In Situ Probing of Adenine Protonation in RNA by ^{13}C NMR

Pascale Legault and Arthur Pardi*

Department of Chemistry and Biochemistry
University of Colorado at Boulder
Boulder, Colorado 80309-0215

Received April 1, 1994

Base protonation likely plays critical structural and functional roles in nucleic acids. Structural studies have revealed that in a large number of RNAs and DNAs, adenine and cytosine pK_a values are significantly perturbed relative to the pK_a values of the mononucleotides.[1-9] NMR provides a direct method for monitoring protonation and therefore is currently the best technique for measuring pK_a values of specific ionizable groups in macromolecules.[10] Protonated adenines and cytosines can be detected directly by ^1H NMR when the added imino proton is in slow exchange with bulk water. However, in many cases hydrogen exchange is too rapid for detection of the imino proton. ^1H NMR can also be used to monitor the pH dependence of nonexchangeable base proton chemical shifts. Although this technique has been used to measure pK_a values of mononucleotides,[11] it is not suitable for accurate determination of pK_a values in folded nucleic acids because the base proton chemical shifts are extremely sensitive to a variety of environmental factors, including electrostatics and conformational changes.[6,12] ^{15}N NMR represents a useful probe for adenine and cytosine protonation because the chemical shift of the imino nitrogen changes by up to 70 ppm upon protonation.[1,13,14] However, the ^{15}N resonances cannot be indirectly detected[15] in all cases,[1] for example, when there is inefficient transfer of magnetization caused by chemical exchange broadening or rapid ^{15}N relaxation. Thus it is essential to have alternate NMR methods for determining the protonation state of nucleic acid bases. Here, we demonstrate that ^{13}C NMR represents a powerful probe of adenine protonation. This technique revealed an unusual pK_a of 6.5 at 25 °C for an adenine close to the cleavage site in a $^{13}C/^{15}N$-labeled lead-dependent ribozyme.[16] The location of a protonated base in the active site of this ribozyme means that this group could play an important structural or functional role in the catalytic reaction.

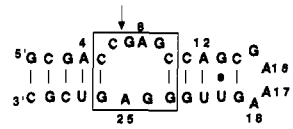

Figure 1. Proposed secondary structure of the leadzyme. The nucleotides required for cleavage are boxed, and the arrow indicates the site of cleavage.[16]

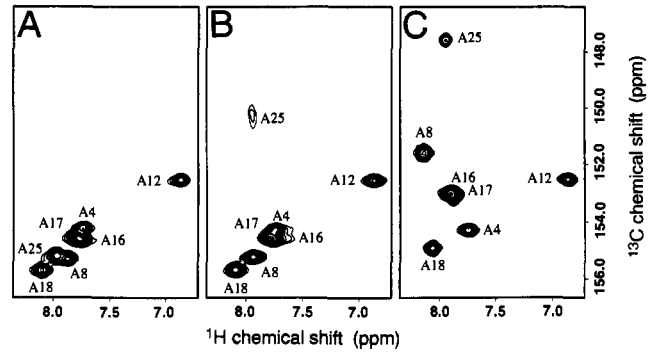

Figure 2. Aromatic C2–H2 region of 2D (^{13}C, ^1H) HMQC spectra[15] at (A) pH 8.3, (B) pH 6.3, and (C) pH 4.4 of a 0.44 mM $^{13}C/^{15}N$-labeled leadzyme in 10 mM sodium phosphate, 100 mM NaCl, and 0.2 mM EDTA in D_2O at 25 °C. The isotopically labeled leadzyme sample was synthesized by in vitro transcription with T7 RNA polymerase and $^{13}C/^{15}N$-labeled NTPs as described previously.[29-31] The pH values were measured in D_2O and adjusted using 0.01–0.1 N NaOD and DCl. After the NMR titration, the integrity of the sample was demonstrated by the observation of a single band using denaturing polyacrylamide gel electrophoresis. NMR spectra were recorded on a Varian VXR-500S spectrometer. The HMQC spectra[15] were recorded with 128 scans, the ^1H carrier at 4.80 ppm, 2048 complex points with a sweep width of 6000 Hz in t_2, the ^{13}C carrier at 145.54 ppm, and 180 complex points with a sweep width of 3000 Hz in t_1. The time of the $1/(2J)$ evolution period[15] was optimized for $J = 209$ Hz. Complex data were collected in t_1 by the hypercomplex-TPPI method,[32] and ^{13}C WALTZ decoupling was applied during t_2. The total time of each HMQC experiment was 8.5 h. The proton chemical shifts were referenced to the internal HOD at 4.80 ppm, and the carbon chemical shifts were referenced to an external standard of TSP at 0.00 ppm. The NMR data were processed with Felix 1.1 (Hare Research Inc.).

We are currently using heteronuclear NMR spectroscopy[17-20] to determine the three-dimensional structure of a small RNA motif, termed the leadzyme, which is specifically cleaved in the presence of lead (Figure 1).[16] Figure 2 shows the aromatic C2–H2 region of 2D (^{13}C, ^1H) HMQC spectra for the 99% $^{13}C/^{15}N$-labeled leadzyme at three different pH values. All seven C2–H2 cross peaks were specifically assigned using a novel application of the heteronuclear HCCH-TOCSY experiment which unambiguously correlates the H8 and H2 within the same adenine residue.[21,22] Unexpectedly, at pH 6.3, the C2 resonance of adenine 25 (A25) shifts significantly upfield compared with the other C2 resonances (Figure 2B). As seen in Figure 3A, the A25 C2 resonance shifts by 8 ppm between pH 4.4 and 8.3, and its chemical shift as a function of pH displays a sigmoidal dependence characteristic of a titration binding curve. The Hill plot in Figure 3B gives a pK_a of 6.5 with a slope of 0.96, indicating that this transition involves protonation of a single site.[23] To verify that the observed change in chemical shift results from

* Author to whom correspondence should be addressed.

(1) Wang, C.; Gao, H.; Gaffney, B. L.; Jones, R. A. *J. Am. Chem. Soc.* **1991**, *113*, 5486–5488.
(2) Boulard, Y.; Cognet, J. A. H.; Gabarro-Arpa, J.; Le Bret, M.; Sowers, L. C.; Fazakerley, G. V. *Nucleic Acids Res.* **1992**, *20*, 1933–1941.
(3) Puglisi, J. D.; Wyatt, J. R.; Tinoco, I., Jr. *Biochemistry* **1990**, *29*, 4215–4226.
(4) Macaya, R. F.; Gilbert, D. E.; Shiva, M.; Sinsheimer, J. S.; Feigon, J. *Science* **1991**, *254*, 270–274.
(5) Dolinnaya, N. G.; Fresco, J. R. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 9242–9246.
(6) Gao, X. L.; Patel, D. J. *J. Am. Chem. Soc.* **1988**, *110*, 5178–5182.
(7) Liu, K.; Miles, H. T.; Frazier, J.; Sasisekharan, V. *Biochemistry* **1993**, *32*, 11802–11809.
(8) Leroy, J.-L.; Gehring, K.; Kettani, A.; Guéron, M. *Biochemistry* **1993**, *32*, 6019–631.
(9) Jaishree, T. N.; Wang, A. H.-J. *Nucleic Acids Res.* **1993**, *21*, 3839–3844.
(10) Fersht, A. *Enzyme Structure and Mechanism*; W. H. Freeman: New York, 1984; p 475.
(11) Sowers, L. C.; Fazakerley, G. V.; Kim, H.; Dalton, L.; Goodman, M. F. *Biochemistry* **1986**, *25*, 3983–3988.
(12) Wüthrich, K. *NMR of Proteins and Nucleic Acids*; John Wiley & Sons: New York, 1986.
(13) Büchner, P.; Maurer, W.; Rüterjans, H. *J. Magn. Reson.* **1978**, *29*, 45–63.
(14) Live, D. H.; Radhakrishnan, I.; Misra, V.; Patel, D. J. *J. Am. Chem. Soc.* **1991**, *113*, 4687–4688.
(15) Mueller, L. *J. Am. Chem. Soc.* **1979**, *101*, 4481–4484.
(16) Pan, T.; Uhlenbeck, O. C. *Nature* **1992**, *358*, 560–563.
(17) Nikonowicz, E. P.; Pardi, A. *Nature* **1992**, *355*, 184–186.
(18) Nikonowicz, E. P.; Pardi, A. *J. Am. Chem. Soc.* **1992**, *114*, 1082–1083.
(19) Pardi, A.; Nikonowicz, E. P. *J. Am. Chem. Soc.* **1992**, *114*, 9202–9203.
(20) Nikonowicz, E. P.; Pardi, A. *J. Mol. Biol.* **1993**, *232*, 1141–1156.
(21) Marino, J. P.; Prestegard, J. H.; Crothers, D. M. *J. Am. Chem. Soc.* **1994**, *116*, 2205–2206.
(22) Legault, P.; Farmer, B. T., II; Mueller, L.; Pardi, A. *J. Am. Chem. Soc.* **1994**, *116*, 2203–2204.

0002-7863/94/1516-8390$04.50/0

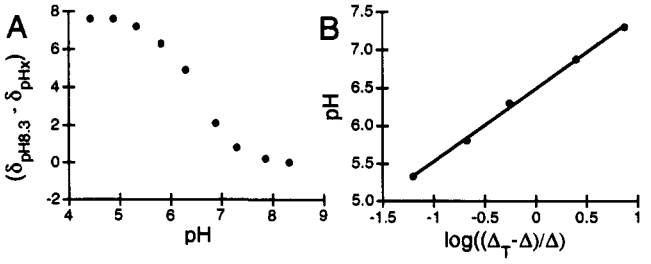

Figure 3. (A) Relative change of the C2 chemical shift ($\delta_{pH8.3} - \delta_{pHx}$) for A25 with pH. (B) Hill plot of the data presented in A. The Hill equation for one-site protonation is $pH = \log((1-\alpha)/\alpha) + pK_a$, where α represents the fraction of the protonated species.[23,33]

protonation of adenine N1, ^{13}C NMR spectra of 99% $^{13}C/^{15}N$-labeled 5′ AMP were obtained as a function of pH.[24-26] These show a similar 8 ppm upfield shift of the C2 resonance upon protonation, and analysis of the Hill plot for the titration data gives a pK_a of 4.0 ± 0.1,[26] which is within experimental error of the previously measured value.[27] The pK_a values of the other six adenines in the leadzyme were measured to be ≤4.3 (data not shown). The pK_a of 6.5 determined for A25 of the leadzyme is 2.5 pH units higher than that for protonation of adenine in 5′ AMP and means that this ribozyme possesses a base with a pK_a near physiological pH.

The unusual ^{13}C chemical shift for the C2 resonance allows facile identification of protonated adenines in RNAs and DNAs. Once identified, the structural features that give rise to these unusual pK_a values can be probed. For example, the unusual ^{13}C chemical shift for A25 C2 allowed straightforward assignment of the ^1H–^1H NOEs involving A25 H2 in a 3D (^1H, ^{13}C, ^1H) NOESY-HMQC spectrum[20] of the leadzyme. This experiment provides critical NOEs that are being used to define the structure of the active site of the leadzyme.[26] These preliminary data indicate that A25 is close to the C6pG7 cleavage site, which means that the protonated adenine identified here could be acting as a general acid catalyst in the cleavage reaction.[28] Another interesting structural characteristic of A25 is observed in the (^{13}C, ^1H) HMQC spectrum at pH 6.3, where there are almost equal populations of the protonated and unprotonated species and the A25 C2 resonance is significantly broadened (Figure 2B). The broadening is due to chemical exchange and provides dynamic information about the active site of the leadzyme. Further NMR studies are being performed to investigate the structural features that give rise to the unusual pK_a for A25 in the leadzyme.

Having a titratable group with a pK_a near physiological pH has important structural and functional implications for RNA, and it will be interesting to see if such "histidine-like" moieties are a common feature of biologically active RNAs. The method described here should prove extremely useful for identification of adenines with unusual pK_a values in other ribozymes.

Acknowledgment. We thank Kathy Morden for critical reading of the manuscript and Jennifer Doudna for the leadzyme template. This work was supported by NIH Grant AI30726 and a NIH Research Career Development Award AI01051 to A.P. P.L. was supported by a NSERC 1967 Science and Engineering scholarship and a FCAR (Fonds pour la Formation de Chercheurs et l' Aide à la Recherche) scholarship. We also thank the W. M. Keck Foundation for their generous support of RNA science on the Boulder campus.

(23) Wyman, J.; Gill, S. J. *Binding and Linkage. Functional Chemistry of Biological Macromolecules*; University Science Books: Mill Valley, CA, 1990; p 330.
(24) Dorman, D. E.; Roberts, J. D. *Proc. Natl. Acad. Sci. U.S.A.* **1970**, *65*, 19–26.
(25) Sierzputowska-Gracz, H.; Gopal, H. D.; Agris, P. F. *Nucleic Acids Res.* **1986**, *14*, 7783–7801.
(26) Legault, P.; Pardi, A. Unpublished results.
(27) Saenger, W. *Principles of Nucleic Acid Structure*; Springer Verlag: New York, 1984; p 556.
(28) Jencks, W. P. *Catalysis in Chemistry and Enzymology*; Dover: New York, 1987.
(29) Milligan, J. F.; Groebe, D. R.; Witherell, G. W.; Uhlenbeck, O. C. *Nucleic Acids Res.* **1987**, *15*, 8783–9798.
(30) Heus, H. A.; Pardi, A. *J. Mol. Biol.* **1991**, *217*, 113–124.
(31) Nikonowicz, E. P.; Sirr, A.; Legault, P.; Jucker, F. M.; Baer, L. M.; Pardi, A. *Nucleic Acids Res.* **1992**, *20*, 4507–4513.
(32) Marion, D.; Ikura, M.; Tschudin, R.; Bax, A. *J. Magn. Reson.* **1989**, *85*, 393–399.
(33) The value of α is calculated from the change in chemical shift relative to the protonated state at a given pH, Δ, divided by the total change in chemical shift between the unprotonated and the protonated state, Δ_T. The Hill equation can then be rewritten as $pH = \log((\Delta_T-\Delta)/\Delta) + pK_a$. Δ_T was initially estimated to be 7.6 ppm, which is the difference in C2 chemical shifts observed between pH 8.3 and 4.4, and was used to obtain the initial value for pK_a. Δ_T was then refined to 7.8 ppm by extrapolation after estimating the percentage of fully protonated and fully unprotonated species at pH 4.4 and 8.3, respectively. The corrected Δ_T value was used in Figure 3B. Linear regression analysis gives a slope of 0.957 and a pK_a of 6.49, with an R value of 0.999. Assuming an error of 0.6 ppm for Δ_T, which is twice the error in the ^{13}C chemical shift measurements, the pK_a of 6.5 is calculated to have an error of ±0.1.