An accessible two-dimensional solution nuclear magnetic resonance experiment on human ubiquitin*

ARTICLE *in* BIOCHEMISTRY AND MOLECULAR BIOLOGY EDUCATION · MARCH 2006

Impact Factor: 0.65 · DOI: 10.1002/bmb.2005.494033022423 · Source: PubMed

2 AUTHORS:

David Rovnyak
Bucknell University
31 PUBLICATIONS **845** CITATIONS
SEE PROFILE

Laura Mcknight
Fred Hutchinson Cancer Research Center
17 PUBLICATIONS **332** CITATIONS
SEE PROFILE

BIOCHEMISTRY AND MOLECULAR BIOLOGY EDUCATION
Vol. 33, No. 2, pp. 117–122, 2005

Laboratory Exercises

An Accessible Two-dimensional Solution Nuclear Magnetic Resonance Experiment on Human Ubiquitin*

Received for publication, August 19, 2004

David Rovnyak‡ and Laura E. Thompson

From the Department of Chemistry, Bucknell University, Lewisburg, Pennsylvania 17837

Solution-state nuclear magnetic resonance (NMR) is an invaluable tool in structural and molecular biology research, but may be underutilized in undergraduate laboratories because instrumentation for performing structural studies of macromolecules in aqueous solutions is not yet widely available for use in undergraduate laboratories. We have implemented an experiment that is ideal for more commonly available 4.8–7.0 Tesla, double-channel NMR instruments that would not usually be used for biomolecular NMR work. We analyzed a commercially available, ^{15}N-enriched human ubiquitin sample with a two-dimensional correlation experiment using indirect ^{1}H evolution and direct ^{15}N detection, which produced spectra with high resolution on a spectrometer operating at 7.0 Tesla (300 MHz ^{1}H resonance frequency). The simplicity of the experiment makes it possible to be configured by undergraduate students with minimal supervision from the instructor. Students gain experience in acquiring multidimensional biomolecular NMR experiments, confirm that ubiquitin is stably folded, and observe the correspondence between specific signals and individual amino acids in ubiquitin.

Keywords: Human ubiquitin, solution-state nuclear magnetic resonance, heteronuclear single-quantum correlation, direct detection.

Solution-state nuclear magnetic resonance (NMR)[1] spectroscopy has become an indispensable tool for life scientists to discover the solubilized structures of biomacromolecules such as proteins, DNA, and RNA [1–4]. Additionally, solution NMR spectra can reveal local and global dynamics of biomacromolecules, localize sites of functional importance, and characterize intermolecular interactions. However, the large scope of NMR applications utilized at the graduate and postgraduate levels of research is often not reflected in undergraduate life sciences laboratory curricula. Difficulties in establishing biomolecular NMR in a curriculum may often be attributed to the cost of purchasing and operating specialized, high-resolution NMR instrumentation and to the considerable expertise required for performing macromolecular NMR studies. We report here a student-accessible, stand-alone experiment that we developed for a biophysical chemistry laboratory to provide hands-on and theoretical reinforcement of introductory concepts in biomolecular NMR, which requires only modestly equipped, pulsed Fourier-transform (FT) NMR instrumentation operating at low fields (4.8–7.0 Tesla). Specifically, we considered instruments configured for routine chemistry applications that generally offer two independent radio frequency (RF) channels and a probe optimized for X-channel (*e.g.* ^{13}C, ^{31}P, ^{15}N) signal detection. Such instruments are often available to undergraduate educators, and it is worthwhile to examine ways to exploit their capabilities for enhancing biomolecular laboratory experiences.

We decided upon several criteria to be satisfied. An experiment should use heteronuclear correlations of an isotopically enriched sample to reflect this standard practice in NMR structural studies; should not use water suppression, shaped pulses, or gradient pulses that cannot be assumed to be available in the instrumentation considered here; should not require a probe change; and should yield spectra of high quality and biological significance. Additionally, undergraduate students, with minor supervision, should be able to configure and run the experiment; an isotopically enriched protein sample must be commercially available; and the experimental time should be reasonable for an upper-level laboratory. We have introduced a ^{15}N-detected ^{15}N{^{1}H} two-dimensional (2D) heteronuclear single-quantum coherence (HSQC) experiment on human ubiquitin into our biophysical chemistry laboratory that

* A website is available at www.facstaff.bucknell.edu/drovnyak/ubiq_hsqc.html, where the data used here may be downloaded, along with the processing scripts used with the nmrPipe program and a set of student exercises; a version of the exercises with a discussion of the answers will be supplied to any faculty making a request to drovnyak@bucknell.edu.

‡ To whom correspondence should be addressed: Department of Chemistry, Bucknell University, Lewisburg, PA 17837. E-mail: drovnyak@bucknell.edu.

[1] The abbreviations used are: NMR, nuclear magnetic resonance; FT, Fourier transform; RF, radio frequency; 2D, two dimensional; HSQC, heteronuclear single-quantum coherence; INEPT, insensitive nuclei enhanced by polarization transfer; HETCOR, heteronuclear correlation; NOE, nuclear overhauser effect; DSS, 2,2-dimethyl-2-silapenta-5-sulfonic acid; 1D, one dimensional; SNR, signal-to-noise ratio; FID, free induction decay.

fulfills these conditions. This experiment yields a well-dispersed set of signals distributed in a 2D plane, where the coordinates of each signal are the ^{1}H and ^{15}N chemical shifts of an amide ^{1}H-^{15}N spin pair. It is noteworthy that this is usually the first multidimensional NMR experiment that researchers conduct when carrying out a full structural study of a protein, and is also the building block of many other biomolecular NMR experiments.

The ^{15}N-detected ^{15}N{^{1}H} HSQC experiment we describe here is less sensitive than the more conventionally applied ^{1}H-detected ^{1}H{^{15}N} HSQC [5, 6]. It was chosen in order to cope with instrumentation that is not optimized for proton detection in aqueous samples. Numerous significant benefits from the strategy of ^{15}N detection (a.k.a. direct detection) make it an important NMR technique, for example, in enabling proteins of high molecular weight or containing paramagnetic centers to become more amenable to study [6–9]. The benefits of direct detection include i) no need to perform suppression of the water proton signal; ii) therefore no spectral distortion due to incompletely suppressed water signals; iii) a long direct acquisition time giving very high resolution in the ^{15}N dimension; and iv) the ability to center the applied proton frequency in the amide region ($\delta(^1H) \sim 8.2$ ppm) rather than at the water frequency ($\delta_{H_2O}(^1H) \sim 4.8$ ppm), which makes it possible to achieve a high spectral resolution in the proton indirect evolution period (see Appendix 1 for explanation). Finally, there is no need for a reverse transfer of coherence from ^{15}N back to ^{1}H at the end of the experiment. During such a "back" step, signal intensity may decrease due to additional T_1 relaxation, and the extra pulses ($\sim$8) usually needed for the back-transfer have typical pulse imperfections such as RF inhomogeneity that contribute to signal losses. Thus, the sensitivity of the directly detected ^{15}N experiment is certainly not prohibitive, as the spectra here demonstrate, and indeed we have been very impressed with the robust nature of this experiment.

In 1–2 h, the ^{15}N{^{1}H} HSQC yields a 2D spectrum of good quality, while a 4–6 h experiment can provide very high quality spectra. In our implementation, students configure, acquire, and process the short experiment in the laboratory period, but are given data also from a longer experiment for comparison.

MATERIALS AND METHODS

Sample—Uniformly ^{15}N-enriched human ubiquitin was purchased from VLI Research (Malvern, PA). The sample consists of 1.0 mM ubiquitin in 50 mM potassium phosphate buffer at pH 5.8. All spectra were obtained at about 293 K. Solubilized ubiquitin should have a shelf life of many years when stored at 4 °C.

Pulse Sequence—All spectra were acquired at room temperature, without sample spinning, on a Bruker ARX300 spectrometer with a two-channel probe optimized for X channel detection. The pulse sequences and data sets are available on the World Wide Web (www.facstaff.bucknell.edu/drovnyak/ubiq_hsqc.html) or upon request to the communicating author. The pulse sequence for the ^{15}N{^{1}H} HSQC is shown in Fig. 1*a* and is a simple extension of an important pulse scheme known as INEPT (insensitive nuclei enhanced by polarization transfer) [10]. The sequence of Fig. 1 is very closely related to the familiar HETCOR (heteronuclear correlation) experiment, and the instructor may adapt the HETCOR pulse program on their current instrumentation to the scheme shown in Fig. 1 (a good student exercise if time permits).

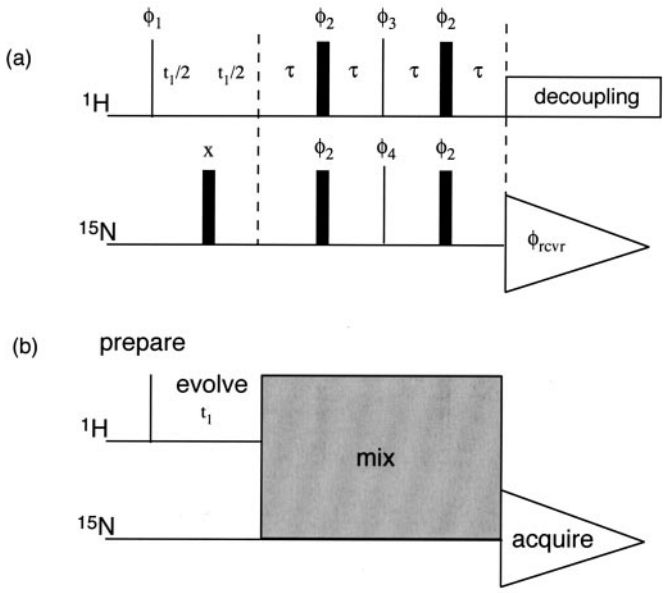

FIG. 1. **Pulse sequence for the ^{15}N-detected HSQC experiment.** The detailed pulse sequence is shown in *a* where the phase cycling is $\phi_1 = 4(x),4(-x)$; $\phi_2 = 8(x),8(-x)$; $\phi_3 = y,-y$; $\phi_4 = 2(x),2(-x)$; $\phi_{rcvr} = x,-x,-x,x,-x,x,x,-x$. The delay τ should be set to $1/4J_{NH}$, where the indirect spin-spin coupling for backbone amide ^{1}H-^{15}N spin pairs is about 92 Hz, giving $\tau = 2.7$ ms. The scheme in *b* shows how the sequence conforms to the basic layout required of all 2D experiments: preparation, evolution, mixing, and acquisition. To perform States acquisition, two spectra must be acquired for every t_1 value, where the phase cycle for the first pulse (ϕ_1) is incremented by 90° for the second spectrum and then reset for the next t_1 value. For States-TPPI acquisition, collect two spectra as described for each t_1 value and also increment the phase cycles of ϕ_1 and ϕ_{rcvr} by 180° when t_1 is incremented.

Coherence transfer by INEPT is a "through-bond" process that relies upon the scalar (a.k.a. "J") coupling to correlate two nuclei, unlike the nuclear overhauser effect (NOE), which is a through-space phenomenon based on the dipole-dipole coupling. The ^{15}N signal obtained by INEPT is enhanced by an approximate factor of 10 *versus* the signal that would be obtained following a single 90° pulse to ^{15}N. The pulse sequence consists of eight total pulses, of either 90° ($\pi/2$) or 180° (π) angle rotations, as shown in Fig. 1*a*. Fig. 1*b* summarizes the key steps of the sequence: first, a $\pi/2$ pulse creates transverse coherence for protons and is the preparation step. The proton coherence is allowed to evolve during an incremented evolution period $t_1/2 - \pi_N - t_1/2$, in which a π pulse on the nitrogen channel refocuses the J_{NH} coupling during t_1. Next, a set of pulses spaced by a time τ directs coherence transfer between directly bonded backbone ^{1}H-^{15}N pairs only using the INEPT scheme, which exploits the 92 Hz J coupling of ^{1}H-^{15}N backbone amide spin pairs that is nearly invariant in proteins. This step is termed mixing because it involves the transfer of coherence among nuclei. Setting the parameter $\tau = 1/4J_{NH} \sim 2.7$ ms will optimize the coherence transfer, while also suppressing signals originating from -NH_2 and -NH_3 groups. The final step is acquisition, which involves digitizing the signal on the ^{15}N RF channel and storing it in a computer. The experiment is repeated over a series of evolution times $t_1 = 0$, Δt_1, $2\Delta t_1$,. . . . We chose the States-TPPI approach to achieve pure phase line shapes and sign discrimination in the indirect evolution period. Further experimental details are given in the figure captions. We performed external referencing with an aqueous solution of 2,2-dimethyl-2-silapenta-5-sulfonic acid (DSS), which gives a single proton resonance defined to be 0 ppm. The proton frequency corresponding to 0 ppm should be multiplied by 0.101329118 to obtain the 0 ppm nitrogen frequency [11]. However, approximate referencing of the ubiquitin spectrum based on

the water resonance ($\delta(^1H, H_2O) \sim 4.80$ ppm at pH 5.5 and 298 K) can be sufficient for teaching purposes.

Data Processing—Processing of the 2D data sets was performed both in the Bruker xwin-nmr software environment and with nmrPipe [12]. The HSQC is highly tolerant to variations in processing. We typically applied a squared 90°-shifted sine bell apodization function to the direct (^{15}N) dimension followed by zero-filling to extend the 512 complex points to 2,048 complex points. In the indirect ^1H dimension we used linear prediction to double the length of the free induction decays (FIDs), followed by a single-shifted sine bell apodization window and zero-filling to 1,024 complex points.

AIMS AND CONDUCT OF THE EXERCISE

It is assumed that basic pulsed FT NMR has been presented in a lecture or prelaboratory component of the class. Also, there is a rich body of knowledge of the biochemistry associated with ubiquitin that is outside the scope of this report, but which may be presented to varying degrees depending upon the instructor's specific goals.

The following sequence of experiments is ordered to promote an active learning approach to multidimensional protein NMR. Students will be challenged to use NMR to i) demonstrate that human ubiquitin adopts a stable fold in solution and ii) demonstrate that multidimensional NMR is needed to measure resolved resonances for backbone amide proton and nitrogen nuclei.

Students should first acquire one-dimensional (1D) proton spectra with and without a presaturation pulse applied at the resonance frequency of the water protons. This corresponds to the sequence (shown with the presaturation pulse)

$$presaturation(x)–90°(\phi)–acquire(\theta)$$

in which we used a 4-s presaturation pulse with fixed transmitter phase (x) at a power level decreased by 50 dB relative to the power used for the 90° pulse. Standard phase cycling for this sequence is $\phi = \{x,y,-x,-y\}$, and $\theta = \{x,y,-x,-y\}$. A Hahn-echo sequence (presat – 90° – τ_1 – 180° – τ_2 – acquire) may give a cleaner baseline and reduced phase errors (for a good discussion on the Hahn-echo and configuring τ_1,τ_2 see p. 177–179 of Ref. 1). Instructor assistance may be needed to set the carrier frequency to the water resonance to achieve reasonable water suppression. A low receiver gain is needed to prevent receiver saturation by the water signal when using only a $\pi/2$ pulse, and little or no signal from the protein is observable. Upon including a presaturation pulse, a significantly higher receiver gain is made possible due to the attenuation of the water signal; many signals from the protein should then be observed. The amide proton region is about 6–11 ppm for most proteins; the region of 6–10 ppm of the ^1H spectrum with presaturation (with ^{15}N-decoupling) that we recorded on ubiquitin is shown in Fig. 2*a*. The signal-to-noise ratio (SNR) and resolution of the signals observed in this region are enhanced by decoupling on the ^{15}N channel during acquisition; however, this is not needed to appreciate that the signals are well-dispersed, which is consistent with a folded protein. The quality of water suppression, by presaturation or otherwise, on a system lacking gradients and using a probe optimized for X-channel spectroscopy may be poor, but will suffice for these initial 1D experiments.

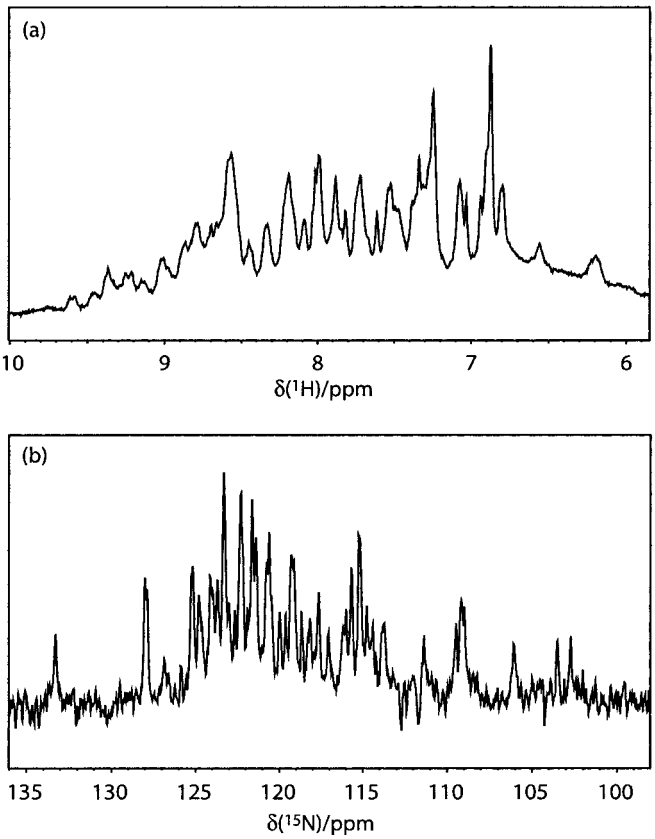

FIG. 2. **1D spectra of human ubiquitin.** *a*, the 6–10 ppm region of the ^1H spectrum (with ^{15}N decoupling using waltz16) using presaturation followed by a single 90° pulse, acquired with 8,192 complex points, 96 transients, a 11.5-ppm spectral width, a recycle time of 4.2 s (0.2-s delay plus 4-s presaturation; presat pulse was 50 dB attenuated relative to the hard pulse), and requiring 9 min of experimental time. *b*, the ^{15}N spectrum (with ^1H decoupling using waltz16) using the sequence of Fig. 1 with $t_1 = 0$ and omitting the first π pulse on the ^{15}N channel, acquired with 1,024 complex points, 512 transients, a 38-ppm spectral width, a recycle time of 1.6 s, and requiring 18 min of experimental time.

At this stage, we asked students how many separate resonances contribute to the total observed signal in the amide region of the proton NMR spectrum with the goal of recognizing that ubiquitin consists of 76 residues and should give rise to a similar number of resonances, yet it is clearly not possible to resolve all of these signals in the amide region of the 1D proton NMR spectrum. This question of the number of resonances to expect is explored in more detail in the exercises (*e.g.* how many proline residues are in the ubiqution). Also, aromatic and side-chain amine protons will overlap partially with the amide proton region (for further reading on protein shifts, see Ch. 8 of Ref. 1). One can next acquire a 1D ^{15}N NMR experiment that detects only backbone amide nitrogen resonances by exploiting an INEPT transfer. The 1D-^{15}N experiment is carried out by using the pulse sequence of Fig. 1 with $t_1 = 0$, and omitting the first π pulse on the ^{15}N channel. The ^{15}N spectrum (with ^1H decoupling) is shown in Fig. 2*b*. This spectrum shows good dispersion, but the SNR is poor and the amide nitrogen signals are still significantly overlapped. These simple 1D experiments lead to the key

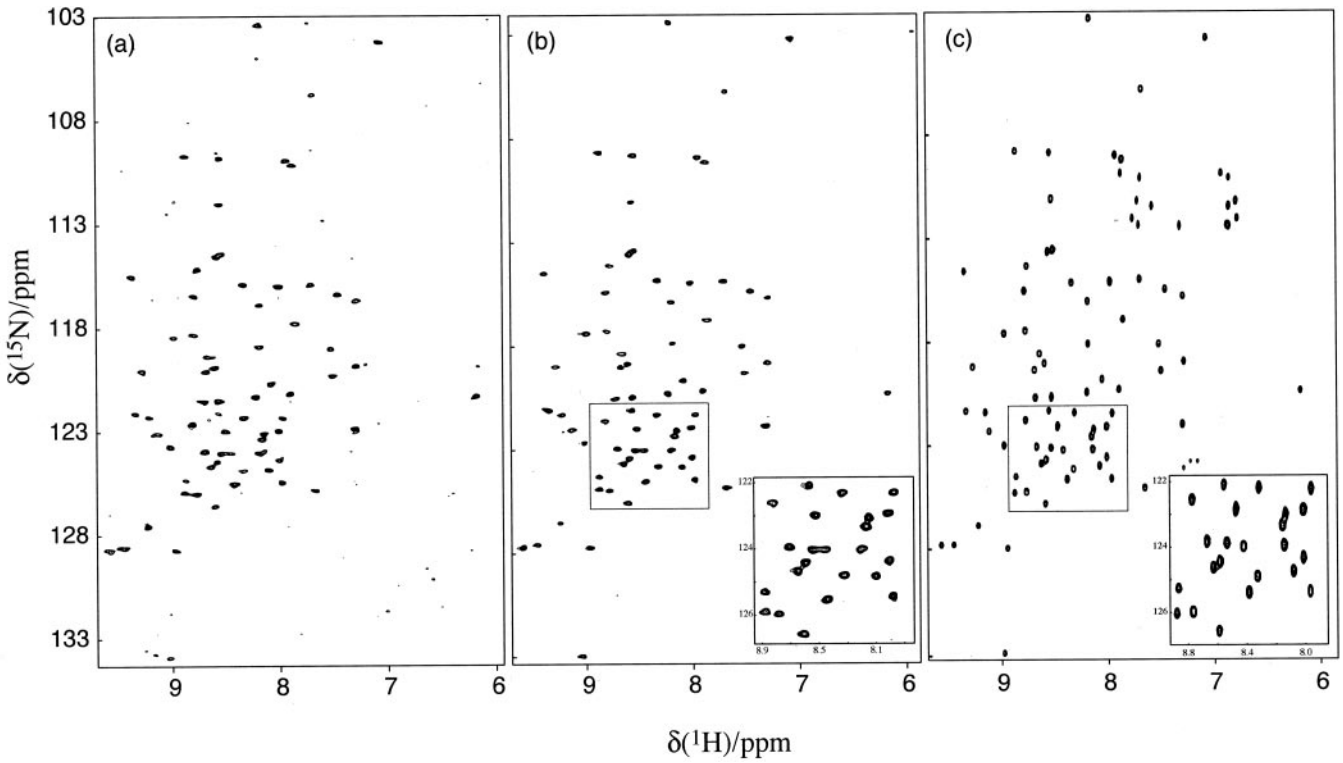

FIG. 3. **HSQC spectra of ^{15}N-enriched human ubiquitin.** Experiments using the ^{15}N{^{1}H} HSQC were acquired at 7.0 Tesla with (*a*) 48 t_1 increments (96 FIDs) and 48 scans per FID requiring 2 h total experimental time and (*b*) 64 t_1 increments (128 FIDs) and 112 scans per FID requiring 6 h total experimental time. The inset in *b* shows an expanded view drawn with lower contour levels of the region indicated by the dashed box to indicate the good resolution of the method. Panel *c* shows data acquired independently using a conventional ^{1}H{^{15}N} HSQC (sensitivity enhanced) from a similarly prepared sample at 11.7 Tesla (500 MHz frequency for ^{1}H) (data set acquired by Richard Harris, University College London, shown with permission). For both *a* and *b*, we used sw(^{1}H) = 4.50 ppm, sw(^{15}N) = 36.0 ppm, ν(^{1}H) = 8.112 ppm, ν(^{15}N) = 117.02 ppm, 512 complex points (real + imaginary) for ^{15}N signal acquisition, and a recycle time of 1.2 s. For *c*, 128 t_1 increments (256 FIDs) and 4 scans per FID were used, requiring 24 min of experimental time.

hypothesis of multidimensional NMR spectroscopy: in the context of this problem, if two amide protons have the same resonance frequency, perhaps they could be distinguished if their attached nitrogen nuclei had distinct chemical shifts. This hypothesis is tested by acquiring a 2D spectrum in which the observed signals occur in a 2D plane and have coordinates given by the ^{1}H and ^{15}N chemical shifts of directly bonded backbone ^{15}N-^{1}H spin pairs.

The ^{15}N-detected HSQC pulse sequence (Fig. 1) will provide such a correlated spectrum. Furthermore, it is possible for students to configure this experiment because they need only determine an appropriate power level and pulse width for the $\pi/2$ pulse on the ^{1}H channel. Students can monitor the amide region of the spectrum as a function of pulse length and note the time at which the signal is nulled as the 180° pulse length, from which the 90° pulse length can be obtained by dividing this time in half. The ^{15}N pulse/power calibrations are also needed, but should be well known for a particular probe/console. Spectral widths, recycle times, and number of increments in t_1 should be previously set by the instructor, and we recommend following the guidelines given in the Fig. 3 caption. This procedure closely mimics the setup of more complex biomolecular NMR experiments. Due to the high degree of linearity in the amplifiers supplied with NMR spectrometers, the configuration of biomolecular NMR pulse sequences can be automated to such a degree that a user can specify only the $\pi/2$ pulse length and corresponding power level for each channel; routines in the pulse program and/or the spectrometer software set parameters for shaped pulses, composite pulse decoupling, and other specialized sequence elements.

A ^{15}N{^{1}H} HSQC spectrum acquired in 2 h is shown in Fig. 3*a*. It is immediately recognized that improved dispersion of signals has been achieved. Students may process the data locally on the spectrometer, or the data may be exported and processed using software such as nmrPipe [12], a commonly used and freely available NMR data processing program. We were able to visually identify 70 resonances in both the 2- and 6-h experiments (Fig. 3, *a*–*b*), and it is remarkable to obtain nearly complete resolution at low field and without the use of more advanced experimental methods. These spectra may be compared with Fig. 3*c*, which shows a ^{1}H{^{15}N} HSQC recorded on a similarly prepared sample at 11.7 Tesla (500 MHz ^{1}H frequency) [13]. The additional signals in Fig. 3*c* originate from side-chain NH_2 groups because this experiment utilized τ = 1/8J, which does not perform multiplicity editing (a good additional experiment for this laboratory, time permitting). In comparing the insets of Fig. 3, it is seen that the signals are particularly well resolved in the nitrogen dimension of the direct detected ^{15}N{^{1}H} experiment. There is less difference in the proton resolution of Fig. 3, *b* and *c*, which is due to the ability to obtain high resolution by centering the proton carrier in the amide proton region (see Appendix 1) when performing direct detection. Res-

onance assignments are provided by the vendor and were in good qualitative agreement with our observed chemical shifts. Small differences are understandable because buffer, salt, and temperature conditions were likely not identical between our sample and that used for the vendor's assignments.

Counting the signals in the 2D-HSQC was an important experience for students. For example, students learned to identify the approximate noise threshold in the data and recognize how to treat regions in which resonances are partially overlapped (*e.g.* inset of Fig. 3*b*). Students should be challenged to understand why they did not count exactly 76 signals.

With prior assignments in hand, students may then observe some examples of the influence of structure on chemical shifts. A feature observed in the NMR spectra of many proteins is the presence of one or perhaps several highly unusual (a.k.a. pathological) chemical shifts, which are often due to unusual features in secondary and tertiary structure and which may point to regions in the protein of special interest. One good example in ubiquitin involves isoleucine 36, which students should attempt to locate on the spectrum (*e.g.* by comparison with the vendor's assignments) and rationalize its unusual shift by examining the structure for human ubiquitin (pdbid: 1D3Z). Exercises for students are described in more detail in the supplemental material.

CONCLUSION

We have described a 2D biomolecular NMR experiment that makes it possible to introduce protein NMR into a biophysical or biochemistry curriculum in a self-contained experimental module. A 2D ^{15}N-detected HSQC experiment provides high quality spectra using modest instrumentation by taking advantage of numerous benefits of detecting the NMR signal on the ^{15}N channel. Students develop a good working knowledge of the ^{1}H-^{15}N HSQC, a cornerstone experiment in protein NMR research, which also represents the initial "out" step of a great number of "out and back" protein NMR experiments that correlate the backbone amide proton resonances with intra- and interresidue nuclei such as N, HA, CA, CB, or C′ [14–18]. In addition to hands-on training in configuring and processing a biomolecular NMR experiment, the goals for student learning in this experiment include recognizing the ability of the ^{15}N-^{1}H HSQC to probe the folded structure of proteins and recognizing the increased resolution that is possible with multidimensional NMR of biomolecules.

This experiment has decreased sensitivity relative to the ^{1}H-detected analog. However, we found this to be a reasonable trade-off for the ability to robustly acquire essentially completely assignable 2D NMR data on an 8.5-kDa protein using instrumentation that is not usually employed for solution protein NMR work, but is available to many educators. Where more advanced instrumentation is available, the proton-detected ^{1}H{^{15}N} HSQC will provide a high SNR in less experimental time and may be substituted for the sequence described here. However, the ^{1}H{^{15}N} HSQC, which may contain about a dozen pulses and utilize more advanced pulse sequence concepts (*e.g.* shaped pulses, pulsed field gradients, etc.), may be less accessible for students as a first experience in biomolecular NMR.

The exercises described here may be explored in more depth for honors study and are certainly not the limit for biomolecular NMR experiments that would be suitable for undergraduate curricula with the ^{15}N-enriched ubiquitin used in this report. Evaluation of other experiments is underway in our laboratory.

Acknowledgments—We are very grateful to Dr. Richard Harris at the Bloomsbury Center of Structural Biology, University College London, for permission to use the ^{1}H{^{15}N} HSQC data (**Fig. 2c**) from the Ubiquitin NMR Resource Page [13]. We also thank Mr. Brian Breczinski for assistance with the operation of the spectrometer, and Bucknell University for purchasing the ^{15}N-enriched ubiquitin sample. L. E. T. acknowledges support from Bucknell University for a summer research assistantship.

REFERENCES

[1] J. Cavanagh, W. J. Fairbrother, A. G. Palmer, N. J. Skelton (1996) *Protein NMR Spectroscopy: Principles and Practice*, Academic Press, Inc., San Diego, CA.
[2] G. Wagner (1997) An account of NMR in structural biology, *Nat. Struct. Biol.* **4**, 841–844.
[3] R. Ishima, D. A. Torchia (2000) Protein dynamics from NMR, *Nat. Struct. Biol.* **7**, 740–743.
[4] G. T. Montelione, D. Zheng, Y. J. Huang, K. C. Gunsalus, T. Szyperski (2000) Protein NMR spectroscopy in structural genomics, *Nat. Struct. Biol.* **7**, (Suppl.) 982–985.
[5] G. Bodenhausen, D. J. Ruben (1980) Natural abundance nitrogen-15 NMR by enhanced heteronuclear spectroscopy, *Chem. Phys. Lett.* **69**, 185–189.
[6] Z. Serber, C. Richter, D. Moskau, J.-M. Bohlen, T. Gerfin, D. Marek, M. Haberli, L. Baselgia, F. Laukien, A. S. Stern, J. C. Hoch, V. Dotsch (2000) New carbon-detected protein NMR experiments using cryoprobes, *J. Am. Chem. Soc.* **122**, 3554–3555.
[7] I. Bertini, I. C. Felli, R. Kummerle, D. Moskau, R. Pierattelli (2004) ^{13}C-^{13}C NOESY: An attractive alternative for studying large macromolecules, *J. Am. Chem. Soc.* **126**, 464–465.
[8] W. Bermel, I. Bertini, I. C. Felli, R. Kummerle, R. Pierattelli (2003) ^{13}C direct detection experiments on the paramagnetic oxidized monomeric copper, zinc superoxide dismutase, *J. Am. Chem. Soc.* **125**, 16423–16429.
[9] Z. Serber, C. Richter, V. Dotsch (2001) Carbon-detected NMR experiments to investigate structure and dynamics of biological macromolecules, *Chembiochem.* **2**, 247–251.
[10] G. A. Morris, R. Freeman (1979) Enhancement of nuclear magnetic resonance signals by polarization transfer, *J. Am. Chem. Soc.* **101**, 760–762.
[11] J. L. Markley, A. Bax, Y. Arata, C. W. Hilbers, R. Kaptein, B. D. Sykes, P. E. Wright, K. Wuthrich (1998) Recommendations for the presentation of NMR structures of proteins and nucleic acids, *Pure Appl. Chem.* **70**, 117–142.
[12] F. Delaglio, S. Grzesiek, G. W. Vuister, G. Zhu, J. Pfeifer, A. Bax (1995) NMRPipe: A multidimensional spectral processing system based on UNIX pipes, *J. Biomol. NMR* **6**, 277–293.
[13] R. Harris (2004) Ubiquitin NMR resource page, available on-line at www.biochem.ucl.ac.uk/bsm/nmr/ubq/; accessed July 17, 2004.
[14] L. E. Kay, M. Ikura, R. Tschudin, A. Bax (1990) Three-dimensional triple resonance NMR spectroscopy of isotopically enriched proteins, *J. Magn. Reson.* **89**, 496–514.
[15] M. Ikura, L. E. Kay, A. Bax (1990) A novel approach for sequential assignment of ^{1}H, ^{13}C, and ^{15}N spectra of proteins: Heteronuclear triple-resonance three-dimensional NMR spectroscopy. Application to calmodulin, *Biochemistry* **29**, 4659–4667.
[16] R. T. Clubb, V. Thanabal, G. Wagner (1992) A constant-time 3-dimensional triple-resonance pulse scheme to correlate intraresidue H-1(N), N-15, and C-13(′) chemical shifts in N-15-C-13-labeled proteins, *J. Magn. Reson.* **97**, 213–217.
[17] G. T. Montelione, G. Wagner (1990) Triple-resonance experiments for establishing conformation-independent sequential NMR connections in isotope-enriched polypeptides by ^{1}H-^{13}C-^{15}N triple-resonance experiments, *J. Magn. Reson.* **87**, 183–188.
[18] R. T. Clubb, V. Thanabal, G. Wagner (1992) A new 3D HN(CA)HA experiment for obtaining fingerprint HN-H-α peaks in ^{15}N- and ^{13}C-labeled proteins, *J. Biomol. NMR* **2**, 203–210.
[19] D. Rovnyak, J. C. Hoch, A. S. Stern, G. Wagner (2004) Resolution and

sensitivity of high field nuclear magnetic resonance spectroscopy, *J. Biomol. NMR* **30**, 1–10.

APPENDIX 1

The relationship among the spectral width, Nyquist theorem, States-type quadrature detection, and spectral resolution is summarized here to save recourse into texts. The time interval, $\Delta t(^1H)$, by which the evolution period is incremented, determines the spectral width in the indirect frequency dimension. The Nyquist theorem indicates that frequencies below $f_{NYQ} = 1/(2\Delta t(^1H))$ will be correctly detected. Because States or States-TPPI methods achieve sign discrimination and pure-phase line shapes in an indirect frequency dimension, the spectral width is $\pm 1/(2\Delta t(^1H))$ centered about the carrier frequency. Decreasing the spectral width means increasing the interval $\Delta t(^1H)$. For the proton carrier centered at 4.8 ppm, as required for ^1H-detected NMR experiments in aqueous samples, the spectral width would need to be 12.4 ppm to detect amide proton signals up to 11 ppm. But if the proton carrier is centered at 8.2 ppm, a spectral width of 5.6 ppm will suffice and will permit the use of longer Δt_1.

For a 2D NMR experiment, FIDs are acquired for N sequentially incremented values of the indirect evolution period: 0, $\Delta t(^1H)$, $2\Delta t(^1H)$,. . . . $(N - 1)\Delta t(^1H)$. The maximum indirect evolution time is $t_a = (N - 1)\Delta t(^1H)$, and the spectral resolution is SR (Hz) $= 1/t_a$, which measures the ability to unambiguously distinguish closely spaced signals. So spectral resolution may be improved by increasing N and/or Δt_1. If the time domain signal length is not modified, such as by zero filling or linear prediction, then $1/t_a$ is also the digital resolution of the frequency spectrum. If the digital resolution is enhanced during processing, the spectral resolution still fundamentally limits the resolving power of the data [19]. In summary, centering the carrier in the amide proton region means that a long time interval for incrementing the indirect proton evolution period is possible (*i.e.* narrow spectral width) and a high spectral resolution can be achieved.