments based on ring-current theories of imino proton NMR shifts.**

Proton NMR studies of the downfield NMR absorption spectra of tRNAs are of interest because the imino protons that resonate in this region are hydrogen bonded and their NMR shifts and solvent exchange rates can yield useful information about structure and dynamics (1–4). Several such NMR lines have been identified by means of chemical modification. Most have been those of protons involved in tertiary interactions, and few specific identifications have been made for protons of the Watson–Crick helical regions of the molecule. It is fairly well established that secondary G·C resonances are in the region 11.5–13.5 ppm and A·U resonances fall below 13 ppm (Fig. 1). Attempts to predict the spectrum by using a model with only a few parameters have not been sufficiently successful to lead to general agreement on assignments (1, 2). One problem with assignments is that both standard A·U and reverse Hoogsteen pairs (Fig. 1) are expected to resonate in the same general region. There are two reverse Hoogsteen tertiary base pairs in yeast tRNA^{Phe} according to x-ray crystallography (5).

When we selectively saturate a line in the A·U region prior to the main observation pulse, we often find that the saturation is transferred to a narrow line in the aromatic region of the spectrum via the nuclear Overhauser effect (NOE) (4). This indicates that the saturated proton and its NOE-coupled mate are spatially close to each other (within ≈3.5 Å). NOE is most usefully detected by difference spectroscopy (Fig. 2). Table 1 lists six NOE that we have observed repeatedly in yeast tRNA^{Phe}. Most of these are expected to originate from standard A·U base pairs because the imino proton of the NOE pair is in the expected A·U region of the spectrum and the upfield NOE is characteristically narrow (<25 Hz). Standard A·U base pairs have an adenine C(2) proton adjacent to the imino proton (Fig. 1). In contrast, we see numerous NOE from lines in the entire imino region to the aromatic-amino region from 6.5 to 9 ppm that are at least 40 Hz broad and that we believe to be imino-amino NOE. Resonances of amino protons should be broader than those of ring carbon protons because the latter have no other nuclear spins nearby.

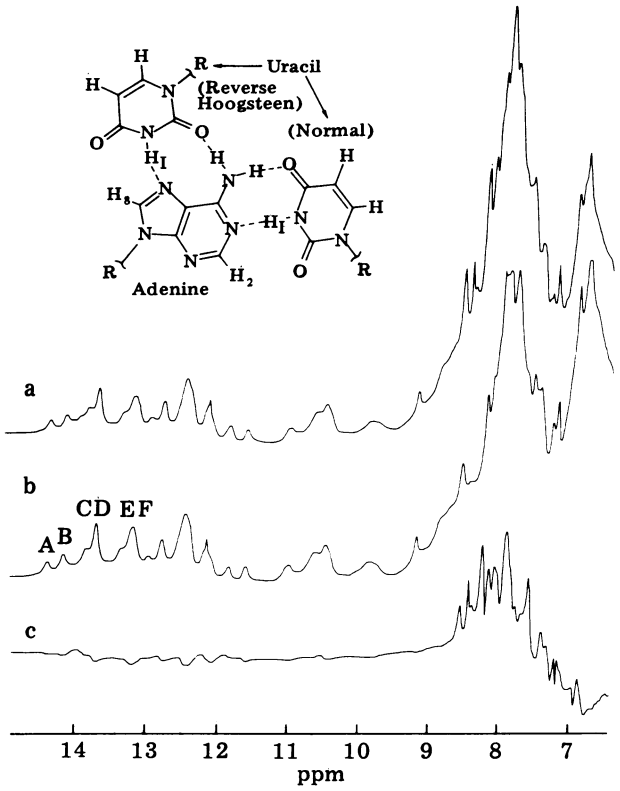

FIG. 1. (*a*) NMR spectrum of yeast tRNA^{Phe} (Boehringer Mannheim) obtained at 24°C, about 12 mg in 0.2 ml of 0.1 M NaCl/1 mM EDTA/10 mM phosphate, pH 7/95% H_2O/5% ^2H_2O. This sample was used to obtain the NOE of Fig. 2*e*. (*b*) Spectrum of tRNA deuterated at the purine C(8) positions; otherwise similar to *a*. This sample was used for the NOE of Fig. 2 *a–d*. (*c*) Difference between *a* and *b*. Spectra *a* and *b* were tilted slightly and renormalized relative to each other to give a good subtraction. (*Inset*) Standard A·U and reverse Hoogsteen A·U base pairs. The imino, C(2), and C(8) protons are designated H_1, H_2, and H_8, respectively.

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Abbreviation: NOE, nuclear Overhauser effect(s).

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Table 1. Probable A·U NOE obtained under the conditions of Fig. 2*

Irradiated imino resonance†	NOE position†	Assignment (tentative)	Predicted C(2) position‡
14.35	7.69	U·A6	7.84
13.88	7.81	A·U5	7.88
13.82	7.34	U·A52	7.43
13.82	7.44	U·A12	7.34
13.2	7.17	U·A7	6.97
13.18	6.82§	A·U29	7.02
		U·A50	7.05

* These lines are all nearly insensitive to $MgCl_2$.
† Positions ppm downfield from 2,2-dimethyl-2-silapentane-5-sulfonate.
‡ Based on the theory of Arter and Schmidt (6), assuming an intrinsic shift of 8.1 ppm (7).
§ Nearly 100% NOE and a decrease in width when $MgCl_2$ is added suggest that this is two overlapping NOE.

Although most of the NOE listed in Table 1 are likely to arise from standard A·U base pairs, they could also arise from unusual base pairs such as reverse Hoogsteen base pairs for which there is an adenine C(8) proton next to the imino proton (Fig. 1) or from the interaction between m^7G46 and G22 (not shown) for which there is a guanine C(8) proton next to an imino proton. The present work resolves this ambiguity.

Isotopic labeling is the method of choice for NMR identification, but it is impractical for labile imino protons. However, if a NOE to a carbon proton is observable, then that carbon can be labeled with deuterium in order to identify the NOE mate. This strategy has already been used in this laboratory for a NMR study of bovine superoxide dismutase (8). Here we use it to distinguish between secondary A·U base-pair NOE and reverse Hoogsteen or the m^7G46-G22 base-pair NOE by deuteron labeling of the C(8) protons of all purines in yeast $tRNA^{Phe}$. We find that all NOE in Table 1 remain in a sample labeled in this way; therefore, all are NOE of standard Watson–Crick A·U base pairs.

MATERIALS AND METHODS

The strategies for labeling the purine C(8) position are based on the fact that the proton at this position is labile at high temperature, both for adenine and for purines in nucleic acids (3). We made a series of attempts to deuterate the C(8) positions of commercial yeast $tRNA^{Phe}$ (Boehringer Mannheim) by heating it in ^2H_2O. The most successful of these attempts was performed on tRNA that was rigorously dialyzed in 0.1 M NaCl/1 mM EDTA. The sample was lyophilized, redissolved in ^2H_2O (which had been treated with Chelex), and heated to 90°C for 1.5 hr. About 2 mM cyclic GMP was added; the C(8) proton resonance of the cyclic GMP disappeared after heating. The tRNA NOE were checked at 20°C and only one, from a methyl proton (see below), disappeared. This NOE reappeared after the sample was heated in H_2O buffer.

This experiment was not entirely satisfying for the following reasons. The NMR spectra observed after these heat treatments were not as clean as before heating although charging assays (of a less concentrated sample, in a preliminary experiment) indicated that the sample should survive heat treatment. We were not sure that the important positively charged base m^1A58 would lose its C(8) proton as rapidly as the other adenosines. Finally, we wished to develop a method that would be useful for tRNAs that would not stand heat treatment as well as does yeast $tRNA^{Phe}$.

Therefore, we grew 100 liters of adenine-requiring yeast cells on C(8)-deuterated adenine. The mutant (strain A-27 *MATa ade5*) lacked glycinamide nucleoside or aminoimidazole nucleoside synthetase activity (or both) and was kindly provided by James Haber. The label was expected to be incorporated at the C(8) position of both adenine and guanine. Adenine was deuterated as follows: 1.25 g of it was mixed with 100 g of ^2H_2O and heated to 100°C for 2 hr in order to dissolve the adenine and then label the C(8) position. The mixture was cooled to 5°C overnight and the precipitate was removed by centrifugation. The supernatant ^2H_2O was immediately used again in the same way, and the procedure was repeated several times. Deuteration was checked by NMR, and the optical absorption spectrum was unchanged. Four grams of labeled adenine was dissolved in 0.8 liter of 50 mM HCl and filtered on Millipore filters before being added to the sterilized 100-liter fermentor. Cells were grown to stationary phase on 20 g of dextrose per liter and 6.7 g of yeast nitrogen base per liter without amino acids (Difco); the yield was nearly 1 kg of cells in 24 hr. The crude tRNA was isolated by phenol extraction followed by DEAE-cellulose chromatography (9), starting with 0.1 M NaCl/0.1 M Tris·HCl, pH 7.5, and increased to 1 M NaCl after extensive washing. The tRNA was then fractionated with benzoylated DEAE-cellulose chromatography, starting with 20 mM acetate, pH 5/10 mM $MgCl_2$/0.2 M NaCl. A linear salt gradient was applied to 1.3 M NaCl, followed by a linear ethanol gradient to 10% ethanol in the same buffer. The $tRNA^{Phe}$ appeared late in the latter gradient. It was precipitated with ethanol, dialyzed against H_2O, and lyophilized. Then 250 A_{258} units were dissolved in 150 μl of dialysis buffer. The sample was dialyzed in a microcell for 3 days against several changes of 0.1 M NaCl/10 mM phosphate, pH 7/20 mM EDTA, followed by several changes of an otherwise identical 1 mM EDTA buffer. The final change contained 5% ^2H_2O for NMR lock. The tRNA accepted 1.6 nmol of phenylalanine per A_{258} unit, or was about 80% pure. A similar amount of Boehringer Mannheim tRNA was dialyzed against the identical buffer as a control; it accepted 1.8 nmol of phenylalanine per A_{258} unit.

An unused portion of the benzoylated DEAE-cellulose effluent and a sample of commercial unfractionated tRNA were degraded enzymatically to the nucleoside monomer as described (10). The retention of C(8) labeling for both adenosine and guanosine was verified by NMR, which showed nearly identical spectra for the two degraded samples except for a lack of adenine and guanine C(8) proton resonances in the degraded deuterated sample.

NMR spectra and NOE were obtained as described by use of 100-msec selective preirradiation before the 214 observation pulse which permits Fourier transform NMR in H_2O buffer (11). Fig. 1 compares the downfield spectra of the two samples, and shows the difference between them.

RESULTS AND DISCUSSION

Fig. 2 *a–c* shows all the NOE listed in Table 1 obtained from the C(8)-deuterated sample. To save time, these observations were made with relatively strong preirradiation power so that a group of several imino lines were saturated in a single experiment. However, we previously obtained action spectra for these NOE at lower power; we can thus associate different parts of composite imino lines with different aromatic NOE. We do not show NOE obtained from the undeuterated sample corresponding to Fig. 2 *a–c* because these are almost identical to those of the deuterated sample.

Irradiation of a methyl resonance at 2.44 ppm gave a strong NOE at 8.4 ppm in the unlabeled sample which was missing in the deuterated sample (Fig. 2 *c* and *d*). The methyl resonance has been identified (12) as that of m_2^2G26, and we believe that

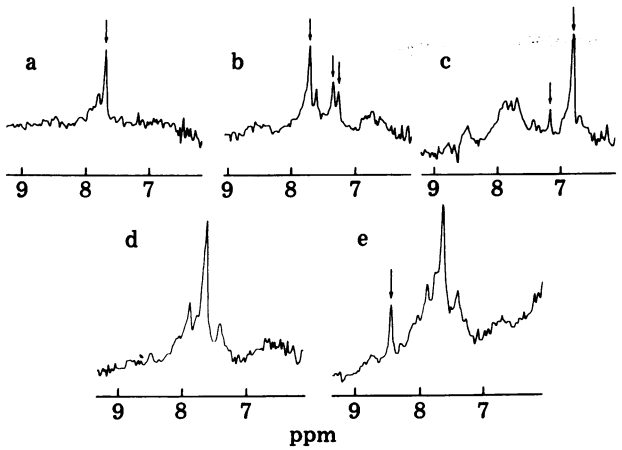

FIG. 2. (*a*) Difference between the aromatic regions of a spectrum obtained with preirradiation of resonance minus that for selective preirradiation of peak A (Fig. 1*b*) for the C(8)-deuterated sample. (*b* and *c*) Similar to *a* except that peaks CD and EF (Fig. 1*b*), respectively, were preirradiated. (*d*) Similar to *a* except that a methyl resonance at 2.44 ppm was preirradiated. (*e*) Similar to *d* except for the fully protonated sample (Fig. 1*a*). In these spectra, NOE discussed in the text or included in Table 1 are indicated by arrows. Temperature was 18°C ± 1°C. In *b* there is a weak apparent NOE at 7.84 ppm which arises because peak A was slightly saturated (as was peak CD) in this experiment.

the NOE at 8.4 ppm is the C(8) proton of m^2G10. Three other NOE from the methyl region to the aromatic region showed the same behavior.

Theories of imino proton ring-current shifts have generally assumed that the net chemical shift for imino protons of A·U base pairs is the sum of an intrinsic A·U shift, which accounts for electron shielding and ring shifts of the donor and acceptor bases, and an added ring-current shift due to diamagnetic shielding by neighboring bases. These theories differ in various ways (1, 2, 13–15) but none predicts a single secondary A·U resonance considerably downfield of all other A·U resonances, as is indicated by the experiment shown in Table 1 and peak A of Fig. 1*b*. There is a second downfield resonance (peak B in Fig. 1*b*) that could conceivably be a secondary A·U base pair. However, this resonance is generally believed to be that of the imino proton of a reverse Hoogsteen base pair, based on experiments on *Escherichia coli* tRNAs (1, 2). We also found a weak NOE (not shown) from this peak to 7.70 ppm which was absent in the C(8)-deuterated sample. This observation tends to support this assignment.

None of the many predictions of imino spectra based on ring-current theories agrees with these results. Some could be said to fit within an accuracy of ±0.25 ppm, but in these cases so many theoretical resonance positions must be assumed to be in error by ±0.2 ppm or more that the "agreement" is most unconvincing. Many of the predictions are inconsistent with other possible correlates such as the predicted C(2) shifts compared to NOE (below) or observed compared to expected solvent exchange rate (4, 16). It seems likely to us that variations in acceptor–donor geometry, induced by tertiary folding, modify the electron density around the imino proton and thereby modify its "intrinsic" shift. Of course, these theories are still useful for providing a base line when independent identification is possible.

Such uncertainty should be largely absent for shifts of carbon protons. Table 1 shows a set of assignments for the observed NOE and predicted shifts for the adenine C(2) protons. These are not unique and differ in one case (A·U12) from a previous assignment by us (16). They were picked primarily to give good agreement between observed NOE and predicted adenine C(2) shifts. We tentatively assigned the imino resonance at 13.88 ppm to the A·U5 base pair because we saw a very weak NOE from peaks previously identified as imino protons of G·U4 to about 13.88 and 7.82 ppm. These assignments are highly tentative and we hope further verification will be possible.

Unfortunately, we have found only one weak NOE that tentatively identifies peak B as a reverse Hoogsteen base pair, as previously assigned (1, 2). Candidates for the other expected reverse Hoogsteen base pair and for the m^7G46-G22 base pair are those few resonances that show no definite NOE in either sample. These are one of the set of four lines CD in Fig. 1 that moves slightly upfield in the presence of Mg^{2+} and exchanges very slowly at 15°C, previously assigned by us (16) to U·A12, or part of peak EF, or upfield from it. The m^7G46-G22 imino proton has been assigned by means of chemical modification to the downfield side of peak EF (17), and the present result does not disagree with this assignment because the m^7G46 imino resonance could overlap one of the resonances showing an NOE in the group of lines EF.

The possibly interesting features of purine C(2) and C(8) proton resonances have been pointed out (18). To our knowledge, the work of Arter and Schmidt (6), based on the assumption of an 11-fold RNA helix and used for the predictions of Table 1, is the only relevant theory for these protons. It would be useful to have predictions of purine carbon proton shifts for various x-ray structures and ring-current assumptions, such as exist for the imino protons. Equally important would be information on how these predictions change as the assumed structure is varied locally in sterically acceptable ways.

In conclusion, we have demonstrated a strategy of exchangeable resonance identification combining NOE with specific biosynthetic deuteration and used it to search for unusual base pairs. The method should be applicable to other tRNAs and other labeling sites, as well as to a variety of protein problems. In yeast $tRNA^{Phe}$, we have only tentatively confirmed one such base pair, but we have eliminated some previously proposed assignments for them.

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