

Article

Simultaneous NMR assignment of backbone and side chain amides in large proteins with IS-TROSY

Aizhuo Liu^{a,*}, Yue Li^a, Lishan Yao^{a,b} & Honggao Yan^a

^aDepartment of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI 48824, USA; ^bDepartment of Chemistry, Michigan State University, East Lansing, MI 48824, USA

Received 5 May 2006; Accepted 25 July 2006

Key words: amide residues, isotope effects, isotopomer selectivity, NMR, simultaneous NMR assignment, TROSY

Abstract

A new strategy for the simultaneous NMR assignment of both backbone and side chain amides in large proteins with isotopomer-selective transverse-relaxation-optimized spectroscopy (IS-TROSY) is reported. The method considers aspects of both the NMR sample preparation and the experimental design. First, the protein is dissolved in a buffer with 50% H₂O/50% D₂O in order to promote the population of semideuterated NHD isotopomers in side chain amides of Asn/Gln residues. Second, a ¹³C'-coupled 2D ¹⁵N-¹H IS-TROSY spectrum provides a stereospecific distinction between the geminal protons in the *E* and *Z* configurations of the carboxamide group. Third, a suite of IS-TROSY-based triple-resonance NMR experiments, e.g. 3D IS-TROSY-HNCA and 3D IS-TROSY-HNCACB, are designed to correlate aliphatic carbon atoms with backbone amides and, for Asn/Gln residues, at the same time with side chain amides. The NMR assignment procedure is similar to that for small proteins using conventional 3D HNCA/3D HNCACB spectra, in which, however, signals from NH₂ groups are often very weak or even missing due to the use of broad-band proton decoupling schemes and NOE data have to be used as a remedy. For large proteins, the use of conventional TROSY experiments makes resonances of side chain amides not observable at all. The application of IS-TROSY experiments to the 35-kDa yeast cytosine deaminase has established a complete resonance assignment for the backbone and stereospecific assignment for side chain amides, which otherwise could not be achieved with existing NMR experiments. Thus, the development of IS-TROSY-based method provides new opportunities for the NMR study of important structural and biological roles of carboxyamides and side chain moieties of arginine and lysine residues in large proteins as well as amino moieties in nucleic acids.

Abbreviations: IS – isotopomer-selective; TROSY – transverse-relaxation-optimized spectroscopy.

Introduction

Amino acid residues with side chain carboxyamides, asparagine (Asn) and glutamine (Gln), play important roles in the structure and function of a

protein via forming either intra- or intermolecular hydrogen bonds, because carboxyamides can be either hydrogen donors or acceptors (Creighton, 1993). X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy are two powerful and mutually complementary tools for determining tertiary structures of proteins at the atomic resolution in the form of static crystals and in solution

*To whom correspondence should be addressed.
E-mail: liua@msu.edu

under near physiological conditions, respectively. In crystallography, uncertainties may arise during the structural refinement, because the electron density maps of the nitrogen and the oxygen atom of a carboxamide group are very similar to each other. On the other hand, in NMR spectroscopy, the geminal protons of a carboxamide NH₂ moiety usually have distinct chemical shifts because of their slow inter-converting rates due to the partial double-bond nature of the side chain C'^δ-N^{δ/ε} (Asn/Gln) amide bond. Hence, the stereospecific assignment of carboxamide resonances is a prerequisite for any further structural and functional NMR studies (Löhr and Rüterjans, 1997; McIntosh et al., 1997; Pervushin et al., 1997a; Cai et al., 2001; Permi, 2001; Higman et al., 2004).

Triple-resonance NMR experiments for the backbone assignment of small to medium sized proteins with uniformly ¹³C/¹⁵N labeling have been established (Bax and Grzesiek, 1993; Cavanagh et al., 1996; Sattler et al., 1999), among which the so-called “out-and-back” type experiments, e.g. HNCA (Kay et al., 1990; Davis et al., 1992; Farmer II et al., 1992; Grzesiek and Bax, 1992; Madsen and Sørensen, 1992; Schleucher et al., 1993; Kay et al., 1994), HN(CO)CA (Bax and Ikura, 1991; Bax and Pochapsky, 1992; Yamazaki et al., 1994) and HNCACB (Wittekind and Mueller, 1993), as well as the “out-and-stay” type experiments, such as CBCA(CO)NH (Grzesiek and Bax, 1992), are most successful ones. In principle, the assignment of side chain amide resonances can be conveniently obtained through the analysis of HNCACB spectrum together with (H)CC(CO)-NH-TOCSY (Grzesiek et al., 1993; Logan et al., 1993) data. Alternatively, side chain amide resonances can be correlated to the side chain carbonyl and aliphatic carbons through selective detection with H₂N-HSQC-based (Farmer II and Venters, 1996) triple-resonance experiments, e.g. 3D H₂NCO (Yamazaki et al., 1993; Vis et al., 1994) and 3D H₂N(CO)CA (Farmer II and Venters, 1996; Schubert et al., 2001). In order to improve the experimental sensitivity, however, proton broad-band decoupling schemes are often employed in indirect dimensions and the experiment is normally optimized for the detection of backbone NH moiety (Bax et al., 1990), resulting in a significant attenuation of side chain NH₂ signals. As a remedy, NOE (Wüthrich, 1986) data are often employed and thus the assignment can only be

achieved in an indirect manner. For larger proteins, to improve the relaxation properties, partially or even fully deuterated samples have to be used and TROSY-based triple-resonance experiments (Pervushin et al., 1997b, 1998; Andersson et al., 1998; Czisch and Boelens, 1998; Meissner et al., 1998; Salzmann et al., 1998, 1999; Weigelt, 1998; Rance et al., 1999; Yang and Kay, 1999; Zhu et al., 1999; Eletsky et al., 2001; Nietlishpach, 2005) are required. Unfortunately, NH₂ resonances could not be detected at all in TROSY-based experiments, because they have been filtered out (Pervushin et al., 1997b).

Pervushin et al. (2000) have demonstrated that by shortening time delays in the normal TROSY experiment NMR signals from ¹⁵NH₂ moieties could be restored. In the previous paper (Liu et al., submitted), however, we have shown that this restoration is rather limited. In contrast, by incorporating broad-band deuterium decoupling schemes into the pulse sequence, TROSY techniques can exclusively detect the semideuterated isotopomers of side chain amides in the mixture solvent of 50% H₂O/50% D₂O with high sensitivities, in particular for large proteins. The TROSY sensitivity enhancement in these semideuterated carboxamide isotopomers, especially for the *E* configuration, is almost equally efficient as in the backbone amide. Because both the electronic distribution and the configuration geometry, thus magnitudes and orientations of dipole-dipole (DD) and chemical shift anisotropy (CSA) interactions, in Asn/Gln side chain amides are very similar to that of backbone peptide bonds (Herzfeld et al., 1987). We took yeast cytosine deaminase (yCD), a 35-kDa protein, as an example and demonstrated that strong side chain amide signals of all 11 Asn/Gln residues were restored, of which only about half of the signals were observable with existing NMR experiments (Liu et al., submitted).

Following up this isotopomer-selective transverse-relaxation-optimized spectroscopy (IS-TROSY) technique, we report here a strategy for the simultaneous assignment of both backbone and side chain amides in large proteins with a suite of IS-TROSY-based triple-resonance experiments. First, a modification in the original 2D ¹⁵N-¹H IS-TROSY experiment leads to an instant stereospecific distinction between the geminal protons of the side chain amide in an Asn/Gln residue. Then, the sensitive 3D IS-TROSY-HNCA experiment

provides correlations between side chain amides and the side chain $^{13}\text{C}^{\beta/\gamma}$ (Asn/Gln) atoms in addition to the conventional backbone $\text{H}-^{15}\text{N}-^{13}\text{C}^{\alpha}$ correlations. The difference in the chemical shifts between $^{13}\text{C}^{\beta}$ of Asn residues and $^{13}\text{C}^{\gamma}$ of Gln residues distinguishes these two types of residues from each other. Last, the 3D IS-TROSY-HNCACB experiment yields complete backbone $\text{H}-^{15}\text{N}-\text{C}^{\alpha}-\text{C}^{\beta}$ connections for all non-proline residues and side chain $\text{H}\{\text{D}\}-^{15}\text{N}-\text{C}^{\beta/\gamma}-\text{C}^{\alpha/\beta}$ connections for Asn/Gln residues. The joint $\text{C}^{\alpha/\beta}$ (for Asn residues) and C^{β} (for Gln residues) resonances from both side chain and backbone amides provide a mutual support for their assignments. The restoration and stereospecific assignment of NH_2 signals with the IS-TROSY strategy constitute the basis for further NMR structural and dynamics studies of these important side chain moieties in large proteins.

Materials and methods

The isotopic labeling and purification of yCD have been previously described (Yao et al., 2005). NMR samples were made by dissolving the lyophilized yCD powder in 100 mM potassium phosphate buffer (pH 7.0, pH meter reading), made with either 93% $\text{H}_2\text{O}/7\%\text{D}_2\text{O}$ or 50% $\text{H}_2\text{O}/50\%\text{D}_2\text{O}$, containing 100 μM NaN_3 and 20 μM DSS (as the internal NMR reference). The final sample volume was about 300 μl in Shigemitsu NMR microcells with the protein concentration (protomer) of ~ 1.8 mM.

NMR samples were initially tested on a Varian INOVA 600 MHz (^1H frequency) NMR spectrometer equipped with a z-gradient triple-resonance probe. Most NMR measurements were performed on a Bruker AVANCE 900 MHz NMR instrument using a TXI triple-resonance probe with three-axis gradients. All NMR data were acquired at 25 $^\circ\text{C}$. The raw data were processed with NMRPipe (Delaglio et al., 1995) and the spectra were analyzed with NMRView (Johnson and Blevins, 1994).

2D $^{15}\text{N}-^1\text{H}$ IS-TROSY

The pulse sequence for the 2D $^{15}\text{N}-^1\text{H}$ IS-TROSY experiment has been described (Liu et al., submitted), which is essentially the conventional

TROSY experiment with the addition of deuterium broad-band decoupling whenever the ^{15}N magnetization is transverse. With a minor modification, it can be conveniently used for the stereospecific assignment of geminal protons of Asn/Gln carboxyamides. Instead of employing an adiabatic ^{13}C 180 $^\circ$ pulse in the middle of the indirect dimension for the decoupling of all carbon atoms, a band-selective inversion pulse was set at 36 ppm (the center of $^{13}\text{C}^{\beta}$ of Asn and $^{13}\text{C}^{\gamma}$ of Gln) to decouple aliphatic carbons only and carbonyl carbons remain coupled to side chain amide ^{15}N atoms. The gradient-selective version (Yang and Kay, 1999; Nietlishpach, 2005) of 2D $^{15}\text{N}-^1\text{H}$ IS-TROSY experiment rendered a higher sensitivity but it also showed stronger non-TROSY interferences (see Figure 1 and Supplementary Material). Due to different signs of the heteronuclear two-bond scalar couplings, $^2J_{\text{HNC}'}$, between each carboxamide proton and the carbonyl carbon, the slope from the lower component (downfield in the ^{15}N dimension) to the upper component of each C' -coupled $^{15}\text{N}-^1\text{H}$ resonance serves as an instant indicator of the proton configuration, either in the *cis* (*Z*) or the *trans* (*E*) configuration relative to the carbonyl oxygen (Cai et al., 2001), just like the carbonyl carbon-coupled $n = 2$ multiplicity-edited $^{15}\text{N}-^1\text{H}$ HSQC experiment (Parella et al., 1997; Cai et al., 2001). These $^2J_{\text{HNC}'}$ couplings are positive (2.5–5.0 Hz) for the *trans* configuration and result in a positive slope in the C' -coupled $^{15}\text{N}-^1\text{H}$ E.COSY pattern, but negative (–5.0 to –2.5 Hz) for the *cis* configuration and lead to a negative slope (Bystrov, 1976).

3D IS-TROSY-HNCA

Figure 2a illustrates the pulse sequence of the 3D IS-TROSY-HNCA experiment. The pulse sequence was derived from the ST2-PT-type (Pervushin et al., 1998) TROSY-HNCA experiment for partially deuterated proteins (Salzmann et al., 1998; Eletsky et al., 2001) with the addition of the deuterium decoupling scheme during the whole $^{15}\text{N}\leftrightarrow^{13}\text{C}$ magnetization transfer period in order to suppress scalar relaxation of the second kind (Abragam, 1961) from the deuterium in $\text{NH}\{\text{D}\}$ isotopomers. Briefly, the magnetization transfer started with the excitation of amide protons at both the backbone and side chains, then, was

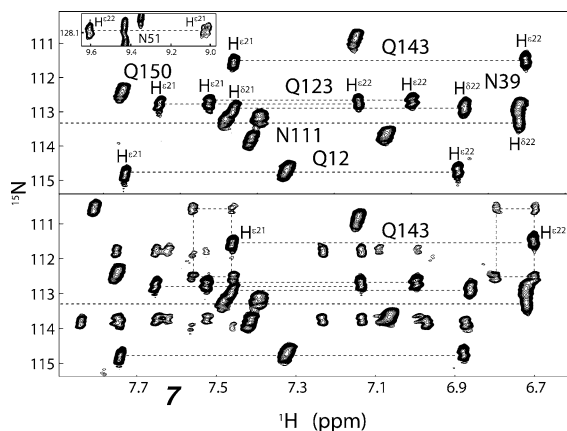


Figure 1. A region of C' -coupled 2D ^{15}N - ^1H IS-TROSY spectra of yCD with uniform $^{13}\text{C}/^{15}\text{N}$ labeling and 75% fractional deuteration. Paired side chain NH{D} resonances of each Asn/Gln residue are linked with a dash line. Stereospecific distinction between the *E* and *Z* carboxamide protons is made based on the observation of the slope of the connection between the two E.COSY components. For yCD, the *E* configuration ($\delta_{21}/\epsilon_{21}$) protons of all Asn and Gln residues have resonances at downfield with respect to the corresponding *Z* configuration ($\delta_{22}/\epsilon_{22}$) protons in the ^1H dimension, with the sole exception of Asn 51 which has the *E* configuration proton at upfield (see the inset in the upper panel). The upper panel is the spectrum recorded with an experimental scheme of the ST2-PT type, while the spectrum in the lower panel was recorded with the gradient-selective type (Nietlishpach et al., 2005) that shows strong non-TROSY interferences for side chain resonances of flexible surface Asn/Gln residues. The non-TROSY peaks of the Gln 143 side chain amide are indicated with dashed rectangles. Both spectra were recorded with 16 scans and a 2 s delay time, $t_{1\text{max}} = 133$ ms and $t_{2\text{max}} = 143$ ms, resulting in the experimental time of 8.5 h for each. Before Fourier transformation, the raw data were zero filled by a factor of 3 in t_1 dimension and 2 in t_2 dimension.

transferred to the attached amide ^{15}N via the INEPT element (Morris and Freeman, 1979). During the following transfer steps of $^{15}\text{N} \rightarrow ^{13}\text{C}^\alpha$ for the backbone and $^{15}\text{N} \rightarrow ^{13}\text{C}^{\beta/\gamma}$ (Asn/Gln) for side chains, the ^2H carrier frequency was set at the center of side chain amide deuterons, 7.3 ppm, to preserve the magnetization of side chain NHD isotopomers in addition to backbone amides. The ^2H carrier frequency was switched to upfield at the center of aliphatic α , β (and γ for Gln) deuterons, 3.0 ppm, before the start of the first evolution period (t_1) of backbone $^{13}\text{C}^\alpha$ as well as side chain $^{13}\text{C}^{\beta/\gamma}$ (Asn/Gln) and then jumped downfield back to 7.3 ppm during the backward $^{13}\text{C}^{\alpha/\beta/\gamma} \rightarrow ^{15}\text{N}$ (t_2) transfer step. In the end, the magnetization was transferred back to both backbone NH and side chain NH{D} amide protons for detection (t_3)

through the ST2-PT element (Pervushin et al., 1998).

3D IS-TROSY-HNCACB

Figure 2b depicts the pulse sequence of the 3D IS-TROSY-HNCACB experiment. It was a natural extension of the IS-TROSY-HNCA experiment by including further magnetization transfer steps of $^{13}\text{C}^\alpha \leftrightarrow ^{13}\text{C}^\beta$ for the backbone and $^{13}\text{C}^{\beta/\gamma} \leftrightarrow ^{13}\text{C}^{\alpha/\beta}$ (Asn/Gln) for side chains, in a way similar to the extension from conventional HNCA to HNCACB and from TROSY-HNCA to TROSY-HNCACB (Salzmann et al., 1999; Eletsky et al., 2001) experiments. In the IS-TROSY-HNCACB experiment, side chain magnetizations were transferred from $^{13}\text{C}^{\beta/\gamma}$ to $^{13}\text{C}^{\alpha/\beta}$ for Asn/Gln residues, while magnetizations of backbone amides of all non-proline residues were from $^{13}\text{C}^\alpha$ to $^{13}\text{C}^\beta$. As a result, for an Asn, $^{13}\text{C}^{\alpha/\beta}$ correlations originated from side chain and backbone amides are 180° out of phase and for a Gln, on the other hand, $^{13}\text{C}^\beta$ correlations originated from side chain and backbone amides have the same phase. This is a very useful feature for the resonance assignment.

Results and discussion

yCD is a 35-kDa homodimeric enzyme with 11 Asn/Gln residues in each protomer (Iretton et al., 2003; Ko et al., 2003). Side chain amide resonances of Asn 51, Gln 55 and Asn 113 were hardly observable in normal 2D ^{15}N - ^1H HSQC spectra and those of Asn 40 and Asn 111 were too weak to be assigned with conventional triple-resonance experiments. In the 2D ^{15}N - ^1H IS-TROSY spectrum, however, side chain amide resonances of all 11 Asn/Gln residues were clearly seen (Liu et al., submitted). Figure 1 shows a region of the $^{13}\text{C}'$ -coupled 2D ^{15}N - ^1H IS-TROSY spectrum of yCD. It is obvious that relative positions of the two carbonyl carbon-coupled E.COSY components of the $^{15}\text{N} - \text{H}^{\delta_{21}}/\text{H}^{\epsilon_{21}}$ (Asn/Gln) correlation is the same in both dimensions, i.e. the connection of the two components shows a positive slope in the 2D spectrum. On the other hand, the connection of the two E.COSY components of the $^{15}\text{N} - \text{H}^{\delta_{22}}/\text{H}^{\epsilon_{22}}$ (Asn/Gln) correlation has a negative slope. Even with a glimpse of Figure 1, one would instantly

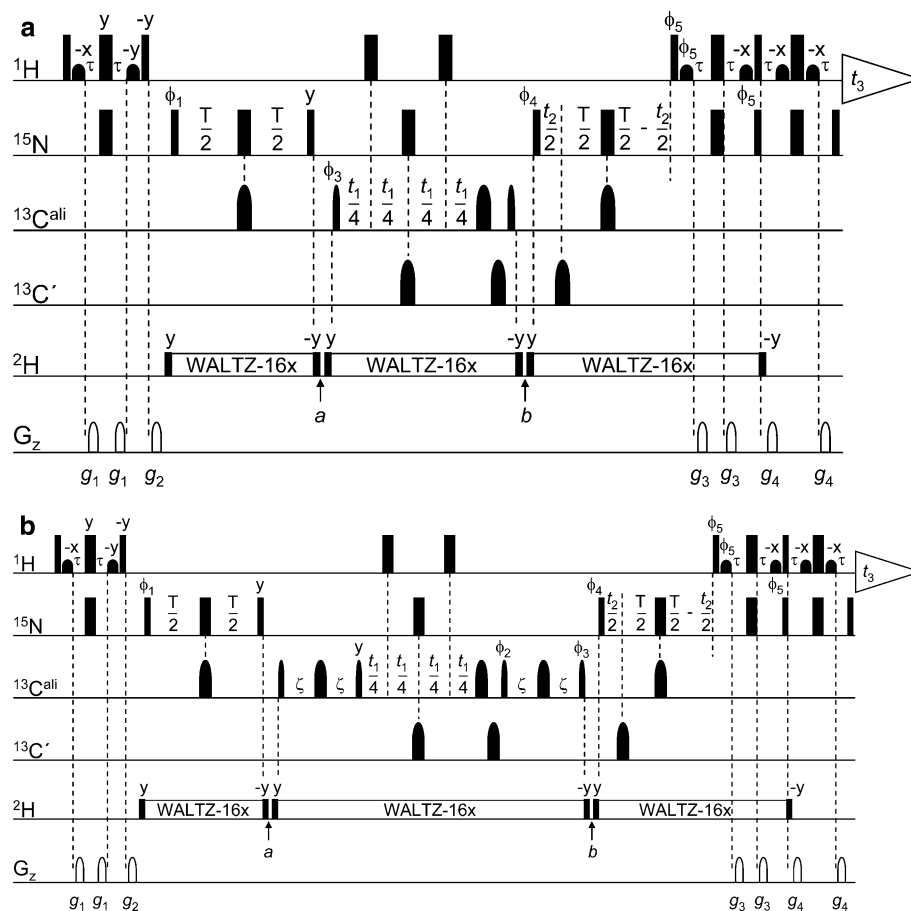


Figure 2. Experimental schemes of (a) 3D IS-TROSY-HNCA and (b) 3D IS-TROSY-HNCACB for the simultaneous resonance assignment of both backbone and side chain amides. Narrow and wide black rectangles stand for 90° and 180° pulses, respectively. The small filled bell-like shapes in ^1H channel are 1.5 ms selective 90° pulses with the profile of the center lobe of sinc function for water flip-back. Narrow and wide shaped bars in ^{13}C channel are band-selective 90° Q5 and 180° Q3 pulses (Emsley and Bodenhausen, 1992) with pulse widths of $390\ \mu\text{s}$ and $227\ \mu\text{s}$, respectively. Deuterium decoupling was achieved with the WALTZ-16 (Shaka et al., 1983) scheme and flanked with 90° pulses at the power level of 1.4 kHz. All pulses have the x phase unless otherwise indicated. Carrier frequencies for ^1H , ^{15}N , $^{13}\text{C}^{\text{ali}}$ (aliphatic carbons) and $^{13}\text{C}'$ pulses were set at 4.82, 118.0, 46 and 176 ppm, respectively. The carrier in ^2H channel was set at 7.2 ppm at the start of the pulse sequence, then switched to 3.0 ppm at time point a and finally jumped back to 7.2 ppm at time point b . The phase cycle scheme was, $\phi_1 = 8x, 8(-x)$; $\phi_2 = 4(-y), 4(y)$; $\phi_3 = 4(x), 4(-x)$; $\phi_4 = y, -y, x, -x$; $\phi_5 = -y$; receiver = $y, -y, -x, x, -y, y, x, -x, -y, y, x, -x, y, -y, -x, x$. Quadrature detection in t_1 was achieved by States-TPPI (Marion et al., 1989) through ϕ_2 and ϕ_3 . Phase-sensitive detection in t_2 was obtained by recording a second FID for each t_2 value with $\phi_4 = y, -y, -x, x$ and $\phi_5 = y$ and data processing as proposed by Kay (Kay et al., 1992). Sine shaped z -axis gradients were applied for 0.8 ms with amplitudes of 18 G/cm (g_1), 42 G/cm (g_2), 10.8 G/cm (g_3) and 26.4 G/cm (g_4). Delays τ , T and ζ were set for 2.5, 24.0 and 3.6 ms, respectively.

appreciate that side chain amides of all Asn/Gln residues but Asn 51 have the $\text{H}^{\delta 21}/\text{H}^{\epsilon 21}$ resonances at down field relative to those of $\text{H}^{\delta 22}/\text{H}^{\epsilon 22}$. The reversed order and unusual chemical shifts of the side chain amide proton resonances of Asn 51 are likely caused by hydrogen bonding interactions and strong ring current effects from the neighboring aromatic ligand and residue His 62. The $\text{H}^{\delta 21}$ proton of Asn 51 forms a hydrogen bond with the O2 atom in the ligand and the $\text{H}^{\delta 22}$ pro-

ton forms a hydrogen bond with the side chain carboxyl group of Asp 155. In addition, both $\text{H}^{\delta 21}$ and $\text{H}^{\delta 22}$ of Asn 51 are within the distance of $3.5\ \text{\AA}$ to His 62 (Ireton et al., 2003; Ko et al., 2003).

There are other NMR experiments available for the stereospecific assignment of Asn/Gln side chain amide protons. Unfortunately, neither $\text{H}_2\text{NCO-E.COSY}$ (Löhr and Rüterjans, 1997) nor EZ-HMQC-NH_2 (McIntosh et al., 1997) experiment is able to detect the missing signals of side

chain amides in yCD, because these experiments are of the constant-time version and not sensitive enough for this size of proteins. The multiplicity-edited ^{15}N - ^1H HSQC experiment (Prella et al., 1997; Cai et al., 2001) is more sensitive but still not enough, because the experimental scheme has an additional filtering step and does not include the TROSY technique. In contrast, the IS-TROSY-based experiments as described here are capable of detecting signals and making stereospecific assignment of side chain amides of all 11 Asn/Gln residues in yCD and have the potential to be applied to large systems with molecular weights beyond 80 kDa, in which no other NMR techniques are available for this purpose (Liu et al., submitted).

Besides backbone ^1H - ^{15}N - $^{13}\text{C}^\alpha$ connections of all non-proline residues, side chain amide $^1\text{H}\{\text{D}\}$ - $^{15}\text{N}^{\delta/\epsilon}$ - $\text{C}^{\beta/\gamma}$ (Asn/Gln) correlations of all 11 Asn/Gln residues can also be clearly identified in the 3D IS-TROSY-HNCA spectrum as shown in Figure 3. It should be pointed out that side chain $^{13}\text{C}^{\beta/\gamma}$ (Asn/Gln) resonances have no any direct correlation with backbone $^{13}\text{C}^\alpha$ in this experiment. Nonetheless, the high sensitivity of the IS-TROSY-HNCA experiment in comparison with the IS-TROSY-HNCACB experiment (see below) greatly helps draw assignment from the latter. More importantly, the difference in the chemical shifts of $^{13}\text{C}^\beta$ (37–40 ppm) of Asn and C^γ (32–35 ppm) of Gln residues has already made an initial distinction between the side chain amides of these two types of residues.

Figure 4 shows the Asn/Gln strips of the 3D IS-TROSY-HNCACB spectrum. Just as in the conventional 3D HNCACB and 3D TROSY-HNCACB experiments, backbone $^{13}\text{C}^\alpha$ resonances have a 180° phase difference with respect to that of $^{13}\text{C}^\beta$, a unique and very useful feature to distinguish $^{13}\text{C}^\alpha$ from $^{13}\text{C}^\beta$ resonances. Moreover, in the IS-TROSY-HNCACB experiment, magnetizations of the side chain amides are transferred from $^{13}\text{C}^\beta$ to $^{13}\text{C}^\alpha$ and from $^{13}\text{C}^\gamma$ to $^{13}\text{C}^\beta$ for Asn and Gln residues, respectively. For Asn residues, $^{13}\text{C}^{\alpha/\beta}$ resonances generated from the backbone have a 180° phase difference from those originated from side chain amides; for Gln residues, $^{13}\text{C}^\beta$ resonances stemmed from both backbone and side chain amides have the same phase. Therefore, the assignment of side chain amides becomes straightforward once the backbone assignment is

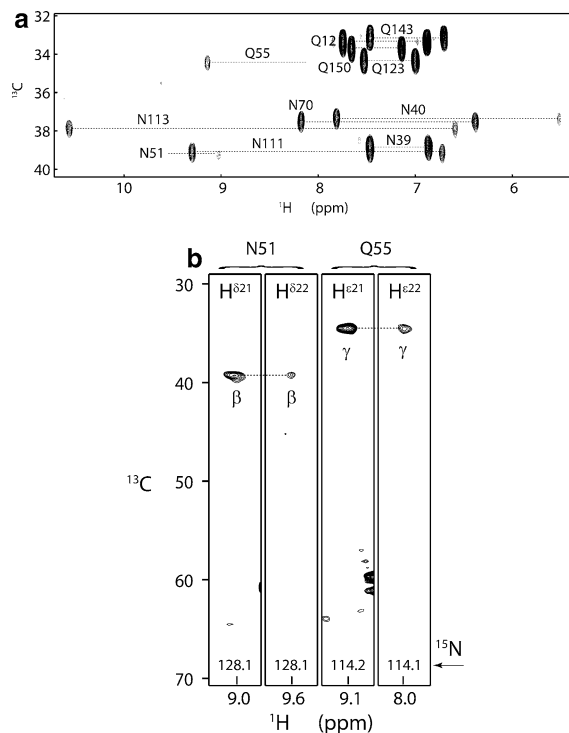


Figure 3. 3D IS-TROSY-HNCA spectra of yCD recorded with the pulse sequence of Figure 2a. Panel (a), an overlay of the 2D ^{13}C - ^1H planes associated with the side chain amides. The paired resonances of the side chain amides of all 11 Asn/Gln residues are connected with dashed lines. The H^{822} resonance of Asn 51 and the H^{822} resonance of Gln 55 are not observable at this plotting level, but can be clearly seen in 2D ^{13}C - ^1H strips of the 3D spectrum as shown in panel (b). The 3D spectrum was recorded with a 2 s delay time and eight transients per FID. Maximum acquisition times in different dimensions were $t_{1\text{max}}(^{13}\text{C}) = 7.4$ ms, $t_{2\text{max}}(^{15}\text{N}) = 14.6$ ms and $t_{3\text{max}}(^1\text{H}) = 71$ ms, resulting in an experimental time of 60.6 h.

available. On the other hand, the side chain resonances of Asn/Gln residues are valuable starting points for the sequential assignment of backbone resonances with the combined analysis of the 3D IS-TROSY-HNCACB and IS-TROSY-HNCA spectra. Furthermore, side chain correlations also help distinguish intra- and inter-residual correlations from each other for the backbone, because resonances from side chains are purely intra. The distinction between intra- and inter-residual resonances is the key step in the amino acid sequential assignment. In fact, for Asn/Gln residues, all resonances except for those of carbonyl carbons have been encoded in the single 3D IS-TROSY-HNCACB spectrum. Figure 4a shows the 2D ^{13}C - $^1\text{H}^{\text{N}}$ strips of the 3D IS-TROSY-HNCACB

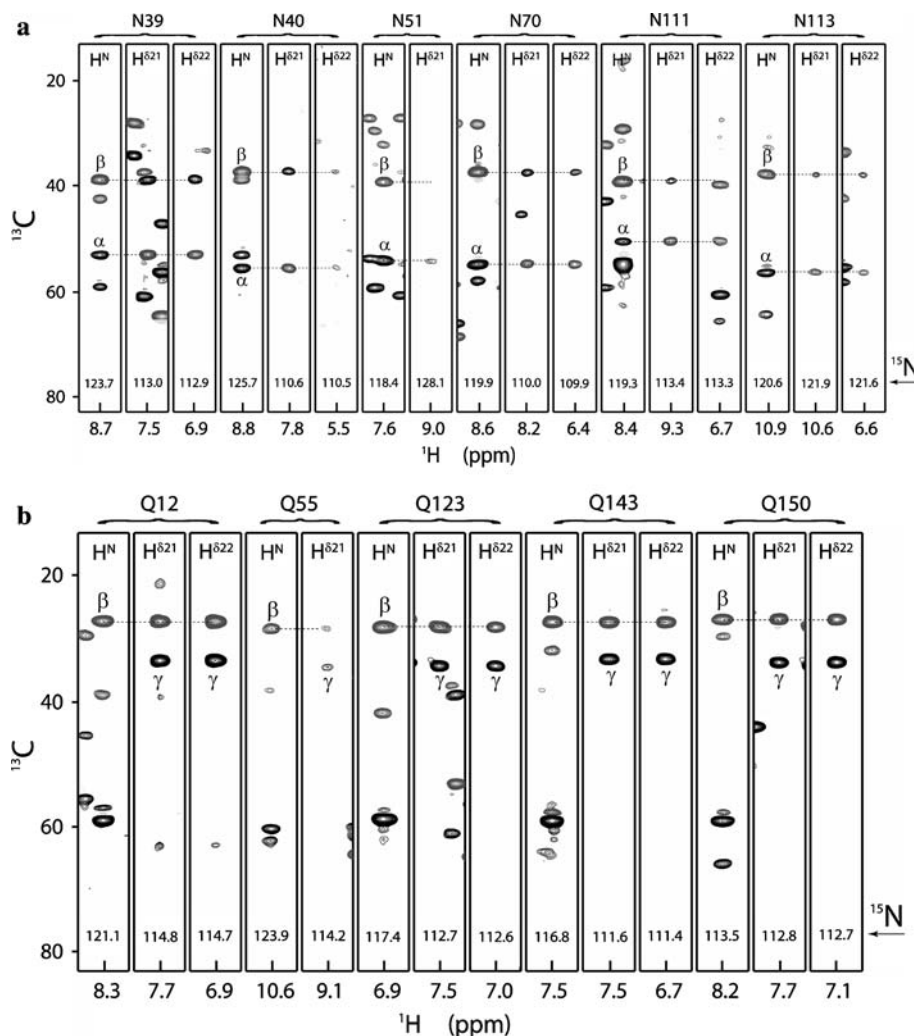


Figure 4. 2D ^{13}C - ^1H strips from the 3D IS-TROSY-HNCACB spectrum of yCD recorded with the pulse sequence depicted in Figure 2b. Panel (a) shows resonances of side chain amides of Asn residues and panel (b) Gln residues. For Asn, backbone and side chain amides of each residue are correlated through both $^{13}\text{C}^\alpha$ and $^{13}\text{C}^\beta$ resonances that have a 180° phase difference. Positive peaks were drawn in dark contours and negative peaks in gray. For Gln, backbone and side chain amides of each residue are correlated through the $^{13}\text{C}^\beta$ resonance which has the same phase. The 3D spectrum was collected with a 1.75 s delay time and 32 scans per FID. Maximum acquisition times in individual dimensions were $t_{1\text{max}}(^{13}\text{C}) = 3.4$ ms, $t_{2\text{max}}(^{15}\text{N}) = 10.0$ ms, $t_{3\text{max}}(^1\text{H}) = 71$ ms, resulting in an experimental time of 117 h.

spectrum of Asn residues in yCD. Correlations between the backbone and side chains of all six Asn residues have been established, although resonances from $\text{H}^{\delta 22}$ of Asn 51 are missing. The $^{13}\text{C}^\beta$ - $\text{H}^{\delta 21}$ resonance of Asn 51 is too weak to be seen at this contour level and the $^{13}\text{C}^\beta$ - $\text{H}^{\delta 22}$ resonance of Asn 111 is also missing due to the overlap with an adjacent negative peak. Figure 4b shows the corresponding strips of Gln residues.

Again, correlations between the backbone and side chains of all five Gln residues have been established, even though resonances from $\text{H}^{\delta 22}$ of Gln 55 are missing. It is worthwhile to point out that the correlation for Gln residues is exclusively based on the $^{13}\text{C}^\beta$ resonances, which may exclude the complete assignment when the chemical shift dispersion is poor. It has not been a limitation for yCD, however, because of its good chemical shift

dispersion. Resonances of carbonyl carbons for both the backbone and side chains can be easily detected and assigned with the most sensitive 3D IS-TROSY-HNCO experiment, which is in essence very similar to the 3D IS-TROSY-HNCA experiment (see Figure 2a) if interconverting the carrier positions between $^{13}\text{C}^\alpha$ and $^{13}\text{C}'$ (see Supporting Information; 2D overlays of the 3D IS-TROSY-HNCO spectrum that contains resonances of both backbone and side chain carbonyl carbons of yCD). Chemical shifts of side chain carbonyl $^{13}\text{C}'$ of Asn and Gln residues of yCD are in two different ranges of 174.3–177.4 ppm and 180.3–181.1 ppm, respectively. Interestingly, side chain $\text{H}^{\delta 21}/\text{H}^{\epsilon 21}$ (Asn/Gln) resonances of yCD are generally stronger than that of $\text{H}^{\delta 22}/\text{H}^{\epsilon 22}$ for the same carboxamide group in all IS-TROSY-based experiments, a phenomenon at least partially reflects the fact that the one-bond scalar couplings $^1J_{\text{HN}}$ for $\text{H}^{\delta 21}/\text{H}^{\epsilon 21}$ are slightly larger than $\text{H}^{\delta 22}/\text{H}^{\epsilon 22}$ (Bystrov, 1976). This piece of information is useful, because it directly supports the stereospecific assignment. However, the major reason for different resonance intensities is likely due to different TROSY effects and dynamics between $\text{N}-\text{H}^{\delta 21}/\text{H}^{\epsilon 21}$ and $\text{N}-\text{H}^{\delta 22}/\text{H}^{\epsilon 22}$ moieties of a carboxamide group.

In the mixture solvent of 50% $\text{H}_2\text{O}/50\%\text{D}_2\text{O}$, there are four possible isotopomers, $\text{NH}^{21}\text{H}^{22}$, $\text{NH}^{21}\text{D}^{22}$, $\text{ND}^{21}\text{H}^{22}$ and $\text{ND}^{21}\text{D}^{22}$ for each carboxamide group. Two of them, $\text{NH}^{21}\text{D}^{22}$ and $\text{ND}^{21}\text{H}^{22}$, are semideuterated and can be exclusively detected with IS-TROSY-type experiments. Assuming that the population of each isotopomer is about equal (LiWang and Bax, 1996; Loh and Markley, 1994), then, the concentration of either $\text{NH}^{21}\text{D}^{22}$ or $\text{ND}^{21}\text{H}^{22}$ is about half of the backbone NH moiety, leading to an intrinsically lower signal intensity for side chain amides compared with the backbone. For backbone amides, too, about half of the population is deuterated and is not detectable. However, this is not necessarily a negative factor in the IS-TROSY NMR technique. Partial deuteration of exchangeable protons in a protein leads to a dilution of the proton density within the molecule and so does the homonuclear DD interactions which otherwise contribute significantly to relaxation rates in biomolecular NMR. Most importantly, a lower proton density is favorable to the TROSY technique (Pervushin et al., 1997b). Actually, we have achieved complete

resonance assignment for all backbone and side chain amides of yCD by using this strategy. In practice, using 20–40% D_2O in the solvent may be enough to restore all resonances of side chain amides for a specific protein sample. In the case of yCD, we have observed all carboxamide signals in the 2D $^{15}\text{N}-^1\text{H}$ IS-TROSY spectrum with an NMR sample under normal solvent conditions in 93% $\text{H}_2\text{O}/7\%\text{D}_2\text{O}$ (see Supporting Information).

Conclusion

In summary, a new strategy for the simultaneous NMR assignment of both backbone and side chain amides in large proteins has been introduced in this work. The method considers aspects of both the NMR sample preparation and the experimental design. First, the protein sample is prepared in a buffer with 50% $\text{H}_2\text{O}/50\%\text{D}_2\text{O}$ to promote the population of semideuterated NHD isotopomers in side chain amides of Asn/Gln residues. Second, a $^{13}\text{C}'$ -coupled 2D $^{15}\text{N}-^1\text{H}$ IS-TROSY spectrum provides an instant stereospecific distinction between the *E* and *Z* configuration protons. Third, a set of isotopomer-selective TROSY-based triple-resonances NMR experiments, e.g. 3D IS-TROSY-HNCA and 3D IS-TROSY-HNCACB, are designed to correlate aliphatic carbons with backbone amides and, for Asn/Gln residues, at the same time with side chain amides. The assignment strategy is similar to that using conventional 3D HNCA/3D HNCACB experiments for small proteins, in which, however, signals from NH_2 groups are often missing due to the use of broad-band proton decoupling schemes and thus NOE data have to be used as a remedy. For large proteins, the use of conventional TROSY experiments makes the side chain amide resonances not observable at all. The application of IS-TROSY experiments in the 35-kDa yCD has established a complete resonance assignment for the backbone and stereospecific assignment for side chain amides, which otherwise could not be achieved with existing NMR experiments. Therefore, the development of IS-TROSY-based method provides a new approach in the NMR exploration of the important structural and biological roles of carboxamides, arginine and lysine side chain groups in large proteins as well as amino moieties in large nucleic acid molecules.

Supporting information available

Figures showing IS-TROSY spectra of yCD (PDF). This electronic supplementary material is available in the online version of this article at <http://dx.doi.org/10.1007/s10858-006-9072-30> and is accessible for authorized users.

Acknowledgements

This work made use of a Bruker AVANCE 900 MHz NMR spectrometer funded in part by Michigan Economic Development Corporation and a Varian INOVA 600 MHz NMR spectrometer funded in part by NSF Grant BIR9512253. The work was supported in part by NIH Grant GM58221 (H.Y.). A.L. was a recipient of IRGP New Faculty Awards at MSU.

References

- Abragam, A. (1961) *Principles of Nuclear Magnetism*, Clarendon Press, Oxford.
- Andersson, P., Annala, A. and Otting, G. (1998) *J. Magn. Reson.*, **133**, 364–367.
- Bax, A. and Grzesiek, S. (1993) *Acc. Chem. Res.*, **26**, 131–138.
- Bax, A. and Ikura, M. (1991) *J. Biomol. NMR*, **1**, 99–104.
- Bax, A. and Pochapsky, S.S. (1992) *J. Magn. Reson.*, **99**, 638–643.
- Bax, A., Ikura, M., Kay, L.E., Torchia, D.A. and Tschudin, R. (1990) *J. Magn. Reson.*, **86**, 304–318.
- Bystrov, V.F. (1976) *Prog. Nucl. Magn. Reson. Spectrosc.*, **10**, 41–81.
- Cai, M., Huang, Y. and Clore, G.M. (2001) *J. Am. Chem. Soc.*, **123**, 8642–8643.
- Cavanagh, J., Fairbrother, W.J., Palmer III, A.G. and Skelton, N.J. (1996) *Protein NMR Spectroscopy: Principles and Practice*, Academic Press, New York.
- Creighton, T.E. (1993) *Proteins: Structures and Molecular Properties* (2nd ed.). Freeman, W. H. and Company, New York.
- Czisch, M. and Boelens, R. (1998) *J. Magn. Reson.*, **134**, 158–160.
- Davis, A.L., Boelens, R. and Kaptein, R. (1992) *J. Biomol. NMR*, **2**, 395–400.
- Delaglio, F., Grzesiek, S., Vuister, G.W., Zhu, G., Pfeifer, J. and Bax, A. (1995) *J. Biomol. NMR*, **6**, 277–293.
- Eletsky, A., Kiehöfer, A. and Pervushin, K. (2001) *J. Biomol. NMR*, **20**, 177–180.
- Emsley, L. and Bodenhausen, G. (1992) *J. Magn. Reson.*, **97**, 135–148.
- Farmer II, B.T. and Venters, R.A. (1996) *J. Biomol. NMR*, **7**, 59–71.
- Farmer II, B.T., Venters, R.A., Spicer, L.D., Wittekind, M.G. and Mueller, L. (1992) *J. Biomol. NMR*, **2**, 195–202.
- Grzesiek, S. and Bax, A. (1992a) *J. Magn. Reson.*, **96**, 432–440.
- Grzesiek, S. and Bax, A. (1992b) *J. Am. Chem. Soc.*, **114**, 6291–6293.
- Grzesiek, S., Anglister, J. and Bax, A. (1993) *J. Magn. Reson.*, **101**, 114–119.
- Herzfeld, J., Roberts, J.E. and Griffin, R.G. (1987) *J. Chem. Phys.*, **86**, 597–602.
- Higman, V.A., Boyd, J., Smith, L.J. and Redfield, C. (2004) *J. Biomol. NMR*, **30**, 327–346.
- Iretton, G.C., Black, M.E. and Stoddard, B.L. (2003) *Structure*, **11**, 961–972.
- Johnson, B.A. and Blevins, R.A. (1994) *J. Biomol. NMR*, **4**, 603–614.
- Kay, L.E., Ikura, M., Tschudin, R. and Bax, A. (1990) *J. Magn. Reson.*, **89**, 496–514.
- Kay, L.E., Keifer, P. and Saarinen, T. (1992) *J. Am. Chem. Soc.*, **114**, 10663–10665.
- Kay, L.E., Xu, G.Y. and Yamazaki, T. (1994) *J. Magn. Reson.*, **A109**, 129–133.
- Ko, T.-P., Lin, J.-J., Hu, C.-Y., Hsu, Y.-H., Wang, A.H.-J. and Liaw, S.-H. (2003) *J. Biol. Chem.*, **278**, 19111–19117.
- LiWang, A. and Bax, A. (1996) *J. Am. Chem. Soc.*, **118**, 12864–12865.
- Logan, T.M., Olejniczak, E.T., Xu, R.X. and Fesik, S.W. (1993) *J. Biomol. NMR*, **3**, 225–231.
- Loh, S.N. and Markley, J.L. (1994) *Biochemistry*, **33**, 1029–1036.
- Löhr, F. and Rüterjans, H. (1997) *J. Magn. Reson.*, **124**, 255–258.
- Madsen, J.C. and Sørensen, O.W. (1992) *J. Magn. Reson.*, **100**, 431–436.
- Marion, D., Ikura, M., Tschudin, R. and Bax, A. (1989) *J. Magn. Reson.*, **85**, 393–399.
- McIntosh, L.P., Brun, E. and Kay, L.E. (1997) *J. Biomol. NMR*, **9**, 306–312.
- Meissner, A., Schulte-Herbrüggen, T., Briand, J. and Sørensen, O.W. (1998) *Mol. Phys.*, **96**, 1137–1142.
- Morris, G.A. and Freeman, R. (1979) *J. Am. Chem. Soc.*, **101**, 760–762.
- Nietlishpach, D. (2005) *J. Biomol. NMR*, **31**, 161–166.
- Parella, T., Sanchez-Ferrando, F. and Virgili, A. (1997) *J. Magn. Reson.*, **126**, 274–277.
- Permi, P. (2001) *J. Magn. Reson.*, **153**, 267–272.
- Pervushin, K., Wider, G. and Wüthrich, K. (1997a) *J. Am. Chem. Soc.*, **119**, 3842–3843.
- Pervushin, K., Riek, R., Wider, G. and Wüthrich, K. (1997b) *Proc. Natl. Acad. Sci. USA*, **94**, 12366–12371.
- Pervushin, K., Wider, G. and Wüthrich, K. (1998) *J. Biomol. NMR*, **12**, 345–348.
- Pervushin, K., Braun, D., Fernández, C. and Wüthrich, K. (2000) *J. Biomol. NMR*, **17**, 195–202.
- Rance, M., Loria, J.P. and Palmer III, A.G. (1999) *J. Magn. Reson.*, **136**, 91–101.
- Salzmann, M., Pervushin, K., Wider, G., Senn, H. and Wüthrich, K. (1998) *Proc. Natl. Acad. Sci. USA*, **95**, 13585–13590.
- Salzmann, M., Wider, G., Pervushin, K., Senn, H. and Wüthrich, K. (1999) *J. Am. Chem. Soc.*, **121**, 844–848.
- Sattler, M., Schleucher, J. and Griesinger, C. (1999) *Prog. Nucl. Magn. Reson. Spectrosc.*, **34**, 93–158.
- Schleucher, J., Sattler, M. and Griesinger, C. (1993) *Andew. Chem. Int. Ed. Engl.*, **32**, 1489–1491.
- Schubert, M., Oschkinat, H. and Schmieder, P. (2001) *J. Magn. Reson.*, **148**, 61–72.

- Shaka, A.J., Keeler, J., Frenkiel, T. and Freeman, R. (1983) *J. Magn. Reson.*, **52**, 335–338.
- Vis, H., Boelens, R., Mariani, M., Stroop, R., Vorgias, C.E., Wilson, K.S. and Kaptein, R. (1994) *Biochemistry*, **33**, 14858–14870.
- Weigelt, J. (1998) *J. Am. Chem. Soc.*, **120**, 10778–10779.
- Wittekind, M. and Mueller, L. (1993) *J. Magn. Reson. B*, **101**, 201–205.
- Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, Wiley, New York.
- Yamazaki, T., Yoshida, M. and Nagayama, K. (1993) *Biochemistry*, **32**, 5656–5669.
- Yamazaki, T., Lee, W., Arrowsmith, C.H., Muhandiram, D.R. and Kay, L.E. (1994) *J. Am. Chem. Soc.*, **116**, 11655–11666.
- Yang, D. and Kay, L.E. (1999) *J. Am. Chem. Soc.*, **121**, 2571–2575.
- Yao, L., Li, Y., Wu, Y., Liu, A. and Yan, H. (2005) *Biochemistry*, **44**, 5940–5947.
- Zhu, G., Kong, X. and Sze, K. (1999) *J. Biomol. NMR*, **13**, 77–81.