Improved Resolution and Sensitivity of Triple-Resonance NMR Methods for the Structural Analysis of Proteins by Use of a Backbone-Labeling Strategy

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Abstract: A novel isotopic labeling strategy is described for the structural analysis of proteins by NMR. Overexpression of a protein in a mammalian cell-line cultured in a medium containing amino acids labeled only in the backbone (N, C^{α}, H^{α}, C′) atoms leads to the formation of exclusively backbone-labeled protein. We demonstrate that the absence of the one bond scalar coupling between the $^{13}C^{\alpha}$ and $^{13}C^{\beta}$ atoms that is observed in uniformly ^{13}C enriched proteins offers a substantial sensitivity and resolution advantage in triple resonance NMR experiments that are commonly used to obtain backbone resonance assignments. This approach is illustrated in application to the β subunit of human chorionic gonadotropin isotopically enriched with ^{13}C (97%), ^{15}N (97%), and ^{2}H (50%) exclusively in the backbone atoms of Phe, Val, and Leu residues.

Introduction

The introduction of multidimensional triple-resonance NMR methods in combination with isotopically enriched proteins has increased dramatically the size limit for protein structure determination by NMR.[1,2] A fundamental limitation on the size of protein whose resonances can be assigned by this approach is the relatively rapid transverse relaxation time of $^{13}C^{\alpha}$ resonances. This gives rise to severe attenuation of the detected signal in experiments of the HNCA type, for example,[1] by virtue of the relatively long defocusing and refocusing delays that are required for evolution under the small $^{1}J_{C}\alpha_{N}$ scalar coupling. While the $^{13}C^{\alpha}$ line widths can be reduced dramatically by deuteration, giving rise to improved sensitivity and resolution,[3] the available resolution remains limited by the one bond scalar coupling between $^{13}C^{\alpha}$ and $^{13}C^{\beta}$. In general it is undesirable to resolve this coupling in experiments of the HNCA type, which gives rise to short acquisition times and poor resolution in the $^{13}C^{\alpha}$ dimension. An elegant approach to overcome this limitation has been described by Wagner and co-workers, involving selective decoupling of $^{13}C^{\beta}$ by application of a band-selective composite pulse train.[4,5] However, this approach introduces two undesirable complications. First, it is in general impossible selectively to decouple serine $^{13}C^{\beta}$'s since they resonate within the $^{13}C^{\alpha}$ region of the spectrum, and second the composite pulse train introduces an undesirable Bloch−Siegert shift on $^{13}C^{\alpha}$ resonances. An alternative approach that would not suffer from these disadvantages involves the overexpression of protein that is isotopically enriched exclusively in the backbone N, C^{α}, H^{α}, and C′ atoms. This would permit optimal resolution and sensitivity to be obtained in triple-resonance NMR experiments, since the undesirable $^{13}C^{\alpha}-^{13}C^{\beta}$ scalar coupling would be absent. Moreover, this approach would be compatible with the use of expression systems using mammalian cell-lines that have already been developed,[6] as these cells require nearly all the naturally occurring amino acids as essential nutrients. In contrast to bacterial expression systems, no isotopic scrambling would thus be expected in these cell types and the expressed protein will be exclusively enriched in the backbone atoms. Here, we evaluate the efficacy of this approach by overexpression of a 26 kDa glycoprotein, the β-subunit of human chorionic gonadotropin, in mammalian (CHO) cells grown in a medium containing backbone-labeled phenylalanine, valine, and leucine. We compare the sensitivity of an HNCA experiment recorded on this material with that recorded on a similar sample of β-subunit containing uniformly labeled phenylalanine, valine, and leucine.

Materials and Methods

Chemical Synthesis of Backbone ^{13}C (97%)-,^{15}N (97%)-, and ^{2}H (50%)-Labeled Phe, Val, and Leu. Uniformly (∼97%) ^{13}C-, ^{15}N-, and (50%) ^{2}H-leucine, phenylalanine, and valine were isolated from a mixture of (∼97%) ^{13}C, ^{15}N, (50%) ^{2}H-amino acids using a 15 × 200 cm column of Dowex 50 × 8H resin (Na^{+} form) eluted with 0.1 M sodium citrate using a step gradient from pH 3.3 to 4.5.[7] Ninhydrin positive fractions were desalted by sequential absorption onto Dowex 50 × 8H resin (Na^{+} form), followed by elution with 2% aqueous

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(1) Ikura, M.; Kay, L. E.; Bax, A. *Biochemistry* **1990**, *29*, 4659−4667.
(2) Bax, A. *Curr. Opinion Struct. Biol.* **1994**, *4*, 738−744.
(3) Grzesiek, S.; Anglister, J.; Ren, H.; Bax, A. *J. Am. Chem. Soc.* **1993**, *115*, 4369−4370.
(4) Matsuo, H.; Kupce, E.; Li, H. J.; Wagner, G. *J. Magn. Reson. Ser. B* **1996**, *113*, 91−96.
(5) Matsuo, H.; Kupce, E.; Wagner, G. *J. Magn. Reson. Ser. B* **1996**, *113*, 190−194.
(6) Lustbader, J. W.; Birken, S.; Pollak, S.; Canfield, R. E.; Chait, B. T.; Ramnarain, S.; Brown, J. M. *J. Biomol. NMR* **1996**, *7*, 295−304.
(7) Moore, S.; Stein, W. H. *J. Biol. Chem.* **1951**, *192*, 663−681.

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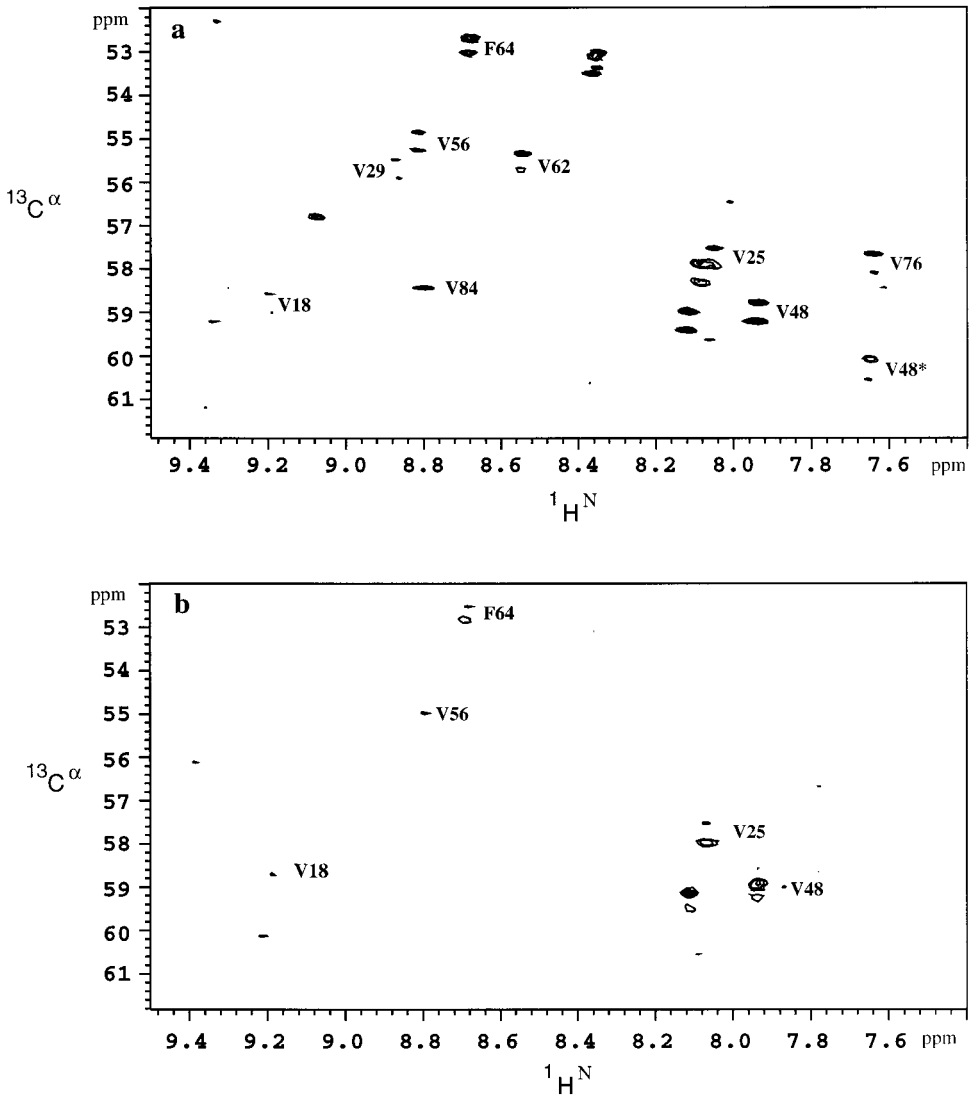

Figure 1. (a) Region taken from the two-dimensional ($F_1 = {}^{13}C^{\alpha}$, $F_2 = {}^{1}H^{N}$) HNCA spectrum (128 transients/t_1 increment) of the β subunit of hCG (100 μM) selectively enriched with ^{13}C, ^{15}N (97%), and ^{2}H (50%) only in the backbone atoms of Phe, Val, and Leu residues. (b) Similar spectrum (32 transients/t_1 increment) of the β subunit of hCG (200 μM) uniformly enriched with ^{13}C, ^{15}N (97%), and ^{2}H (50%) in all Phe, Val, and Leu residues. Those resonances for which resonance assignments are available are labeled. Note that there is a minor resonance for V48 (marked with an asterisk) due to cis−trans isomerism at Pro 50.

ammonia onto Dowex 1 × 8 resin (OH^- form), and elution with dilute acetic acid. After evaporation of volatile components, the amino acids were crystallized from aqueous ethanol.

The synthesis of backbone (∼ 97%) ^{13}C, ^{15}N, (50%) ^{2}H-labeled leucine, phenylalanine, and valine was essentially by the method of Oppolzer [8] and will be described in detail elsewhere. Briefly, $^{13}C_2$,^{15}N-glycine (Isotec Inc.) was converted in two steps to (2*R*)-*N*-(diphenylmethylene)[1,2-$^{13}C_2$,^{15}N] glycine ethyl ester. Treatment of this material with (2*R*)-bornane-10,2-sultam in the presence of aluminum hydride, followed by workup with D_2O, gave the chiral derivative (2*R*)-*N*-(diphenylmethylene)[1,2-$^{13}C_2$,^{15}N, 2-^{2}H]glycylbornane-10,2-sultam. This material was divided into three parts, each one treated with *n*-butyllithium, then each treated separately with 2-iodopropane, benzyl bromide, and 1-iodo-2-methylpropane to give fully protected backbone (∼ 97%) ^{13}C-, ^{15}N-, (50%) ^{2}H-labeled valine, phenylalanine, and leucine, respectively. These materials were deprotected by sequential treatment with lithium hydroxide and hydrochloric acid, and desalted and crystallized as described above.

Preparation of Growth Medium. CHO-SSFM-1 serum free CHO cell medium was obtained from the manufacturer (Life Technologies) with all amino acid and carbohydrate components omitted. Unlabeled components were added as follows: alanine, aspartic acid, asparagine, glutamic acid, glycine, tryptophan, serine (10−50 mg/L); histidine, methionine (50−100 mg/L); threonine, tyrosine, isoleucine, cysteine (100−200 mg/L); arginine, proline, lysine (200−400 mg/L); and glutamine (600 mg/L), glucose 4 g/L. Leucine, phenylalanine, and valine, labeled as required, were added at concentrations of 190, 100, and 140 mg/L, respectively.

Overexpression and Purification of the β-Subunit of hCG. Cultures were maintained in tissue culture flasks in Ham's F-12 (JRH Biosciences, Kenexa, KS), 2.5% bovine calf serum, 2.5% fetal calf serum, and 400 mg/mL G418 (Life Technologies) in a humidified 37 °C incubator with 5% CO_2. Isotopically labeled hCG β-subunit was obtained by expression of hCG by transfected CHO cells [6,9] grown with media containing appropriately labeled phenylalanine, valine, and leucine. The expressed labeled hCG was purified into subunits by reverse-phase HPLC. The hCG was dissociated by incubating in 6 M guanidine−HCl, 0.1 M sodium acetate at pH 4.0 for 1−3 h at 37 °C. The subunits were separated on a Vydac C4, 300A column, 1 cm × 22 cm (Vydac, Hesperia, CA). The column was run at 2 mL/min in a mobile phase of 0.1% TFA (Buffer A) and 0.1% TFA in 100% acetonitrile (Buffer B). The gradient was held at 0% B for 10 min and then increased to 40% B in 60 min.

(8) Oppolzer, W. J. *J. Chem. Soc., Perkin Trans.* **1996**, *1*, 2503.

(9) Lustbader, J. W.; Wu, H.; Birken, S.; Pollak, S.; Gawinowicz Kolks, M. A.; Pound, A.; Canfield, R. E. *Endocrinology* **1995**, *136*, 640−650.

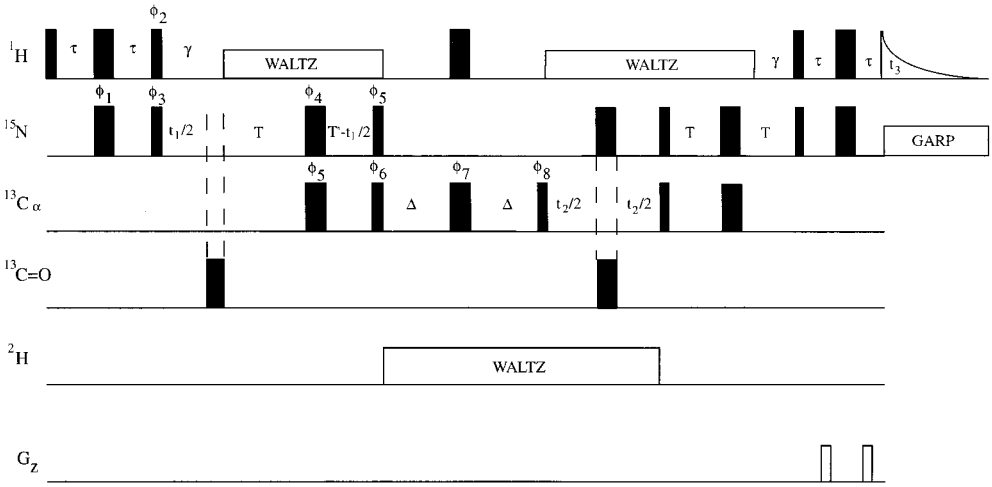

Figure 2. Pulse scheme for the acquisition of two- or three-dimensional HNCA spectra with suppression of correlations to $^{13}C^{\alpha}[-^{1}H^{\alpha}]$. Narrow bars represent 90° pulses and wide bars represent 180° pulses. Pulses are applied along the $+x$ axis except where indicated, i.e., $\phi_1 = x,-x$; $\phi_2 = y,-y$; $\phi_3 = x$; $\phi_4 = 4(x),4(y),4(-x),4(-y)$; $\phi_5 = 16(x),16(-x)$; $\phi_6 = x,x,-x,-x$; $\phi_7 = x$; $\phi_8 = x$; receiver $= 2(x,-x,-x,x,-x,x,x,-x)$, $2(-x,x,x,-x,x,-x,-x,x)$. The power and duration of $^{13}C^{\alpha}$ pulses is adjusted to give a null in their excitation profile at the $^{13}C'$ frequency and vice versa. The final 180° ^{1}H pulse is of composite type, which together with the two gradient pulses along the z axis gives rise to WATERGATE H_2O suppression.[19] Quadrature in the $^{13}C^{\alpha}$ dimension is achieved by altering the phases ϕ_6, ϕ_7, and ϕ_8 in a States-TPPI manner.[20] Similarly quadrature in the ^{15}N dimension is achieved by altering ϕ_3. The delay Δ is set to $1/(2^1J_{CH})$, and the other delays in the sequence are set according to the original scheme devised by Grzesiek and Bax,[10] namely $\tau = 2.25$ ms, $T = 11$ ms, and $\gamma = 5.55$ ms.

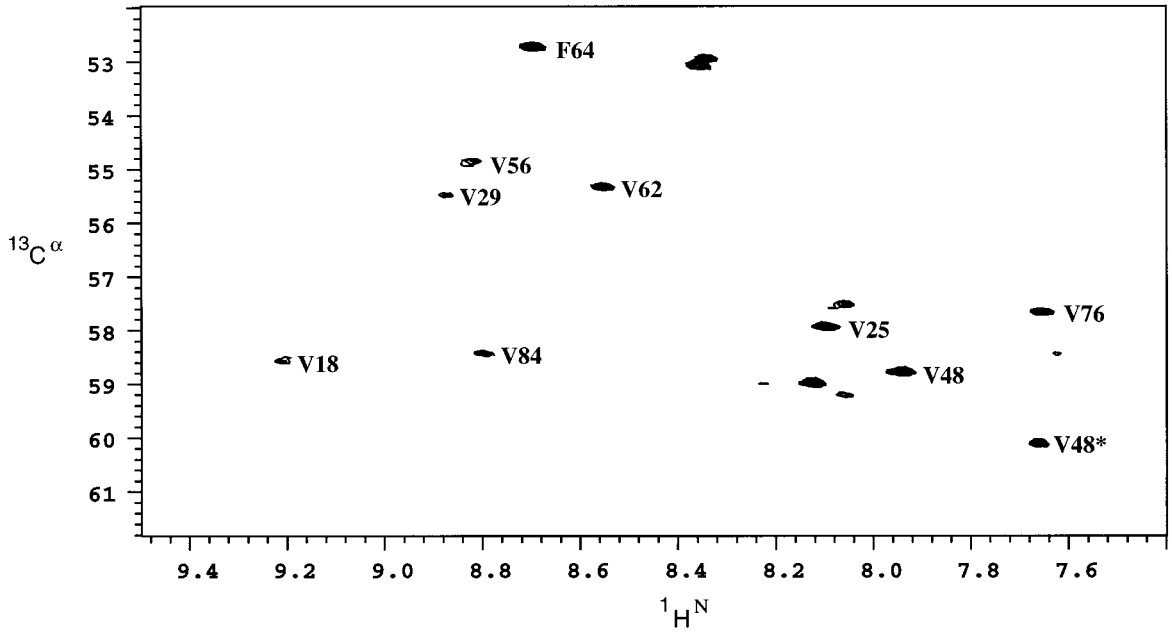

Figure 3. Region taken from the two-dimensional ($F_1 = {}^{13}C^{\alpha}$, $F_2 = {}^{1}H^{N}$) HNCA spectrum acquired under similar conditions to Figure 1a but utilizing the pulse sequence of Figure 2 to suppress correlations to $^{13}C^{\alpha}[-^{1}H^{\alpha}]$.

NMR Experiments. Two-dimensional HNCA spectra were acquired using the three-dimensional constant-time HNCA pulse scheme with composite pulse proton decoupling described by Grzesiek and Bax,[10] by keeping the ^{15}N-evolution time constant. Deuterium decoupling was accomplished in the ^{13}C dimension by application of a 1 kHz WALTZ16 decoupling field whose carrier was located at 4 ppm. Spectra were acquired with spectral widths of 1800 and 3600 Hz with 1024 complex points and 512 complex points in the ^{13}C and ^{1}H dimensions, respectively. Prior to two-dimensional Fourier transformation, data were apodized with cosine-bell functions in each dimension followed by zero-filling once in the ^{13}C dimension. In total 32 transients were acquired per t_1 increment in the HNCA spectrum of the uniformly labeled β-subunit, whereas 128 transients were acquired per t_1 increment in the HNCA spectrum of the backbone-labeled β-subunit. In this manner the effective signal-to-noise ratio of each spectrum is comparable given the different concentrations of the two samples (100 μM for the backbone-enriched sample and 200 μM for the uniformly enriched sample).

(10) Grzesiek, S.; Bax, A. *J. Magn. Reson.* **1992**, *96*, 432−440.

Results and Discussion

To assess the effectiveness of the backbone labeling strategy in improving the sensitivity and resolution of a typical triple-resonance experiment, a two-dimensional HNCA experiment was recorded ($F_2 = {}^{1}H^{N}$, $F_1 = {}^{13}C^{\alpha}$) on a sample of hCG β-subunit containing ^{13}C (97%), ^{15}N (97%), and ^{2}H(50%) isotopic enrichment exclusively in the backbone atoms of Phe, Val, and Leu residues (Figure 1a). At this low concentration (100 μM), only a fraction of the expected 26 $^{1}H^{N}-^{13}C^{\alpha}$ correlations are observed, and these are predominantly in regions of defined secondary structure. With the exception of glycine,

there is no evidence of isotope scrambling to other amino acid residue types. The majority of the unobservable correlations correspond with residues in loop regions or in the long flexible COOH terminus of the β-subunit, with consequent broad $^1H^N$ line widths due to fast exchange with solvent water. Each correlation involving a given $^1H^N$ proton gives rise to two $^{13}C^\alpha$ correlations corresponding to $^{13}C^\alpha[-^1H]$ and $^{13}C^\alpha[-^2H]$, the latter appearing at higher field due to the deuterium isotope effect.[3] By comparison, a similar two-dimensional HNCA spectrum of β-subunit containing full ^{13}C (97%), ^{15}N (97%), and ^2H(50%) isotopic enrichment of Phe, Val, and Leu residues is shown in Figure 1b. It is seen that only the most intense resonances in Figure 1a are observable, and the sensitivity is demonstrably lower: as anticipated, signals are approximately 2-fold stronger in Figure 1a. In addition to this useful gain in sensitivity, the resolution in the $^{13}C^\alpha$ (F_2) dimension is higher in the spectrum of Figure 1a due to the absence of the ~40 Hz one-bond scalar coupling between $^{13}C^\alpha$ and $^{13}C^\beta$. It is commonplace to limit the acquisition time in the $^{13}C^\alpha$ dimension of the HNCA spectrum such that this undesirable splitting is not resolved. In contrast, the acquisition time in the $^{13}C^\alpha$ dimension of HNCA spectra of backbone-labeled material can be extended to a point that is limited only by the $^{13}C^\alpha$ transverse relaxation times. Since the transverse relaxation times of deuterated carbons are substantially longer than their protonated counterparts, with larger proteins the $^1H^N-^{13}C^\alpha$ correlations serve no practical purpose and can be removed from the spectrum by use of a simple modification to the conventional HNCA spectrum as shown in Figure 2. It should be noted that this sequence will not function correctly with uniformly labeled material due to evolution under the $^{13}C^\alpha-^{13}C^\beta$ coupling. The resulting two-dimensional HNCA spectrum showing only $^1H^N-^{13}C^\alpha[-^2H^\alpha]$ correlations is shown in Figure 3. This spectrum shows excellent resolution in the $^{13}C^\alpha$ dimension. Indeed, a pair of currently unassigned resonances at δ^1H^N~8.35 and $\delta^{13}C^\alpha$~53, which appear to be a single resonance in the conventional low-resolution HNCA spectrum (data not shown), are well resolved in the spectrum of Figure 3 despite a difference in $^{13}C^\alpha$ resonance position of less than 12 Hz.

Conclusions

Specifically isotopically enriched proteins can be expressed with no isotopic scrambling in mammalian cells cultured in media containing the appropriate labeled amino acids. Use of protein isotopically ^{13}C, ^{15}N, and ^2H enriched only in the backbone atoms results in the abolition of the undesirable one bond $^{13}C^\alpha$ and $^{13}C^\beta$ scalar coupling and improves both sensitivity and resolution of triple-resonance NMR experiments for backbone assignment. While alternative methods have been described for removing this coupling, such as constant-time experiments [11,12] and selective $^{13}C^\beta$ decoupling,[4,5] the former suffer from a substantial loss in sensitivity and the latter do not achieve uniform decoupling for all residue types. In contrast, the present approach offers optimal resolution and sensitivity for all residue types, which is important in the context of automated spectral analysis and assignment. Many other experiments would also benefit from the absence of the $^{13}C^\alpha-^{13}C^\beta$ scalar coupling during the relevant evolution periods. Recent examples include cross-correlation type experiments for the measurement of protein dihedral angles ψ.[13−15] Moreover, it will be possible to exclude the [^{13}C] constant time evolution period in TROSY-HNCA experiments,[16] thus offering a useful gain in sensitivity. The very narrow $^{13}C^\alpha$ line widths that can be obtained with the backbone-labeling approach will also facilitate the measurement of residual dipolar couplings between $^{13}C^\alpha-^{15}N$ and $^{13}C^\alpha-^{13}C'$ using IPAP methods.[17] In combination with selective deuteration of appropriate side chains, and with measurement of one-bond residual dipolar couplings in weakly oriented samples, this approach offers a route to the automated determination of the global fold of larger proteins.[18] Efforts are underway to synthesize all of the natural amino acids in backbone-labeled form.

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(11) Santoro, J.; King, G. C. *J. Magn. Reson.* **1992**, *97*, 202−207.
(12) Yamazaki, T.; Lee, W.; Arrowsmith, C. H.; Muhandiram, D. R.; Kay, L. E. *J. Am. Chem. Soc.* **1994**, *116*, 11655−11666.
(13) Reif, B.; Hennig, M.; Griesinger, C. *Science* **1997**, *276*, 1230−1233.
(14) Yang, D. W.; Konrat, R.; Kay, L. E. *J. Am. Chem. Soc.* **1997**, *119*, 11938−11940.
(15) Yang, D. W.; Gardner, K. H.; Kay, L. E. *J. Biomol. NMR* **1998**, *11*, 213−220.
(16) Salzmann, M.; Pervushin, K.; Wider, G.; Senn, H.; Wüthrich, K. *J. Biomol. NMR* **1999**, *14*, 85−88.
(17) Ottiger, M.; Delaglio, F.; Bax, A. *J. Magn. Reson.* **1998**, *131*, 373−378.
(18) Clore, G. M.; Starich, M. R.; Bewley, C. A.; Cai, M.; Kuszewski, J. *J. Am. Chem. Soc.* **1999**, *121*, 6513−6514.
(19) Sklenár, V.; Piotto, M.; Leppik, R.; Saudek, V. *J. Magn. Reson.* **1993**, *A 102*, 241−245.
(20) Marion, D.; Ikura, M.; Tschudin, R.; Bax, A. *J. Magn. Reson.* **1989**, *85*, 393−399.