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Measurement of one-bond $^{1}H^{\alpha}$-$^{13}C^{\alpha}$ couplings in backbone-labelled proteins

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Abstract

NMR dipole-dipole couplings between protein backbone nuclei ($^{1}H^{\alpha}$, $^{13}C^{\alpha}$, ^{15}N, $^{1}H^{N}$,$^{13}C'$) offer enormous scope for the rapid determination of protein global folds. Here, we show that measurement of one-bond splittings in the protein backbone is facilitated by use of protein that is selectively isotopically enriched only in the backbone atoms. In particular, $^{1}H^{\alpha}$-$^{13}C^{\alpha}$ couplings can be measured simply and with high sensitivity by use of conventional heteronuclear single quantum correlation (HSQC) techniques.

Introduction

Residual dipolar couplings offer unique conformational restraints in structure determination by NMR (Saupe, 1968; Bothner-By, 1995; Tolman et al., 1995; Tjandra and Bax, 1997a; Hansen et al., 1998b; Kiddle and Homans, 1998). The realization that most macromolecules can be partially aligned in dilute liquid-crystalline media comprised of phospholipid bicellar (Sanders II and Schwonek, 1992; Tjandra and Bax, 1997a; Losonczi and Prestegard, 1998; Ottiger and Bax, 1998, 1999; Wang et al., 1998a) or lamellar (Prosser et al., 1998) phases, or by phage particles (Clore et al., 1998; Hansen et al., 1998a), has dramatically increased the number of systems in which these dipolar couplings can be measured. Recently, Bax and co-workers (Delaglio et al., 2000) demonstrated that it is possible to determine protein global folds using solely backbone dipolar couplings together with molecular fragment replacement. Thus, if backbone residual dipolar couplings can be determined in an accurate and rapid manner, there exists the possibility of determining protein global folds in a fraction of the time required using NOE-based methods. In this regard, Wang et al. (1998b) have introduced an approach for simultaneous measurement of backbone $^{1}H^{N}$-^{15}N, $^{1}H^{N}$-$^{13}C'$ and ^{15}N-$^{13}C'$ residual dipolar couplings using the ‘IPAP’ ^{1}H-^{15}N HSQC approach (Wang et al., 1998b). By analogy, the IPAP ^{1}H-^{13}C HSQC approach would appear to be a convenient method for measurement of residual dipolar couplings involving $^{1}H^{\alpha}$ and $^{13}C^{\alpha}$. However, this approach is complicated by the fact that $^{13}C^{\alpha}$ nuclei in all amino acids except glycine are scalar coupled to $^{13}C^{\beta}$, which causes an undesirable increase in complexity of HSQC spectra. Well-known methods have been devised to overcome this problem, such as constant-time acquisition in the $^{13}C^{\alpha}$ dimension (Santoro and King, 1992; Tjandra and Bax, 1997b) or band-selective decoupling (Kupce and Wagner, 1996; Matsuo et al., 1996a,b). However, the former approach gives rise to a significant sensitivity loss that is unacceptable for larger proteins, whereas the latter approach is not effective for all residue types. Here, we show that $^{1}H^{\alpha}$-$^{13}C^{\alpha}$ couplings

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can be measured in a straightforward manner using the IPAP-HSQC approach with proteins that are isotopically enriched only in backbone ($^{1}H^{\alpha}$, $^{13}C^{\alpha}$, ^{15}N, $^{1}H^{N}$,$^{13}C'$) nuclei. We illustrate this approach with a sample of ubiquitin fully enriched with ^{13}C, ^{15}N and fractionally enriched with ^{2}H in the backbone nuclei of Glu, Asn, Val, Lys, Ala, Ile, Asp, Gln.

Materials and methods

Chemical synthesis of backbone ^{13}C (97%),^{15}N (97%),^{2}H (50%)-labelled Glu, Asn, Val, Lys, Ala, Ile, Asp, Gln

$^{13}C_2$,^{15}N-glycine (Isotec Inc.) was converted in two steps to ethyl N-(diphenylmethylidene)[1,2-$^{13}C_2$,^{15}N]glycinate. Treatment of this material with (2R)-bornane-10,2-sultam (Oppolzer et al., 1989) in the presence of aluminium hydride, followed by workup with D_2O, gave the chiral derivative (2R)-{N-(diphenylmethylidene)[1′,2′-$^{13}C_2$, ^{15}N, 2′-^{2}H]glycyl}bornane-10,2-sultam. This material was divided into seven parts, each one treated with n-butyl lithium, then each treated separately with ethyl 3-bromopropionate, iodoacetamide, 2-iodopropane, 1-bromo-4-phthalimido-butane, methyl iodide, (R)-(−)-2-butyl-p-nitrobenzenesulphonate, and ethyl bromoacetate to give fully protected backbone 50% ^{2}H, ^{13}C,^{15}N-labelled glutamic acid, asparagine, valine, lysine, alanine, isoleucine and aspartic acid, respectively. These materials were deprotected by sequential treatment with lithium hydroxide and hydrochloric acid, and desalted by sequential absorption onto Dowex 50 × 8H resin (H^{+} form), elution therefrom with 2% aqueous ammonia onto Dowex 1 × 8 resin (OH^{-} form), and elution therefrom with dilute acetic acid. After evaporation of volatile components, the amino acids were crystallised from aqueous ethanol. Glutamine was then prepared from a portion of the backbone 50% ^{2}H, ^{13}C,^{15}N-labeled glutamic acid via treatment with glutamine synthetase (Sigma) (Hansen et al., 1992).

Overexpression and purification of ubiquitin

cDNA corresponding to the 3′-terminus of the *E. coli* ribosome binding site-human ubiquitin-glycine-serine-(histidine)$_6$ was isolated from a human cDNA library by two stage PCR using primer sets ATCTTCGTGAAGACC and TCTAAGACGAGCAC, and CACAGAATTCATTAAAGAGGAGAAATTAACCATGCAGATCTTCGTGAAGACCCTGAC and GACTGGATCCACCTCTAAGACGGAG for the first and second rounds, respectively. The PCR product was ligated into pGEM (Promega) and the resulting plasmid was amplified by transformation and culture of DH5α cells.

The required cDNA was digested with EcoR1 and BamH1, and ligated into similarly digested pQE60 (Qiagen). The resulting plasmid was amplified by transformation and culture of DH5α cells and then used to transform M15 cells for protein expression. A glycerol stock of the M15 cells transformed with the vector containing the gene for human ubiquitin-glycine-serine-(histidine)$_6$ was used to inoculate 1 litre of medium comprising 500 mg alanine, 400 mg arginine, 400 mg aspartic acid, 50 mg cysteine, 400 mg glutamine, 650 mg glutamic acid, 550 mg glycine, 100 mg histidine, 230 mg isoleucine, 230 mg leucine, 420 mg lysine.HCl, 250 mg methionine, 130 mg phenylalanine, 100 mg proline, 2.1 g serine, 230 mg threonine, 170 mg tyrosine, 230 mg valine, 500 mg adenine, 650 mg guanosine, 200 mg thymine, 500 mg uracil, 200 mg cytosine, 1.5 g sodium acetate (anhydrous), 1.5 g succinic acid, 750 mg NH_4Cl, 850 mg NaOH, 10.5 g K_2HPO_4 (anhydrous), 2 mg $CaCl_2{\cdot}2H_2O$, 2 mg $ZnSO_4{\cdot}7H_2O$, 2 mg $MnSO_4.H_2O$, 50 mg tryptophan, 50 mg thiamine, 50 mg niacin, 1 mg biotin, 20 g glucose, 4 ml 1 M $MgSO_4$, 1 ml 0.01 M $FeCl_3$, 15 mg ampicillin, 50 mg kanamycin and cultured at 37 °C. When cell density had reached an OD of 1.2, the cells were harvested by centrifugation, rinsed with PBS, recentrifuged and resuspended in a medium of the above proportions but in which backbone 50% ^{2}H, ^{13}C,^{15}N-labeled glutamic acid, glutamine, asparagine, valine, lysine, alanine, isoleucine and aspartic acid were substituted for the unlabelled materials. After 30 min, protein expression was induced by addition of IPTG to a final concentration of 0.1 mmol. After 6 h the cells were harvested by centrifugation, resuspended in PBS, and broken by 4 passes through a French press. The desired E, Q, N, V, K, A, I, D backbone 50% ^{2}H,^{13}C,^{15}N-labelled human ubiquitin-glycine-serine-(histidine)$_6$ fusion protein was then isolated by absorption onto, and elution from, nickel 2^{+} resin (Qiagen), followed by reverse phase chromatography (aqueous isopropanol/TFA gradient) and lyophilisation. The yield of pure protein was 60 mg per litre of culture medium.

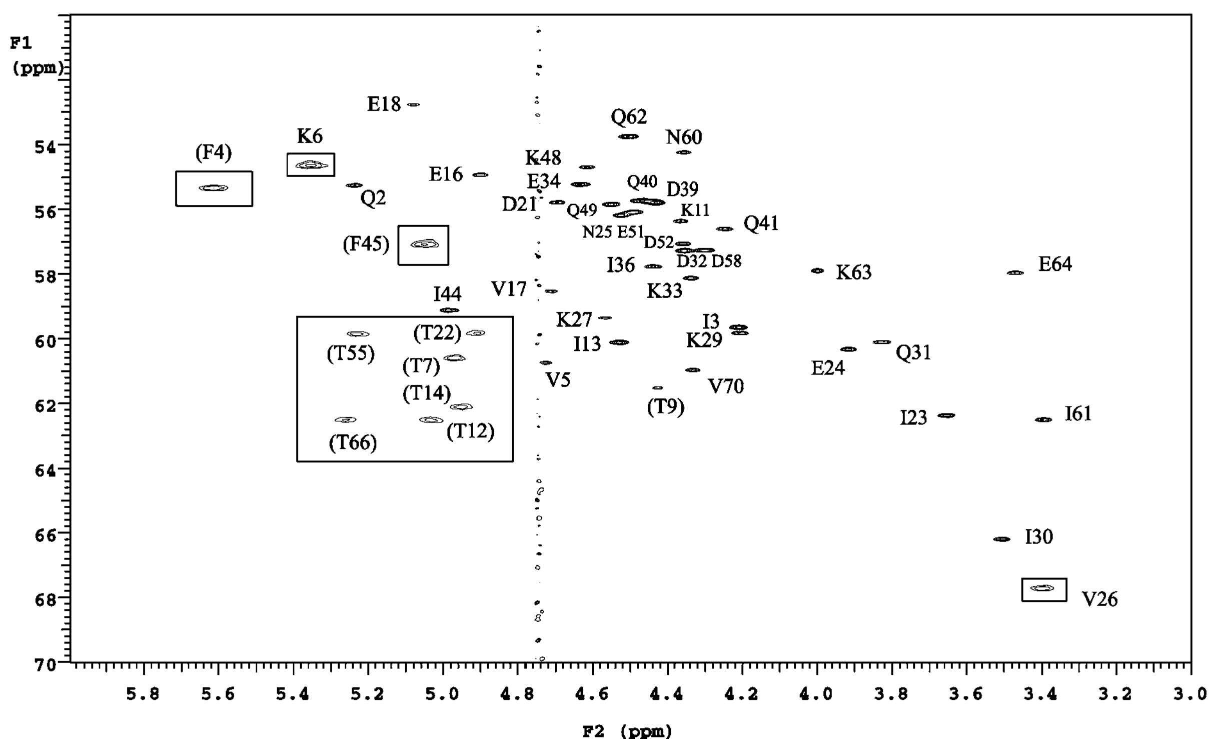

Figure 1. Region of the ^{1}H-^{13}C HSQC spectrum of ubiquitin 97% ^{13}C,^{15}N, 50% ^{2}H enriched in backbone nuclei of Glu, Asn, Val, Lys, Ala, Ile, Asp, Gln. Selected assignments based on literature data are shown. In addition, assignments of resonances that appear due to isotopic scrambling are shown in parentheses. Resonances shown boxed are displayed at a vertical scale magnified by a factor of four.

NMR experiments

A backbone-labelled sample of ubiquitin (prepared as above) in 200 μl 87% H_2O/13% D_2O buffer (pH = 5) was used for all experiments, at a concentration of 0.8 mM. The spectra were recorded on a 500 MHz Varian Unity Inova spectrometer. Each of the correlation experiments was acquired with spectral widths of 2900 Hz and 2000 Hz in the ^{13}C and ^{1}H dimension, respectively. The ^{13}C, ^{1}H HSQC spectrum was recorded with acquisition times t_1 = 98 ms and t_2 = 128 ms using a refocussed HSQC sequence combined with a water suppression approach described by Kay (1993). The ^{13}C,^{1}H IPAP-HSQC spectra were acquired with acquisition times t_1 = 64 ms and t_2 = 128 ms in either the ^{13}C′-coupled or ^{13}C′-decoupled mode using the protocol of Ottiger et al. (1998) to select individual components of the one-bond $^{13}C^{\alpha}$-$^{1}H^{\alpha}$ multiplets. Sixteen transients or 32 transients per t_1 increment were accumulated for the ^{13}C′-decoupled mode or the ^{13}C′-coupled mode, respectively.

While measurement of $^{13}C^{\alpha}$-$^{1}H^{\alpha}$ couplings in uniformly ^{13}C-enriched proteins can be made accessible by use of constant-time HSQC experiments (Santoro and King, 1992; Tjandra and Bax, 1997b), the efficient relaxation of $^{13}C^{\alpha}$ during the constant-time period gives rise to significant loss in sensitivity that is unacceptable for larger proteins. Indeed, by comparison with the current approach, constant-time acquisition gives rise to a substantial sensitivity loss even for ubiquitin, as illustrated in Figure 3.

Results and discussion

The conventional ^{1}H, ^{13}C′-decoupled ^{13}C-^{1}H HSQC spectrum of backbone-V,E,K,N,D,Q,A,I-labelled ubiquitin is shown in Figure 1. We chose to record this and all other spectra in H_2O solution for compatibility with methods for the measurement of $^{1}H^{N}$-^{15}N, $^{1}H^{N}$-^{13}C′ and ^{15}N-^{13}C′ couplings (Wang et al., 1998b) using a single sample. As anticipated, ^{1}H-^{13}C correlations are only observed in the $^{13}C^{\alpha}$ region of the spectrum, indicating that there is no isotopic scrambling of ^{13}C into side-chain positions. All expected ^{1}H-^{13}C correlations are observed, and additional weaker correlations are also detected that could be assigned to Phe and Thr residues as a result of isotopic scrambling (bracketed

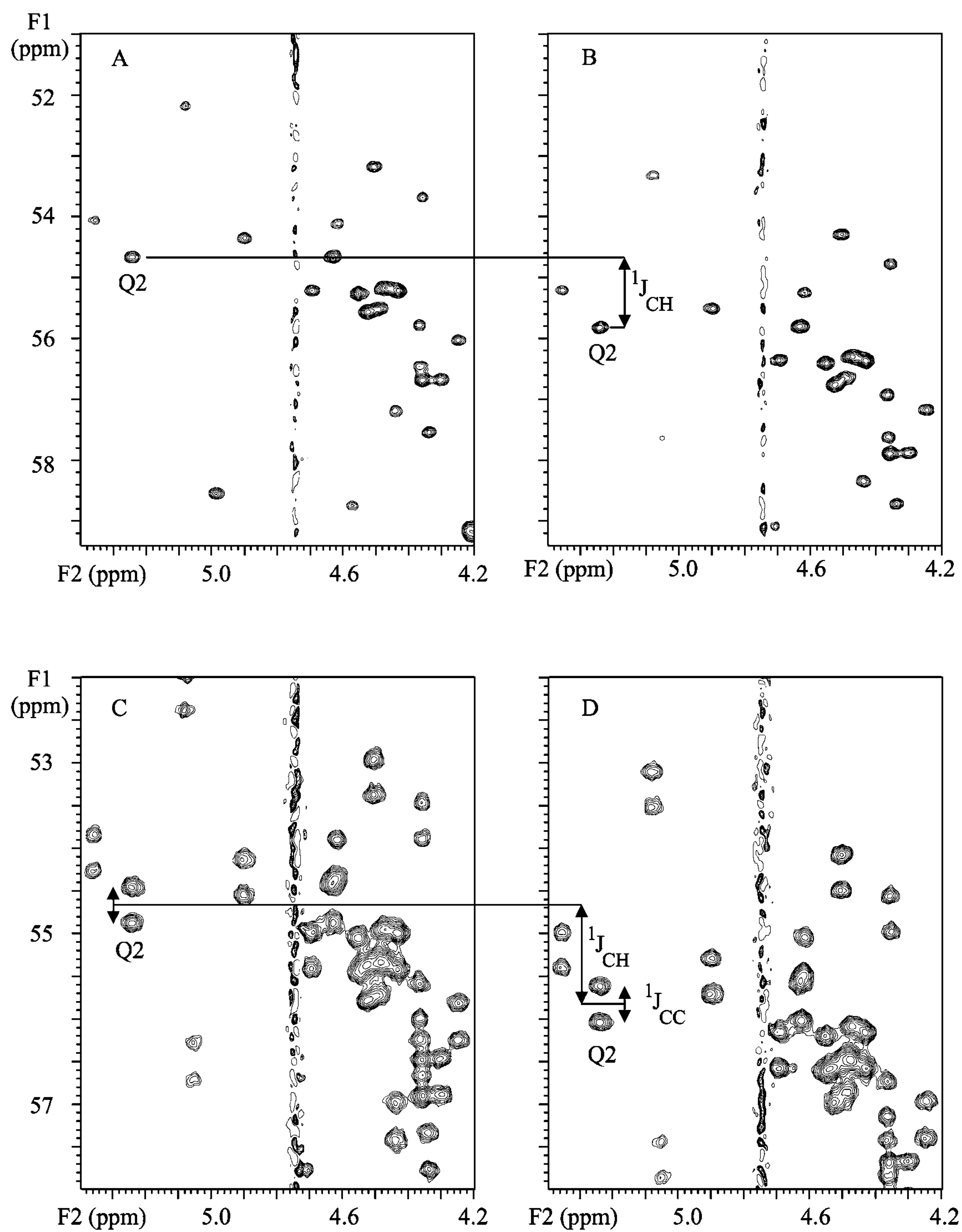

Figure 2. Sections of the IPAP-[^{1}H-^{13}C]-HSQC spectrum of ubiquitin ^{13}C,^{15}N,^{2}H enriched in backbone nuclei of Glu, Asn, Val, Lys, Ala, Ile, Asp, Gln, recorded in the presence (A,B) and absence (C,D) of ^{13}C′ decoupling. (A and C) Upfield ^{1}H$^\alpha$-^{13}C$^\alpha$ multiplet components; (B and D) downfield multiplet components.

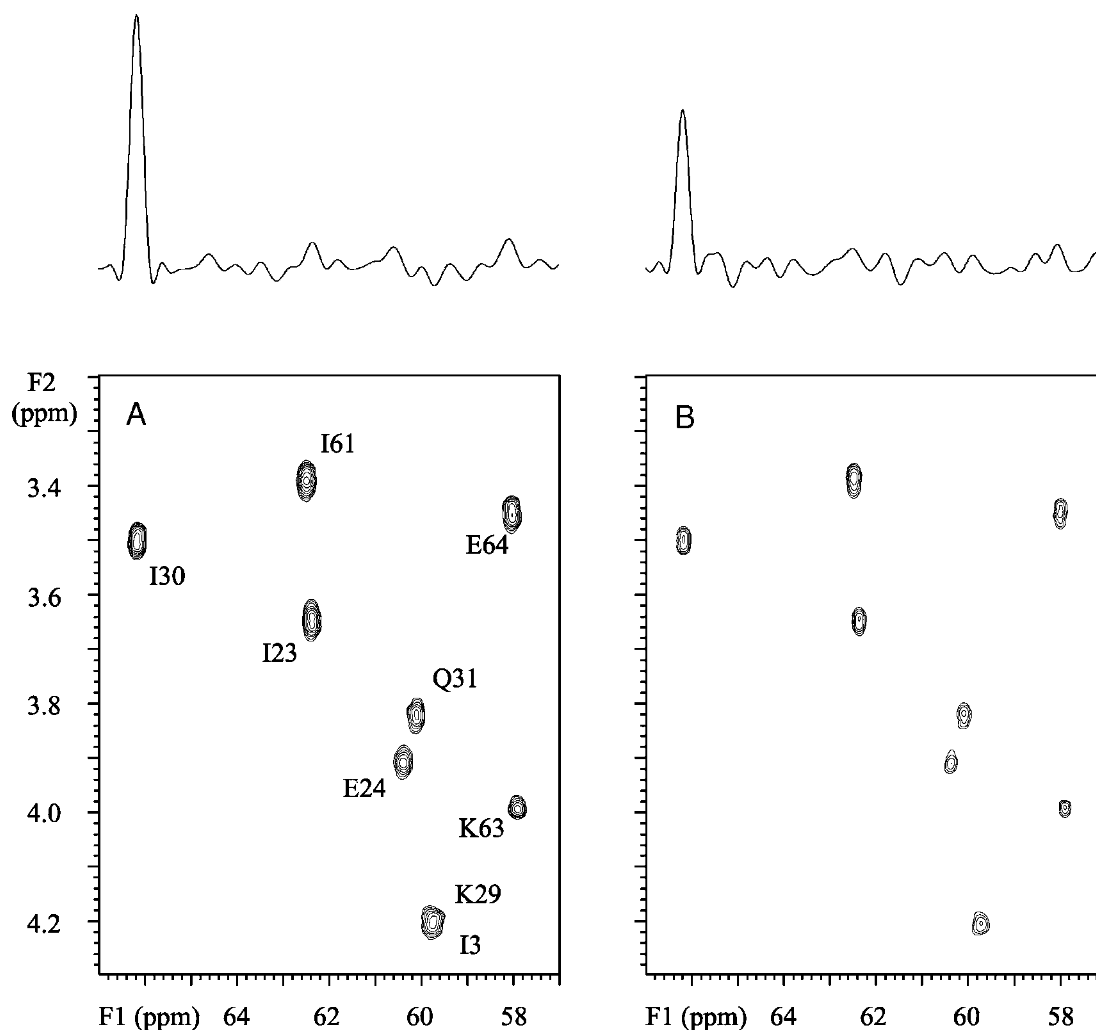

Figure 3. Sections from ^{1}H,^{13}C HSQC spectra of ubiquitin: (A) ^{13}C-^{1}H HSQC spectrum of backbone Glu, Asn, Val, Lys, Ala, Ile, Asp, Gln enriched ubiquitin, ^{13}C acquisition time = 22 ms. (B) Constant-time HSQC spectrum of the same sample, ^{13}C acquisition time = 22 ms, constant-time evolution period 2T = 26.6 ms. The cross section shown corresponds to Ile 30, $\delta_H = 3.5$. In both cases, solvent suppression was achieved with mild presaturation of the water resonance. Both spectra were ^{13}C′ and ^{15}N decoupled in F1 and ^{13}C$^{\alpha}$ decoupled in F2.

assignments in Figure 1). It is difficult to speculate on the source of this scrambling without further investigation. However, we note that there is a direct biosynthetic route from aspartate to threonine, and that the backbone atoms of phenylalanine could derive from amino acid catabolism through the tricarboxylic acid cycle via phosphoenolpyruvate. In general all anticipated correlations are not of uniform intensity, due to the variable degree of backbone-^{2}H enrichment of amino acids used in growth media. The absence of ^{1}H-^{13}C correlations within amino acid residue side chains enables the acquisition of a ^{13}C$^{\alpha}$-^{1}H$^{\alpha}$ IPAP-HSQC spectrum with optimal spectral width in the ^{13}C$^{\alpha}$ dimension, without resorting to 'folding' of resonances. Measurement of ^{13}C$^{\alpha}$-^{1}H$^{\alpha}$ couplings can thus be undertaken at high digital resolution as shown in Figure 2A,B. In order to determine the reproducibility of the method, the experiment was repeated twice. Good correlation between measured values was obtained (not shown) with a pairwise root-mean-squared-deviation (rmsd) of 0.8 Hz. By recording the ^{13}C$^{\alpha}$-^{1}H$^{\alpha}$ IPAP-HSQC spectrum in the ^{13}C′-coupled mode, it is possible to measure both ^{13}C$^{\alpha}$-^{1}H$^{\alpha}$ and ^{13}C$^{\alpha}$-^{13}C′ couplings in a single experiment, at the expense of a doubling in the number of resonances (Figure 2C,D). The doubling of resonances observed

in Figure 2B could of course be overcome by recording a $^{1}H^{\alpha}$ decoupled version of this experiment, with IPAP separation of the individual $^{13}C^{\alpha}$-$^{13}C'$ doublets. However, the longer defocussing times would in this case give rise to an unacceptable loss in sensitivity for all but small proteins.

As discussed in detail by Yang et al. (1998), possible sources of error in the measurement of coupling constants derive from cross-correlated spin relaxation occurring during the relevant evolution period. In the case of the $^{13}C^{\alpha}$-$^{1}H^{\alpha}$ coupling, the major source of relaxation interference concerns non-zero cross-correlated relaxation involving the coupling of $^{13}C^{\alpha}$ with $^{1}H^{\alpha}$ and another, proximal proton that is also coupled to $^{13}C^{\alpha}$. While it is difficult to eliminate these effects, the influence of cross-correlated relaxation in the present scheme would be predicted to introduce significantly smaller errors than schemes involving constant-time evolution (Yang et al., 1998).

In conclusion, we have described an approach for the measurement of one-bond $^{13}C^{\alpha}$-$^{1}H^{\alpha}$ and $^{13}C^{\alpha}$-$^{13}C'$ couplings in a protein that is isotopically enriched exclusively in backbone nuclei. Since these couplings can be measured effectively with HSQC-based techiques, optimal sensitivity should be maintained for intermediate size proteins due to the absence of significant defocussing and refocussing delays that characterise more complex experiments. Clearly, for practical applications it will be necessary to overexpress protein containing all naturally occurring amino acids in backbone labelled form. However, chemical synthesis of these is achievable using an approach analogous to that described under Materials and methods, and is currently in progress.

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