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High-level $^{2}H/^{13}C/^{15}N$ labeling of proteins for NMR studies

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Summary

The protein human carbonic anhydrase II (HCA II) has been isotopically labeled with ^{2}H, ^{13}C and ^{15}N for high-resolution NMR assignment studies and pulse sequence development. To increase the sensitivity of several key $^{1}H/^{13}C/^{15}N$ triple-resonance correlation experiments, ^{2}H has been incorporated into HCA II in order to decrease the rates of ^{13}C and $^{1}H_N$ T_2 relaxation. NMR quantities of protein with essentially complete aliphatic ^{2}H incorporation have been obtained by growth of *E. coli* in defined media containing D_2O, [1,2-$^{13}C_2$, 99%] sodium acetate, and [^{15}N, 99%] ammonium chloride. Complete aliphatic deuterium enrichment is optimal for ^{13}C and ^{15}N backbone NMR assignment studies, since the ^{13}C and $^{1}H_N$ T_2 relaxation times and, therefore, sensitivity are maximized. In addition, complete aliphatic deuteration increases both resolution and sensitivity by eliminating the differential ^{2}H isotopic shift observed for partially deuterated CH_nD_m moieties.

Introduction

Recent advances in NMR spectrometers, multidimensional and multinuclear NMR pulse sequences, and molecular biology have made it possible to utilize NMR spectroscopy to obtain three-dimensional solution structures of proteins and other biological macromolecules at atomic resolution. In addition, high-resolution NMR techniques can often be used to study dynamical features and interactions of these molecules under physiologically relevant pH and buffer conditions. Proteins as large as 30 kDa can now be successfully assigned and studied (Fogh et al., 1994; Remerowski et al., 1994) using ^{13}C and ^{15}N isotope labeling techniques (Ikura et al., 1990; Venters et al., 1991) and elegant NMR pulse sequences that have been developed over the last several years (Bax and Grzesiek, 1993; Muhandiram and Kay, 1994).

NMR pulse sequences developed for ^{13}C, ^{15}N and ^{1}H assignment in proteins depend exclusively on large one-bond heteronuclear couplings and spread through-bond correlations into two, three or four dimensions. These experiments work extremely well on peptides and lower molecular weight proteins, but rapidly lose sensitivity as the rotational correlation times of the protein increase above 10 ns due mainly to fast ^{13}C and $^{1}H_N$ T_2 relaxation. The most significant contribution to ^{13}C T_2 relaxation is the strong dipolar coupling between a ^{13}C spin and its directly bonded proton(s) (Grzesiek et al., 1993; Yamazaki et al., 1994a,b). A significant contribution (~40%) (Farmer II, B.T. and Venters, R.A., unpublished results; Markus et al., 1995) to $^{1}H_N$ T_2 relaxation arises from dipolar coupling to surrounding aliphatic protons. These relaxation effects taken together cause backbone assignment experiments such as HNCA and HN(CA)HA to decrease in sensitivity and experiments such as HNCACB to yield no observable C^{β} resonances for large proteins. Replacement of ^{1}H with ^{2}H on non-glycine C^{α} nuclei can be calculated to increase the ^{13}C T_2 relaxation time approximately 12.5-fold and the $^{1}H_N$ T_2 relaxation time approximately 1.7-fold (Farmer II, B.T. and Venters,

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R.A., unpublished results) for rotational correlation times consistent with the size and packing of HCA II (Liljas et al., 1972). The magnitude of this ^{13}C T_2 increase has recently been measured experimentally for a 37 kDa Trp repressor complex (Yamazaki et al., 1994b) and agrees with our calculated results for a system with a rotational correlation time of 13.4 ns (Kay, L.E., private communication). These increases in ^{13}C and $^{1}H_N$ T_2 relaxation times allow many otherwise marginal heteronuclear 3D and 4D pulse sequences, which are important in current assignment strategies, to be executed successfully on proteins with rotational correlation times larger than that of HCA II. However, resonance assignment of deuterated proteins necessitates the development of new sequences and new assignment strategies due to the absence of all aliphatic protons and the introduction of the ^{2}H isotopic shift on the ^{13}C chemical shifts (Farmer and Venters, 1995).

We have previously described a technique for uniform ^{13}C and ^{15}N labeling of proteins (Venters et al., 1991) using media containing [1,2-$^{13}C_2$, 99%] sodium acetate as the sole carbon source and [^{15}N, 99%] ammonium chloride as the sole nitrogen source. Here we extend this procedure to include ^{2}H labeling of proteins to any desired incorporation level, including essentially complete aliphatic deuteration. For NMR studies of proteins with slow rotational correlation times (> 10 ns), the backbone carbon and nitrogen nuclei can be most easily assigned using a sample with essentially complete ^{13}C, ^{15}N and ^{2}H labeling, followed by exchange of all amide ^{2}H nuclei with ^{1}H. NMR experiments which correlate the intra- and interresidue carbon and nitrogen nuclei in the protein with the amide protons can then be used for resonance assignment. We have optimized the growth conditions and have prepared NMR quantities of ^{13}C, ^{15}N and ^{2}H labeled human carbonic anhydrase II (HCA II), a 29 kDa zinc metalloenzyme. Using this sample we have been able, for the first time, to successfully obtain complete HN-CACB data on HCA II. Such data are essential for the sequential assignment of a protein the size of HCA II, providing both amino acid type classifications and interresidue connectivities.

Materials and Methods

High-level expression of HCA II in *E. coli* (Nair et al., 1991) has been achieved by the construction of vectors (pACA) which contain the protein gene subcloned behind a phage T7 RNA polymerase promoter vector (Rosenberg et al., 1987). Protein production is then achieved by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG), inducing a chromosomal copy of T7 RNA polymerase (behind a *lac* UV promoter) in the cell line BL21(DE3), which in turn starts transcription of the protein gene (Studier and Moffatt, 1986). With this promoter system, more than 15% of the total cellular protein is HCA II after induction. HCA II was purified using sulfonamide affinity chromatography with slight modifications to the published procedures (Khalifah et al., 1977). The cells grown in D_2O media did not completely lyse using 25 mg/100 ml lysozyme and 0.1% tritonX-100. After the initial lysis step, the resulting cell solution was centrifuged at 18 k and 4 °C for 30 min. The cell pellet was resuspended and further lysed using a French press, yielding additional HCA II activity (40% of total) in the supernatant. The supernatants from these lysis steps were combined for further fractionation. HCA II activity was measured by assaying enzyme-catalyzed hydrolysis of *p*-nitrophenyl acetate at 348 nm (Verpoorte et al., 1967).

Uniform ^{15}N and ^{13}C labeled HCA II was obtained by growing BL21(DE3)pACA *E. coli* in defined media containing 3 g/l sodium [1,2-$^{13}C_2$, 99%] acetate as the sole carbon source and 1 g/l [^{15}N, 99%] ammonium chloride as the sole nitrogen source (Venters et al., 1991). In addition, the defined media contained M9 salts (Sambrook et al., 1989), 2 mM $MgSO_4$, 1 μM $FeCl_3$, 10 ml/l vitamin mixture (containing 10 mg/100 ml each of biotin, choline chloride, folic acid, niacinamide, D-pantothenate and pyridoxal, and 1 mg/100 ml riboflavin), 5 mg/l thiamine, 100 μM $CaCl_2$, 50 μM $ZnSO_4$, and 50 μg/ml ampicillin. The activity and NMR spectra of the protein labeled by this technique are identical to those obtained from protein produced from defined media containing labeled glucose.

Triply ^{2}H, ^{13}C and ^{15}N labeled protein was obtained using the media described above, except that H_2O was replaced by D_2O. Stock reagents were prepared in D_2O and filter sterilized. To minimize $^{2}H/^{1}H$ exchange, the media were used immediately after preparation and were never autoclaved. In order to obtain maximum sensitivity in heteronuclear 3D experiments, it is essential that all amide ^{2}H be exchanged with ^{1}H. To achieve this, deuterated HCA II was unfolded in the presence of H_2O by incubation in 3 M guanidine–HCl at pH 7.5 and room temperature for 1 h, followed by a rapid 20-fold step dilution with 0.1 M tris sulfate at pH 7.5 and subsequent refolding for 2 h (Carlsson et al., 1973).

To successfully implement complete replacement of protonated solvent with deuterated solvent and, subsequently, to obtain high levels of deuterium incorporation, one must select *E. coli* which can tolerate high levels of D_2O. Therefore, a single colony of BL21(DE3) cells containing the plasmid described above was inoculated into a 10 ml media culture of LB containing 25 mg/ml ampicillin and incubated at 37 °C until A_{600} = 0.5–0.7. At this point, the cells were diluted 20-fold into LB/amp media containing 30% D_2O and incubated at 37 °C until A_{600} = 0.5–0.7. This process was repeated with LB media containing 60% D_2O, and finally with LB media containing 90% D_2O. Cells were then flash frozen in 50% sterile glycerol and stored at −20 °C for future use. Subsequent

to these initial studies, we found that it was possible to dilute cells directly from H_2O LB media into LB media containing high levels of D_2O (> 90%) without the need for the intermediate steps and without sacrificing protein yield. D_2O tolerant cells were then diluted 50-fold into defined media containing 2 g/l glucose and 90% D_2O, incubated at 37 °C until A_{600} = 0.5–0.7, and then a frozen glycerol suspension was prepared. To prepare HCA II, an aliquot of this latter glycerol suspension containing BL21-(DE3) pACA cells was streaked on minimal/glucose/ampicillin agar plates containing 90% D_2O, and a single colony was used to inoculate a flask containing minimal/glucose/ampicillin/ 98.8% D_2O media and incubated at 37 °C. When A_{600} = 0.5–0.7, an aliquot was diluted 50-fold into minimal/acetate/ampicillin/D_2O media. When A_{600} = 0.3–0.5, HCA II production was induced by addition of IPTG to a final concentration of 500 μM and incubated for an additional 10–16 h at 34 °C. Cells were pelleted by centrifugation (5 k, 20 min, 4 °C).

[$^2H_3$, 98%] sodium acetate, [1,2-$^{13}C_2$, 99%] sodium acetate, [^{15}N, 99%] ammonium chloride, and D_2O were obtained from Cambridge Isotope Laboratories (Andover, MA). All NMR experiments on fully protonated HCA II and initial ^1H-^{15}N HSQC experiments on deuterated HCA II were carried out on a three-channel Varian Unity 600 spectrometer, using a $^1H/^{13}C/^{15}N$ triple-resonance probe equipped with an actively shielded B_z gradient coil. HNCACB experiments on the fully deuterated HCA II were carried out on a four-channel Varian UnityPlus 600 spectrometer, equipped with the same style probe, capable of broadband ^2H decoupling and lock sample/hold. Mass spectrometry data were collected on a Fisons-VG Quattro BQ triple quadrupole mass spectrometer equipped with a pneumatically assisted electrostatic ion source, operating at atmospheric pressure. Protein samples were extensively dialyzed against ion-free buffers before lyophilization. Lyophilized protein samples were reconstituted in a mixture of D_2O or H_2O and acetonitrile (1:1) containing perdeuterated acetic acid (1% v/v) and introduced by loop injection into the LC stream of similar composition at a flow rate of 6 μl/min. Mass spectra were acquired in the multichannel analyzer mode between m/e 700–1400 with a scan time of 10 s. The mass scale was calibrated with horse heart myoglobin (M_r 16951.48) with a resolution corresponding to a peak width at half height of 1.4 Da for m/e 893. The mass spectra were transformed to a molecular mass scale using MaxEnt software. This algorithm uses the method of maximum entropy to enhance resolution and signal-to-noise ratio from multiply charged electrospray mass spectra.

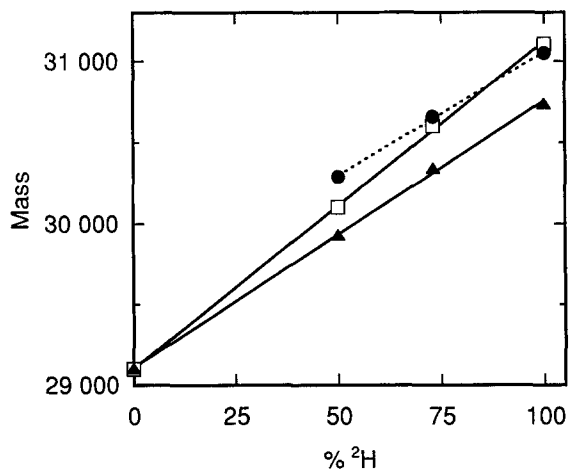

Fig. 1. Mass spectrometry data of HCA II prepared from *E. coli*, grown in media with varied percentages of D_2O and reconstituted in a mixture of D_2O (●) or H_2O (▲) and (1:1) acetonitrile containing 1% v/v acetic acid. A curve representing the calculated mass of HCA II at various uniform deuterium incorporation levels (□) is also presented. The samples reconstituted in H_2O were allowed to exchange for 1 h prior to mass determination. The mass differences between the samples in H_2O and D_2O represent the exchangeable protons under the conditions used.

Results and Discussion

In order to prepare $^2H/^{13}C/^{15}N$ labeled HCA II, we optimized growth conditions in defined acetate media for maximum protein yields using BL21(DE3) pACA *E. coli* cells selected for growth in D_2O. Conditions optimized included A_{600} at time of induction, induction time, growth temperature, antibiotic levels, and pH. Doubling times for these cells were found to increase from 7.2 h in H_2O/acetate to 8.6 h in 98.8% D_2O/acetate media. Optimal protein yields were obtained using the conditions found to be optimal for acetate growths in H_2O (Venters et al., 1991) with two exceptions: maximum protein yield was achieved when the cells were induced at A_{600} = 0.3–0.5 and when induction times were increased from 8 to 16 h. In addition to the increased doubling time in D_2O media, an appreciable lag was observed upon dilution of cells from minimal/glucose/D_2O media into minimal/acetate/D_2O media. Therefore, it was not unusual for the cell growths to require 48–72 h from inoculation to cell harvest. Nonetheless, more than 50 mg of HCA II per liter of media were obtained in this manner. This is only a slightly decreased (33%) yield compared with fully protonated media (75 mg).

To determine the upper limit of deuterium incorporation, we isolated milligram quantities of ^2H labeled HCA II prepared from cells grown in defined media containing 98.8% D_2O and [$^2H_3$, 98%] sodium acetate as the sole carbon source, using the optimized procedures described above. To quantitate the level of deuterium incorporation in this sample we analyzed the molecular mass of purified HCA II by mass spectrometry. The molecular mass of the fully protonated sample prepared in minimal/acetate/H_2O media was measured (see Materials and Methods) to be 29 102 ± 2.4, in excellent agreement with the theoretical

mass of 29 098.9 based on the amino acid sequence and the natural abundance of all isotopes. At low pH the protein contains 2018 protons. If all of these protons are exchanged for deuterons, the mass of the protein should increase by 2030.5 mass units. The mass of protein produced in 98.8% D_2O and [$^2H_3$, 98%] sodium acetate media was measured using fully deuterated solvents and was found to be 31 133 ± 13, an increase of 2034 ± 15 mass units, indicating essentially complete deuteration of the protein.

Furthermore, we purified HCA II from BL21(DE3) pACA cells grown in minimal [$^1H_3$] acetate media containing either 50%, 75%, or 98.8% D_2O to examine the feasibility of obtaining HCA II with selected levels of deuterium incorporation. The masses of these proteins were determined both in deuterated solvents and after exposure to protonated solvents for 1 h (Fig. 1). The molecular mass of HCA II isolated from cells grown in minimal acetate and 98.8% D_2O was 31 050 ± 20 in deuterated solvent and 30 740 ± 21 after exposure to protonated solvent for 1 h. This indicates that, simply by growing cells in D_2O, very high levels of deuteration (96% of nonexchangeable protons) may be achieved, and that approximately 310 protons (16%) are rapidly exchangeable in HCA II under these conditions. In addition, the mass of HCA II (after exchanging with protonated solvent for 1 h) isolated from cells grown in minimal acetate media containing 50% and 75% D_2O was 29 930 ± 21 and 30 340 ± 25, respectively, indicating that the levels of deuterium incorporation of nonexchangeable protons were 49% and 73%. This demonstrates that the level of deuterium enrichment is linearly dependent on the percentage of D_2O present in the growth media, allowing for the preparation of HCA II with any desired level of deuteration. In *E. coli*, acetate is converted into acetyl-CoA,

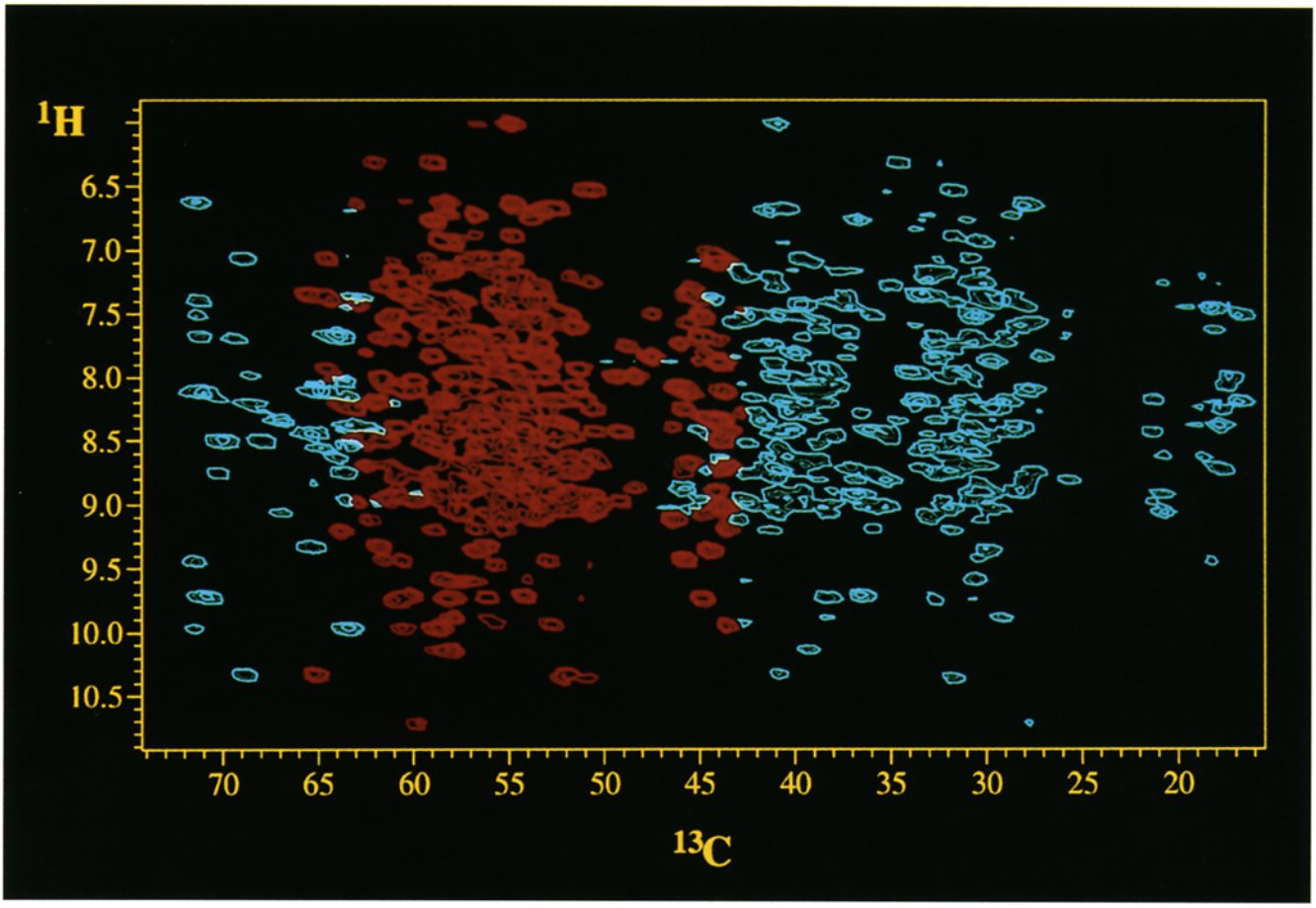

Fig. 2. $^{13}C/^1H$ skyline projection along the ^{15}N axis of HNCACB data obtained on a 1.6 mM $^2H/^{13}C/^{15}N$ labeled HCA II sample in 100 mM phosphate buffer at pH 6.8. The sample was deuterated to 96% on aliphatic carbons. Red cross peaks correspond to positive contours from C^α nuclei; cyan cross peaks correspond to negative contours from C^β nuclei. The data were collected at 30 °C on the four-channel Varian UnityPlus spectrometer described in the Materials and Methods section. The total acquisition time was 36 h. Other experimental conditions included: $t_{90}(^1H)$ = 6.5 μs, $t_{90}(^{13}C)$ = 13.8 μs, $t_{90}(^{15}N)$ = 46 μs, $sw(^1H)$ = 11 001.1 Hz, t_3 = 70 ms, $sw(^{13}C)$ = 9000.9 Hz, t_1(max) = 5 ms, $sw(^{15}N)$ = 2040, t_2(max) = 17.65 ms, γ_D = 760 Hz for broadband ^2H WALTZ-16 decoupling (Shaka et al., 1983), γ_H = 4.03 kHz for ^1H broadband WALTZ-16 decoupling, and γ_N = 1.16 kHz for broadband ^{15}N WALTZ-16 decoupling. The ^1H carrier was on-resonance with the H_2O signal and the ^2H carrier was on-resonance with the D_2O signal. The 3D data were processed on an SGI 4D/440VGX computer using an extensively modified version of FELIX 1.0 (Hare Research, Inc.).

which is then used directly by the cells in the tricarboxylic acid cycle to obtain energy for the biosynthesis of amino acids (Gottschalk, 1986). For this reason, the incorporation of the ^2H label should be essentially random for a protein expressed in *E. coli* grown on $^1H_3$-acetate and 98.8% D_2O. Glucose, however, is utilized to synthesize amino acids, especially aromatic ones, through different metabolic pathways which are less likely to produce random ^2H labeling (LeMaster, 1994).

High ^2H incorporation is ideal for high-resolution NMR assignment studies. At 96% ^2H enrichment, nearly full advantage in ^{13}C and ^1H_N T_2 relaxation is realized; therefore, experiments correlating backbone atoms with the amide protons in larger proteins are at near optimum sensitivity. One drawback, however, is that a second $^{13}C,^{15}N$ labeled sample is required at a lower ^2H enrichment level (Pachter et al., 1992) to complete the ^1H assignments, using such experiments as HCCH-TOCSY (Kay et al., 1993) and 4D $^{13}C/^{13}C$-separated NOESY (Vuister et al., 1993). Nonetheless, this approach has advantages over a compromise solution using a single sample containing 60–70% deuteration for all assignment experiments, since the fully and partially deuterated samples can be optimized for their intended use. It should be noted here that the hydrogen-bonded amide protons in a protein produced in completely deuterated solvent will remain deuterated after purification. For maximum sensitivity of all residues in the protein, it is essential that these ^2H amides be exchanged with ^1H. For HCA II this involved unfolding the protein, using 3 M guanidine-HCl followed by a rapid 20-fold step dilution and subsequent refolding (Carlsson et al., 1973). We can recover 94% of fully active protein with this procedure.

Using the optimal conditions outlined above, we have twice grown and induced 1 liter of BL21(DE3) pACA *E. coli* cells in [1,2-$^{13}C_2$, 99%] sodium acetate, [^{15}N, 99%] ammonium chloride, and 98.8% D_2O media, obtaining 50 mg of HCA II per liter of cells. We used a portion of this protein to prepare a 1.6 mM, ^{13}C, ^{15}N and ^2H labeled HCA II sample in 100 mM phosphate buffer at pH 6.8 for NMR studies. Figure 2 presents the $^{13}C/^1H$ projection of an HNCACB data set collected on the ^2H labeled sample described above. It should be noted here that we were unable to obtain these data on a protonated sample, due to fast C^α and H_N relaxation. Utilizing the ^2H sample we obtained this HNCACB data set with an excellent signal-to-noise ratio in 36 h of acquisition time. These and other results will be discussed in greater detail elsewhere.

These data indicate that 96% ^2H enrichment can be achieved in proteins expressed in *E. coli* by growing selected cells in D_2O media; complete aliphatic deuteration can be achieved if ^2H labeled acetate substrate is also included in the media. After amide $^2H/^1H$ exchange, complete deuteration provides optimum signal-to-noise enhancement in 3D 'out-and back' heteronuclear assignment experiments which use the amide proton for detection. In addition, any level of ^2H enrichment can be selected simply by adjusting the level of D_2O in the growth media. Deuterium substitution greatly enhances the sensitivity of heteronuclear 3D NMR experiments and permits studies of proteins with rotational correlation times longer than 10 ns.

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