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Improved RNA Structure Determination by Detection of NOE Contacts to Exchange-Broadened Amino Protons

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Abstract: A 2D ^{15}N-correlated NOESY experiment is described which makes it possible to obtain distance information for exchange-broadened amino protons in nucleic acids. Little or no NOE distance information is normally obtained for guanine and adenine amino protons because of rotation of the amino group around the C−N bond which leads to extreme broadening of the amino proton resonances. Here NOEs involving amino protons are detected via efficient transfer of heteronuclear magnetization between the amino protons and nitrogens, which is accomplished by application of a train of closely spaced 180° echo pulses. This technique has been applied to a uniformly ^{15}N-labeled lead-dependent ribozyme known as the leadzyme. The 2D ^{15}N-correlated NOESY experiment allowed 19 NOE constraints to amino protons to be identified that had not been previously observed in standard NOE spectra. Molecular dynamics calculations were performed with and without these additional amino NOE constraints and demonstrated that these amino constraints significantly improved the structure of the GAAA tetraloop in the leadzyme. Such NOE constraints involving amino protons will likely improve structure determinations of many other nucleic acid motifs.

Introduction

Multinuclear, multidimensional NMR techniques are routinely used to generate high resolution solution structures of isotopically labeled proteins.[1,2] With recent advances in synthesis of uniformly ^{13}C-/^{15}N-labeled RNA and DNA oligomers, these techniques are also being used to help simplify the resonance assignment and structure determination of nucleic acids.[3−5] One difficulty in RNA and DNA structure determinations is the lower density of protons in nucleic acids compared with proteins. Since NOE-derived proton−proton distance constraints are the primary data for the solution structure determination of a macromolecule,[6] the lower density of protons means that the structures of nucleic acids are generally not as well defined as those of similar molecular weight proteins. Furthermore, the majority of the protons in a nucleic acid are on sugar rings, so many of the observed NOEs only yield information on the conformation of the ring. Thus the conformation of the sugar ring is often over-determined from NOE and J coupling constant data whereas the bases and phosphates are less well defined. This low proton density, and the clustering of protons in the sugar ring, means that any new sources of structural information for the bases will have a significant impact on the quality of NMR

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(1) Clore, G. M.; Gronenborn, A. M. In *NMR of Proteins*; Clore, G. M., Gronenborn, A. M., Eds.; CRC Press: Boca Raton, 1993; p 1.
(2) Clore, G. M.; Gronenborn, A. M. *Science* **1991**, *252*, 1390.
(3) Batey, R. T.; Inada, M.; Kujawinski, E.; Puglisi, J. D.; Williamson, J. R. *Nucleic Acids Res.* **1992**, *20*, 4515.
(4) Nikonowicz, E. P.; Sirr, A.; Legault, P.; Jucker, F. M.; Baer, L. M.; Pardi, A. *Nucleic Acids Res.* **1992**, *20*, 4507.
(5) Zimmer, D. P.; Crothers, D. M. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 3091.
(6) Wüthrich, K. *NMR of Proteins and Nucleic Acids*; John Wiley & Sons: New York, 1986.

0002-7863/95/1517-11043$09.00/0

structure determinations in nucleic acids. One as yet untapped source of structural information involves extracting NOE distance constraints from exocyclic amino protons on the purine bases. The G and A amino protons in nucleic acids are usually highly exchange-broadened by internal rotation about the carbon−nitrogen bond. Thus in most nucleic acids, no useful NOE data can be obtained for these amino protons because they have very large line widths (often >100 Hz) under standard conditions.

Here we present a 2D ^{15}N-correlated NOESY experiment[7] for obtaining NOE interactions involving exchange-broadened amino protons in nucleic acids. In this 2D experiment, instead of labeling the frequency of the exchange-broadened amino protons, their attached amino nitrogen frequencies are labeled in the t_1-evolution period. Cross peaks are then observed between amino nitrogens and protons within NOE distance of the amino protons. Since the amino nitrogens have sharp resonance lines, these NOE cross peaks are observed with high sensitivity. This 2D ^{15}N-correlated NOESY experiment was performed on a uniformly ^{15}N-labeled lead-dependent ribozyme known as the leadzyme.[8−12] Structure calculations were performed that demonstrate how the additional distance constraints obtained for these amino protons significantly improve the structural precision of the GAAA tetraloop in the leadzyme.

Methods

The 99% ^{15}N-labeled leadzyme with the sequence given in Figure 1 was synthesized by *in vitro* transcription with T7 RNA polymerase using chemically synthesized DNA templates and 99% ^{15}N-labeled NTPs as previously described.[4,9,13] The NMR sample was 1.8 mM RNA, 10 mM sodium phosphate, 0.1M NaCl, 0.2 mM EDTA, pH 5.5 in 90% H_2O/10% D_2O. The NMR experiments were performed on a 600-MHz Varian Unityplus spectrometer at 5 °C. The TPPI-States method was used for quadrature detection.[14,15] All NMR data were transferred to Silicon Graphics computers and processed with the program FELIX (Biosym Inc.). Detailed descriptions of the NMR acquisition and processing parameters are given in the figure legends.

Results and Discussion

In an A-form double helix with standard Watson−Crick base pairs the A, C, and G amino protons are close to many other base and sugar protons and thus represent important NOE data for generating three-dimensional structures of nucleic acids.[16] The C amino protons usually show many NOE interactions, but little or no NOE data is generally obtained for the G and A amino protons.[6,17,18] This is a result of the extreme broadening for the G and A amino proton resonances caused by rotation of the amino group around the C−N bond. This is less of a problem for the C amino protons since the additional double bond character in the C−N bond leads to slower rotation, and therefore less exchange broadening.

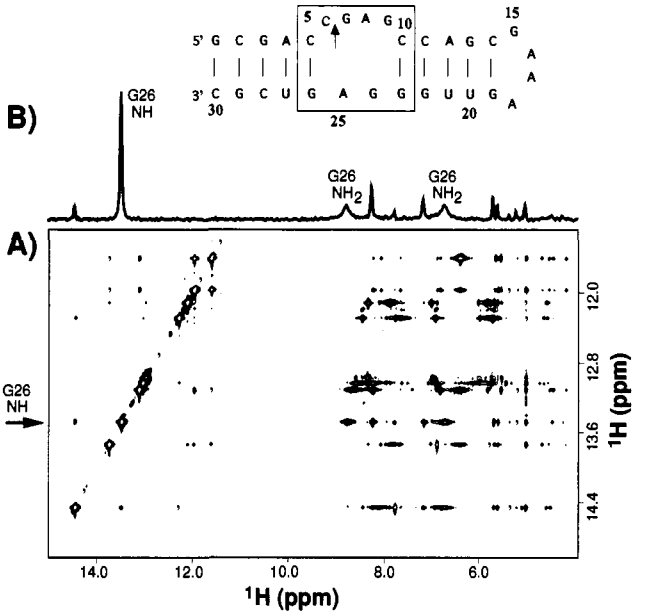

Figure 1. (a) The imino proton to aromatic proton region of a 2D (^{1}H, ^{1}H) NOESY spectrum of the leadzyme in 90% H_2O/10% D_2O at 5 °C, collected with a flip-back enhanced WATERGATE pulse train[21,23] replacing the NOESY read pulse. Spectral widths in ω_1 and ω_2 were 12602.4 and 14000.7 Hz, respectively, 640 complex t_1 points were collected with 32 scans per FID, the relaxation delay was 1.3 s, the mixing time was 0.15 s, and total data collection time was 18 h. The parameters for the water flip back and WATERGATE sequence are similar to those given in Figures 2 and 3. (b) 1D cross section along ω_2 for the G26 imino proton (13.48 ppm) illustrating the large line widths for the G amino protons. The position of this 1D cross section is indicated by the arrow in the 2D spectrum. Above the spectra is a schematic representation of the sequence and secondary structure of the leadzyme.[12] The box indicates the nucleotides required for site-specific cleavage, and the arrow points to the cleavage site.

2D (^{1}H, ^{1}H) NOESY Experiments on the Leadzyme. Figure 1a shows the imino to aromatic proton region of a 2D (^{1}H, ^{1}H) NOESY spectrum of the leadzyme in 90% H_2O/10% D_2O. The pulse sequence used is a modification of the standard proton NOESY pulse sequence[19] where a selective EBURP 90° [20] flip-back pulse[21,22] is applied to the H_2O resonance at the end of the NOESY mixing time, and then a WATERGATE water suppression sequence is used as the detection pulse.[23] Furthermore, in order to minimize quadrature detection artifacts in ω_1, the relative phase of the RF pulses flanking the evolution period was set to ±45°. These modifications ensure that the bulk water proton magnetization remains in a fully polarized state throughout the experiment which results in increased sensitivity for solvent exchangeable protons relative to standard NOESY experiments. Very similar water flip-back NOESY experiments have been independently developed by others.[22,24,25] Some of the G imino protons in this NOESY spectrum show cross peaks to extremely broad resonances (>100 Hz line widths) that can be tentatively assigned to G amino protons (see Figure 1, parts a and b). Although intrabase imino proton to amino proton cross peaks could be observed for some of the

(7) Gronenborn, A. M.; Bax, A.; Wingfield, P. T.; Clore, G. M. *FEBS Lett.* **1989**, *243*, 93.
(8) Legault, P.; Pardi, A. *J. Am. Chem. Soc.* **1994**, *116*, 8390.
(9) Legault, P. Ph.D. Thesis, University of Colorado, Boulder, 1995.
(10) Legault, P.; Pardi, A. *J. Magn. Reson. Ser. B* **1994**, *103*, 82.
(11) Legault, P.; Jucker, F. M.; Pardi, A. *FEBS Lett.* **1995**, *362*, 156.
(12) Pan, T.; Uhlenbeck, O. C. *Nature* **1992**, *358*, 560.
(13) Milligan, J. F.; Groebe, D. R.; Witherell, G. W.; Uhlenbeck, O. C. *Nucleic Acids Res.* **1987**, *15*, 8783.
(14) Bax, A.; Ikura, M.; Kay, L. E.; Zhu, G. *J. Magn. Reson.* **1991**, *91*, 174.
(15) Marion, D.; Ikura, M.; Tschudin, R.; Bax, A. *J. Magn. Reson.* **1989**, *85*, 393.
(16) Saenger, W. *Principles of Nucleic Acid Structure*; Springer-Verlag: New York, 1984.
(17) Varani, G.; Tinoco, I., Jr. *Q. Rev. Biophys.* **1991**, *24*, 479.
(18) Wijmenga, S. S.; Mooren, M. M. W.; Hilbers, C. W. In *NMR of Macromolecules*; Roberts, G. C. K., Ed.; Oxford University Press: Oxford, 1993; p 217.
(19) Kumar, A.; Ernst, R. R.; Wüthrich, K. *Biochem. Biophys. Res. Commun.* **1980**, *95*, 1.
(20) Geen, H.; Freeman, R. *J. Magn. Reson.* **1991**, *93*, 93.
(21) Grzesiek, S.; Bax, A. *J. Am. Chem. Soc.* **1993**, *115*, 12593.
(22) Mori, S.; Abeygunawardana, C.; Johnson, M. O.; Vanzijl, P. C. M. *J. Magn. Reson. Ser. B* **1995**, *108*, 94.
(23) Piotto, M.; Saudek, V.; Sklenar, V. *J. Biomol. NMR* **1992**, *2*, 661.
(24) Fulton, D. B.; Hrabal, R.; Ni, F., presented at the 36th Experimental NMR Conference; Boston, April 1995.
(25) Lippens, G.; Dhalluin, C.; Wieruszeski, J. M. *J. Biomol. NMR* **1995**, *5*, 327.

guanine residues (the intrabase G imino proton to G amino proton distance is 2.2 Å), no interresidue, and therefore structurally useful, NOE cross peaks could be detected for any G or A amino protons in the leadzyme. These results illustrate a major limitation in standard NMR studies of nucleic acids; a large percentage of the amino protons are so highly exchange broadened that no NOE structural data can be obtained for them.

2D ^{15}N-Correlation NOESY Experiments on the Leadzyme. The goal here was to devise a procedure that prevents the decay of the exchange-broadened proton signal. This can be achieved by efficient transfer of amino proton magnetization to the amino nitrogen, subsequent evolution of amino ^{15}N coherence in t_1, followed by an efficient back transfer of the t_1-modulated ^{15}N coherence to the amino protons. At the end of this back transfer, proton M_z-magnetization is restored rapidly to allow NOE cross relaxation to occur to other protons in proximity of the amino protons. In the resulting 2D ^{15}N-correlated NOESY spectrum, cross peaks are observed at the amino ^{15}N resonance frequencies. These cross peaks are much more intense than their counterparts in a corresponding 2D (^{1}H, ^{1}H) NOESY spectrum, because the line widths of ^{15}N-amino resonances are narrow and appear unaffected by rotational exchange of their directly bonded amino protons.

The sensitivity of this approach critically depends on the ability to transfer amino proton Zeeman magnetization to ^{15}N single quantum coherence without incurring significant loss in signal intensity. There are two known techniques for preserving spin coherence in the presence of exchange broadening: first, the magnetization of interest can be aligned along a coherent RF field by application of hetero-TOCSY spin locking pulses.[26-31] The decay of magnetization due to chemical exchange is minimized when the RF field strength significantly exceeds both the rate of exchange and the difference in chemical shifts of the two exchanging amino protons.[32] Alternatively, spin coherence can be preserved in the presence of chemical exchange by the application of a string of closely spaced 180° RF pulses (echo pulses) such as the so-called Carr Purcell–Meiboom–Gill (CPMG) pulse train.[33] We have applied these two approaches and found that the CPMG pulse train gave superior results in the ^{15}N-labeled leadzyme (~50% more signal in the region which is effectively locked by the applied RF field). A detailed comparison of these two techniques will be presented elsewhere; however, the major disadvantages of using hetero-TOCSY for transferring coherence between protons and nitrogens in amino groups are as follows: (i) the inherent loss in sensitivity because the full polarization of the two amino protons cannot be transferred to nitrogen transverse magnetization;[34] (ii) the optimal hetero-TOCSY transfer period is about 40% longer than the corresponding INEPT period;[35] (iii) effective hetero-TOCSY transfer can be achieved only in a relatively narrow spectral range. Hence, with a typical TOCSY RF field of 3.3 kHz it is difficult to achieve efficient polarization transfer for all types of amino nitrogens which resonate from ≈70 ppm (G-amino) to ≈100 ppm (C-amino).

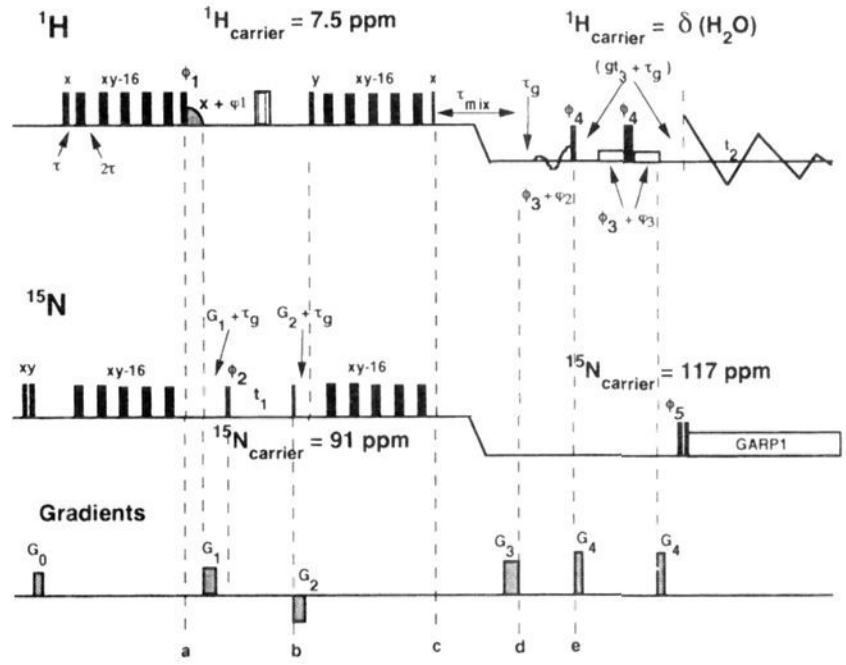

Figure 2. The 2D ^{15}N-correlated NOESY pulse sequence that employs synchronous proton nitrogen CPMG pulse trains during proton–nitrogen polarization transfer intervals. Narrow and wide rectangles represent 90° and 180° pulses, respectively, and the proton pulse in the center of t_1 represents a composite 90$_x$ 240$_y$ 90$_x$ population inversion pulse. All unmarked pulses have x-phase, $\Phi_1 = y, -y$, $\Phi_2 = 2(x), 2(-x)$, $\Phi_3 = -x$, $\Phi_4 = x$, $\Phi_5 = 4(x), 4(-x)$, receiver = $x, -x, -x, x$; $\varphi_1 = -16°$, $\varphi_2 = -3°$, $\varphi_3 = -2°$. The phase shifts φ_1–φ_4 are system dependent and to optimize water flip-back and solvent peak suppression it is important to adjust the phases of all selective proton pulses with a small angle phase shifter where deviations of as little as 0.5° can have a significant effect on the quality of solvent peak suppression. A frequency shifted selective right-handed half Gauss 90° pulse[37] with a duration of ≈3 ms was employed at point a to selectively flip the water signal to $-z$. An EBURP1 selective 90° pulse[20] with a duration of ≈7 ms was employed immediately before point e; the selective soft square pulses flanking the 180° proton pulse in the WATERGATE sequence[23] should have a duration of 1.2–1.4 ms; τ_g is the gradient recovery delay and was 0.4 ms. The proton carrier frequency was set to 7.5 ppm in the first half of the experiment and switched on-resonance with H_2O during the NOESY mixing period. The nitrogen carrier frequency was set to 93 ppm during the first half of the experiment and switched to 116 ppm during the mixing period (τ_{mix}). The phases of the 180° pulses in the CPMG pulse trains are successively incremented according to the xy-16 phase cycling scheme {2(x,y), 2(y,x), 2($-x$,$-y$), 2($-y$,$-x$)}.[36]

For these reasons, we employed synchronous proton–nitrogen CPMG pulse trains during the proton–nitrogen INEPT transfer. This is achieved by placing a string of echo elements {τ–180°-(H,N)–τ} in the $1/(2*{}^1J_{NH})$ INEPT period. The rate of decay of amino proton magnetization due to chemical exchange is a function of the CPMG pulse interval τ (which is one-half of the separation between two adjacent 180° pulses), so the shorter the echo interval τ the better the suppression of exchange broadening.[32] However, it is important to note that there is an upper limit of RF power that the NMR probe can absorb. Thus spectra were acquired using a train of 24 RF pulses with τ = 114 μs, a ^{15}N 180° pulse of 51 μs, and a ^{1}H 180° pulse of 15.4 μs on which the xy-16 phase cycling scheme was superimposed for both the proton and nitrogen pulses.[36]

In the 2D ^{15}N-correlated NOESY sequence given in Figure 2 the proton H_x-magnetization is transferred to H_zN_y transverse magnetization by an INEPT sequence containing the synchronous proton–nitrogen CPMG-pulse train. Since the amino protons exchange with water, in order to maximize sensitivity care has been taken to preserve the proton polarization of the bulk H_2O. For this reason, the INEPT period is followed by a right-half Gauss 90° pulse which selectively rotates the H_2O

(26) Yamazaki, T.; Pascal, S. M.; Singer, A. U.; Forman-Kay, J. D.; Kay, L. E. *J. Am. Chem. Soc.* **1995**, *117*, 3556.
(27) Dewerell, C.; Morgan, R. E.; Strange, J. H. *Mol. Phys.* **1970**, *18*, 553.
(28) Hartmann, S. R.; Hahn, E. L. *Phys. Rev.* **1962**, *128*, 2042.
(29) Zuiderweg, E. R. P. *J. Magn. Reson.* **1990**, *89*, 533.
(30) Ernst, M.; Griesinger, C.; Ernst, R. R.; Bermel, W. *Mol. Phys.* **1991**, *74*, 219.
(31) Krishnan, V. V.; Rance, M. *J. Magn. Reson. A* **1995**, in press.
(32) Bloom, M.; Reeves, L. W.; Wells, E. J. *J. Chem. Phys.* **1965**, *42*, 1615.
(33) Meiboom, S.; Gill, D. *Rev. Sci. Instrum.* **1958**, *29*, 688.
(34) Mueller, L.; Ernst, R. R. *Mol. Phys.* **1979**, *38*, 963.
(35) Kellogg, G. W.; Schweitzer, B. I. *J. Biomol. NMR* **1993**, *3*, 577.
(36) Gullion, T.; Baker, D. B.; Conradi, M. S. *J. Magn. Reson.* **1990**, *89*, 479.

coherence to $-M_z$.[37] The pulsed-field gradient G1 eliminates all residual transverse coherence including untransferred amino proton coherence and residual transverse H_2O magnetization. This dephasing of all residual transverse magnetization ensures that the H_2O Zeeman polarization remains inverted until it is flipped back to $+z$ by the composite proton 180° pulse in the middle of the nitrogen evolution period t_1. The nitrogen evolution period is terminated by a 90° pulse that converts one component of the transverse t_1-modulated nitrogen coherence along z at point b. In the subsequent interval, a pulsed field gradient G2 purges unwanted coherence. After a recovery interval τ_g (0.2−0.4 ms), a proton 90° pulse converts the surviving $H_zN_z\cos(\delta_i t_1)$ polarization into $H_xN_z\cos(\delta_i t_1)$ which reverts to proton transverse magnetization $H_y\cos(\delta_i t_1)$ during the subsequent reverse INEPT-CPMG period from where it is rotated into Zeeman polarization at point c, which marks the beginning of the NOESY mixing period. Radiation damping[38] during the subsequent NOESY mixing period ensures that most of the H_2O signal rotates back toward the equilibrium $+z$ direction and near the end of the mixing period a pulse field gradient eliminates all residual transverse coherence. In order to preserve the H_2O polarization, the NOESY read pulse at point e is extended into a water flip-back enhanced WATERGATE pulse cascade.[21,23]

Figure 3 depicts the 2D ^{15}N-correlated NOESY spectrum on the ^{15}N-labeled leadzyme in 90% H_2O/10% D_2O. In this experiment amino and imino nitrogen resonances are correlated with protons that are within 4−5 Å of their attached amino or imino protons. Thus NOE cross peaks for amino and imino protons of guanines and adenines are indirectly observed by frequency labeling of their bonded nitrogens. Many of the NOEs to the C amino protons and the U and G imino protons had been previously assigned from analysis of standard NOESY spectra.[9] For example, standard intra base pair and sequential NOEs are observed for the G and U imino protons of the duplex regions of the leadzyme (Figure 1).[6,17,18] The open cross peaks in the left portion of the spectrum represent folded NOE cross peaks to the G and U imino protons with the most intense peaks being auto peaks for the G and U imino proton−nitrogen pairs. The peaks resonating with nitrogen frequencies of 94−100 ppm originate from cytosine amino groups and most of these peaks were previously observed in standard NOESY experiments. However, few of the peaks with nitrogen frequencies of 70−88 ppm are observed in standard NOESY experiments or when the CPMG pulse train is replaced with a simple INEPT interval. The cross peaks at these nitrogen frequencies arise from NOEs involving exchange-broadened G and A amino protons. Resonance assignments of the new amino NOEs in the 2D ^{15}N-correlated NOESY spectrum were made by identification of cross peaks between protons with unique chemical shifts combined with the known structural data for the leadzyme[9] (data not shown). The intense dark cross peaks in the upper left corner of the spectrum represent intra-base G amino to imino cross peaks that were used to help assign the chemical shifts for the G amino nitrogens. The cross section in Figure 3b shows a very strong cross peak to the previously assigned U21 imino proton, indicating formation of this A-U base pair, and assigns the A12 amino nitrogen frequency to 82.2 ppm. The auto peaks corresponding to the amino nitrogen to bonded amino proton are weak due to the large line widths for the amino protons. The A12 amino protons also have NOE interactions with the neighboring base pairs. As seen in Figure 3b sequential NOEs are observed from the A12 amino to the U20 and G22 imino protons as well as the C11 amino protons. Such NOEs to neighboring base pairs are expected in an A-form helix and these additional NOE constraints should improve the local helical structure for this part of the molecule.

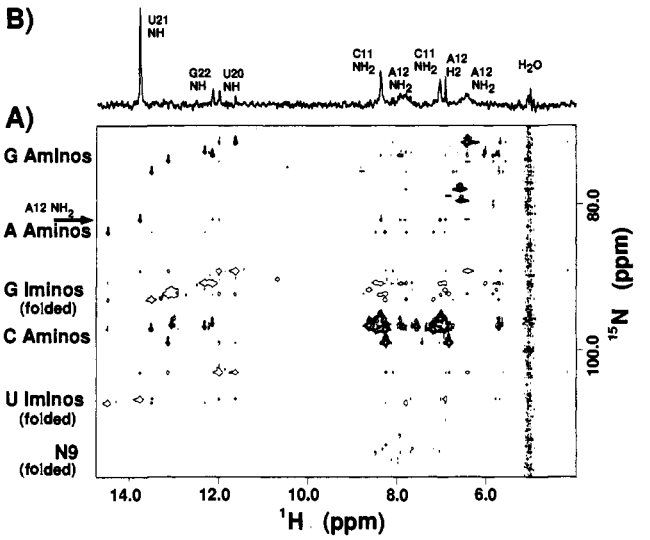

Figure 3. (a) Contour plot of the 2D ^{15}N-correlated NOESY spectrum for the leadzyme in 90% H_2O/10% D_2O at 5 °C using the pulse sequence depicted in Figure 2. Spectral widths in ω_1 and ω_2 were 3300 and 14000.7 Hz, respectively, 220 complex t_1 points were collected with 144 scans per FID, the relaxation delay was 1.3 s, τ_{mix} was 0.15 s, and the total data collection time was 29 h. Gradient amplitudes and durations were as follows: G0 = (12 G/cm, 2.0 ms), G1 = (16 G/cm, 2.0 ms), G2 = (−16 G/cm, 0.76 ms), G3 = (−32 G/cm, 3.0 ms), G4 = (−32 G/cm, 0.4 ms), selective 90° pulse at point a: frequency shifted right-half Gauss,[37] duration = 3.37 ms, small angle phase correction = −16°, selective 90° pulse preceding point e: EBURP1,[20] duration = 7.09 ms, small angle phase correction = −3°, selective square 90° pulses flanking the proton echo pulse at point f: duration = 1.290 ms, small angle phase correction = −2°. Proton and nitrogen RF pulse amplitudes were 32.5 and 9.8 kHz, respectively, 28 synchronous ^{1}H, ^{15}N 180° pulse pairs (^{1}H 180° = 15.4 μs and ^{15}N 180° = 51 μs) were placed both in the INEPT and reverse INEPT period with τ = 119.5 μs. ^{15}N decoupling during t_2 was achieved with GARP1 and a decoupling field of 2 kHz with the ^{15}N carrier at 116 ppm. Subtraction of a 2nd order polynomial fit of the low-frequency H_2O signal was performed on the t_2 FIDs using an algorithm obtained from Dr. Lewis E. Kay, University of Toronto. 90° and 70° shifted sinebell square functions were applied in t_1 and t_2, respectively, prior to Fourier transformation, t_1 and t_2 FIDs were zero-filled to 1024 and 4096 points, respectively, prior to Fourier transformation. The 2D ^{15}N-correlated NOESY spectrum was acquired such that some of the nitrogen resonances are folded in ω_1 but since the first t_1 point was half the dwell time, the folded and unfolded peaks have opposite sign.[14] To differentiate between the folded (negative) and unfolded peaks only two contours are plotted for the folded peaks whereas the unfolded peaks are plotted with up to 6 contours. (b) 1D cross section along ω_2 at the nitrogen frequency for the A12 amino nitrogen. The position of this 1D cross section is indicated by the arrow in the 2D spectrum.

It has been previously shown that both intra- and interstrand NOEs can be observed between G imino protons and ribose 1′ protons in A-form RNA helices.[39] These NOEs are only found at long mixing times because spin diffusion allows transfer of magnetization from the G imino proton to the G amino protons and then to the ribose 1′ proton. However, in the 2D ^{15}N-correlated NOESY spectrum presented here these G amino

(37) Keeler, J. In *Multinuclear Magnetic Resonance in Liquids and Solids—Chemical Applications*; Granger, P., Harris, R. K., Eds.; Kluwer Academic: Boston, 1990; p 201.

(38) Abragam, A. *The Principles of Nuclear Magnetism*; Oxford: Oxford, 1978.

(39) Heus, H. A.; Pardi, A. *J. Am. Chem. Soc.* **1991**, *113*, 4360.

Table 1. New Amino Distance Constraints for the Leadzyme Obtained from the 2D ^{15}N-Correlated NOESY Spectrum[a]

residue	atom	residue	atom
G3	N2	A4	H2
A4	N6	G3	H1
A4	N6	C5	$N4H_2$
A4	N6	G26	H1
A4	N6	U27	H3
A4	N6	C28	$N4H_2$
A12	N6	C11	$N4H_2$
A12	N6	U21	H3
A12	N6	G22	H1
C14	N4	G15	H1
A16	N6	A17	H2
A17	N6	G15	H1′
A17	N6	A16	H8
A17	N6	A18	H2
A18	N6	G15	H1′
A18	N6	G19	H1
G19	N2	G13	H1
G22	N2	A12	H2
G26	N2	U27	H3

[a] Only distance constraints involving amino protons that had not been previously identified in standard NOESY experiments are listed here.[9] The G NH_2 to H1′ constraints were not included in this list because they were previously inferred from G NH to H1′ NOEs[39] (see text). The constraints were defined in XPLOR as distances between the amino nitrogen and the other proton involved in the NOE cross peak with distance bounds of 1.8 to 7 Å, except for NOEs involving two amino protons which were given an upper bound of 8 Å.

to ribose 1′ proton NOEs are observed directly, without complications due to spin diffusion.[40]

Table 1 lists the assigned NOEs involving the A, C, and G amino protons that were obtained from the 2D ^{15}N-correlated NOESY spectrum and that had not been previously observed in standard NOESY spectra. A few cross peaks could not be unambiguously assigned in this 2D ^{15}N-correlated NOESY experiment, due to overlap of the proton resonances. These ambiguities can be overcome by extending this 2D experiment into a third dimension by correlating the resonance of the acceptor protons with their directly bonded carbons and/or nitrogens. This extension to 3D experiments will be useful for crowded regions or for larger RNAs.

One feature of the amino proton cross peaks in the 2D ^{15}N-correlated NOESY spectrum is that they all appear as asymmetric doublets in ω_1, which is a result of a deuterium isotope effect on the nitrogen chemical shifts.[41] In a RNA sample containing 10% D_2O/90% H_2O the abundance of fully protonated and singly deuterated amino groups is 81% and 18%, respectively. Thus each cross peak for the amino groups has a deuterium isotope shifted satellite peak of ≈22% relative intensity.

In Figure 3a there are number of weak cross peaks that appear at nitrogen frequencies of 111 to 116 ppm. These cross peaks arise from through-bond H8 to N9 connectivities for some of the G and A residues. These peaks are folded in ω_1 and have actual nitrogen chemical shifts of 165 to 170 ppm. The cross peaks are quite weak since the INEPT delay was optimized for the large $^1J_{HN}$ coupling constant and thus gives poor transfer for the smaller (<10 Hz) $^2J_{H8-N9}$ coupling constants. These long-range two-bond heteronuclear correlations have been previously used to assign the N9 resonances in the leadzyme.[9]

Structure Determination of the Leadzyme. Almost complete assignment of the proton and protonated carbon and nitrogen resonances for the leadzyme have been made by application of a variety of 2D and 3D heteronuclear NMR experiments on ^{13}C- and/or ^{15}N-labeled molecules.[9,42] Three-dimensional structures for the leadzyme were generated by molecular dynamics calculations using distance and torsion angle constraints obtained from the NMR experiments. In order to assess how these additional amino proton NOEs affect the final structures, two sets of structures were generated for the leadzyme, one with and one without the amino proton NOEs. Restrained molecular dynamics and energy minimization calculations were performed on the leadzyme using the XPLOR program.[43] The calculations started from a set of structures for the leadzyme that had random backbone torsion angles. After simulated annealing molecular dynamics refinement against 414 NOE, torsion angle, and hydrogen bonding constraints, 25 structures with low energy and distance violations were used for further studies. Two sets of more extensive molecular dynamics and energy minimization calculations were then performed; one set used the previously assigned 414 constraints and the second set also included the 19 new amino NOE constraints obtained from the 2D ^{15}N-correlated NOESY spectrum. No attempt was made to quantify the size of the amino NOEs, instead conservative distance bounds of 1.8 to 7 Å were employed, except for NOEs involving two amino protons where an upper bound of 8 Å was employed.

One of the primary goals in the NMR study of the leadzyme is to help understand the structural features that give rise to its lead-dependent site-specific cleavage.[12] Since the active site of the leadzyme is composed of a purine-rich internal loop containing 3 G and 2 A residues (see Figure 1), we hoped that additional NOE information could be obtained for the amino protons in these residues. Unfortunately we did not observe any NOEs for the amino protons on the 5 purine residues in the internal loop of the leadzyme. This absence of NOEs could be a result of these amino protons being in fast exchange with H_2O, could be a result of such extensive exchange broadening of the amino protons that there was not efficient transfer of heteronuclear magnetization even with the CPMG pulse sequence, or could arise from the known dynamics of this internal loop.[9]

However, the 2D ^{15}N-correlated NOESY spectrum provided many new structural constraints for the GAAA loop which is a member of the family of highly stable GNRA tetraloops.[44,45] The structures of GNRA RNA tetraloops have been previously determined by both NMR spectroscopy and X-ray crystallography[46-49] and the known structural features of this loop are also present in the GAAA tetraloop in the leadzyme. The structures determined without the amino NOE constraints had an average pairwise root-mean-square (rms) deviation for the heavy atoms of 3.0 Å for residues 14 to 19. The additional 19 distance constraints derived from the 2D ^{15}N-correlated NOESY spectrum lower this rms deviation to 1.9 Å. As seen in Table 1, 8 of these distance constraints involve protons in the GAAA tetraloop and its closing CG base pair. Figure 4 shows these amino distance constraints for the GAAA tetraloop and six of these constraints involve the A amino protons on

(40) Kalk, A.; Berendsen, H. *J. Magn. Reson.* **1976**, *24*, 343.
(41) Wang, C.; Gao, X. L.; Jones, R. A. *J. Am. Chem. Soc.* **1991**, *113*, 1448.
(42) Legault, P.; Farmer, B. T.; Mueller, L.; Pardi, A. *J. Am. Chem. Soc.* **1994**, *116*, 2203.
(43) Brünger, A. T. *X-PLOR 3.1: A System for X-ray Crystallography and NMR*; Yale University Press: New Haven, 1992.
(44) Antao, V. P.; Tinoco, I., Jr. *Nucleic Acids Res.* **1992**, *20*, 819.
(45) Woese, C. R.; Winker, S.; Gutell, R. R. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 8467.
(46) Heus, H. A.; Pardi, A. *Science* **1991**, *253*, 191.
(47) Orita, M.; Nishikawa, F.; Shimayama, T.; Taira, K.; Endo, Y.; Nishikawa, S. *Nucleic Acids Res* **1993**, *21*, 5670.
(48) Szewczak, A. A.; Moore, P. B.; Chan, Y. L.; Wool, I. G. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 9581.
(49) Pley, H.; Flaherty, K. M.; McKay, D. B. *Nature* **1994**, *372*, 111.

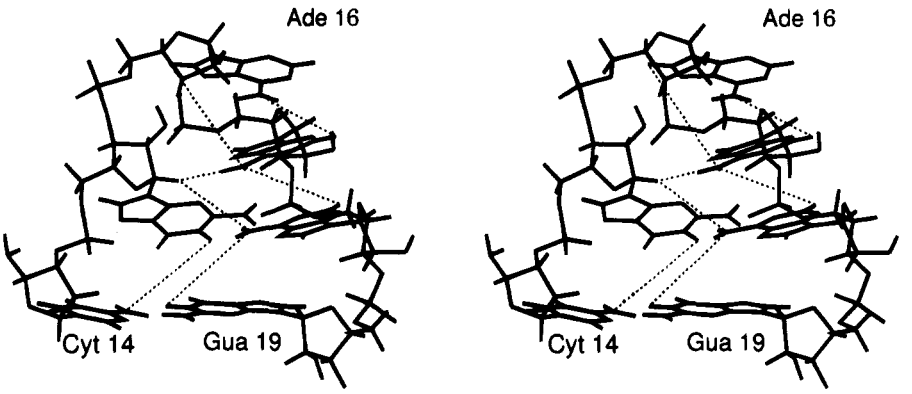

Figure 4. Stereoview of one structure of the GAAA tetraloop in the leadzyme illustrating the amino NOEs that were obtained from the 2D ^{15}N-correlated NOESY spectrum in Figure 3a. The dashed lines represent constraints used in the structure determinations that were derived from NOEs involving amino protons that are not observed in standard NOESY experiments due to chemical exchange broadening of the amino proton resonances.

residues 16−18. It is predicted from this picture that these extra distance constraints would improve the quality of the structure that can be determined for the leadzyme, and this is borne out in the significantly lower rms deviation for the structures that included these constraints. Structure refinements are currently being carried out with additional dihedral angle constraints in order to improve further the structure determination of the leadzyme.

Conclusion

We have presented a new 2D ^{15}N-correlated NOESY experiment that allowed us to identify NOE contacts originating from exchange-broadened amino protons by frequency labeling their attached amino nitrogens. Most of these NOEs are unobservable in standard NOESY experiments due to the very large line widths of the G and A amino protons. A CPMG pulse train was used to efficiently transfer polarization between amino protons and amino nitrogens even in the presence of rotational exchange of the amino protons. Using this experiment 19 new NOEs involving G and A amino protons were identified for the ^{15}N-labeled leadzyme. These NOE constraints led to significantly improved structures for the GAAA tetraloop in the leadzyme and will likely improve structure determinations of many other nucleic acid motifs.

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