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Article

Amino-acid type identification in ^{15}N-HSQC spectra by combinatorial selective ^{15}N-labelling

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Abstract

The efficiency of cell-free protein synthesis combined with combinatorial selective ^{15}N-labelling provides a method for the rapid assignment of ^{15}N-HSQC cross-peaks to the 19 different non-proline amino-acid types from five ^{15}N-HSQC spectra. This strategy was explored with two different constructs of the C-terminal domain V of the τ subunit of the *Escherichia coli* DNA polymerase III holoenzyme, τ_C16 and τ_C14. Since each of the five ^{15}N-HSQC spectra contained only about one third of the cross-peaks present in uniformly labelled samples, spectral overlap was much reduced. All ^{15}N-HSQC cross-peaks of the backbone amides could be assigned to the correct amino-acid type. Availability of the residue-type information greatly assisted the evaluation of the changes in chemical shifts observed for corresponding residues in τ_C16 vs. those in τ_C14, and the analysis of the structure and mobility of the C-terminal residues present in τ_C16 but not in τ_C14.

Abbreviations: τ_C14 – residues 499–625 of the τ subunit of *E. coli* DNA polymerase III with an additional N-terminal methionine; τ_C16 – same as τ_C14, but including the C-terminal 18 residues 626–643 of τ.

Introduction

^{15}N-HSQC spectra provide well-resolved fingerprint information and are the cornerstone of backbone resonance assignments of ^{15}N-labelled proteins. With the advent of high-yield cell-free protein synthesis systems, the preparation and NMR spectroscopic analysis of selectively ^{15}N-labelled proteins has become both fast and inexpensive (Kigawa et al., 1995; Ozawa et al., 2005a). While maximal information could be obtained from 19 different samples, where each of the 19 non-proline residues is selectively ^{15}N-labelled (Yamazaki et al., 1991; Ozawa et al., 2004), the same information can be retrieved with much less effort by the use of combinatorial ^{15}N-labelling, where several amino acids are simultaneously ^{15}N-labelled in a limited number of samples (Shortle 1994; Parker et al., 2004). Combinatorial isotope labelling results in ambiguities only if chemical shift degeneracies lead to perfect superposition of the cross-peaks of two or more amino-acid residues.

Here we present a different combinatorial ^{15}N-labelling scheme which delivers complete residue-type identifications for the ^{15}N-HSQC cross-peaks from all 19 non-proline residues and where each ^{15}N-HSQC spectrum produces only a third of the peaks generated by uniformly ^{15}N-labelled protein. It was used for the structural

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characterization of the C-terminal 16 kDa domain of the τ subunit, τ_C16, from the *Escherichia coli* replisome (Gao and McHenry, 2001). The protein is toxic to *E. coli* and could not be purified in stable form due to pronounced sensitivity with respect to proteolysis, whereas a shorter 14 kDa fragment, τ_C14, that is missing the C-terminal 18 residues of τ_C16, was sufficiently stable to allow the determination of its three-dimensional structure by NMR spectroscopy (X.-C. Su, S. Jergic and G. Otting, unpublished). We show that five combinatorially ^{15}N-labelled samples, freshly prepared of each of the two proteins, readily identifies the ^{15}N-HSQC cross-peaks of the C-terminal 18 residues, allowing their characterization from spectra containing only about one third of the cross-peaks that would be observed for a uniformly ^{15}N-labelled protein.

Materials and methods

Sample preparation

Selectively ^{15}N-labelled samples of τ_C14 and τ_C16 were synthesized in a cell-free *E. coli* coupled transcription–translation system, following a previously described protocol (Ozawa et al., 2005b), modified by heat treatment of the S30 extract prior to use as recommended by Klammt et al. (2004). Protein synthesis was programmed with plasmids pKO1296 and pSH1062 (K. Ozawa, S.M. Hamdan and N.E. Dixon, unpublished), which contain genes encoding τ_C14 and τ_C16, respectively, in the phage T7-promoter vector pETMCSI (Neylon et al., 2000). T7 RNA polymerase was provided by non-competitive transcription/translation using plasmid pKO1166 (Ozawa et al., 2005b). Five ^{15}N-labelled samples were prepared of each protein following the combinatorial labelling scheme of Table 1, using a dialysis system with 0.7 ml of reaction mixture in 7 ml of outside buffer. ^{15}N-labelled amino acids (Cambridge Isotope Laboratories, Andover, MA, USA) were supplied in concentrations of 0.05, 0.15, 0.35 or 1 mM, depending on the K_m values of the respective aminoacyl-tRNA synthetases (Ozawa et al., 2004), except for Ala, which was supplied at 2 mM concentration to account for the large number of alanine residues present in the amino-acid sequence of T7 RNA polymerase and Glu, which was supplied at 1.5 mM for improved protein yields. Unlabelled amino acids were supplied at a concentration of 1 mM. The reaction buffer contained 208 mM potassium glutamate except for the preparation of ^{15}N-Glu labelled samples where only 1.5 mM of ^{15}N-labelled glutamate was present. The reactions were carried out for 8 h at 37 °C. The product mixtures were subsequently centrifuged at $30000\times g$ for 60 min. Preparations 1–4 were dialyzed overnight at 4 °C against 2 l of NMR buffer (10 mM sodium phosphate, 100 mM NaCl, 1 mM DTT, 0.1 mM NaN_3, pH 6.8), using the same beaker of buffer for all four samples, whereas the fifth sample was prepared later and dialyzed against freshly prepared NMR buffer. Except during NMR measurements the samples were kept frozen to minimize proteolytic digestion.

NMR spectroscopy

Following addition of D_2O to 10% (v/v), ^{15}N-HSQC spectra were recorded at 25 °C on a Bruker AV800 NMR spectrometer equipped with a ^{1}H/^{15}N/^{13}C-triple-resonance cryoprobe. All spectra were recorded using $t_{1max} = 32$ ms, $t_{2max} = 158$ ms and total recording times of 2.5 and 5 h for the τ_C16 and τ_C14 samples, respectively. The program Sparky (Goddard and Kneller, 2004) was used to overlay differently coloured contour plots of the NMR spectra recorded of the different samples. $^3J(H^N, H^\alpha)$ coupling constants of τ_C16 were measured with the CT-HMQC-HN experiment (Ponstingl and Otting, 1998), using $T = 7.5$ ms, $t_{1max} = 20$ ms, $t_{2max} = 256$ ms, water suppression by SWET (Wu and Otting, 2005) and Watergate (Piotto et al., 1992) and a total recording time of 7.3 h per sample. Measured $^3J(H^N, H^\alpha)$ coupling constants were uniformly increased by 10% to account for different relaxation rates of antiphase and in-phase magnetization (Ponstingl and Otting, 1998).

Results

Cell-free protein synthesis

The protein yields obtained by cell-free protein synthesis were approximately 2 mg/ml of reaction mixture for samples 1–4 and 1 mg/ml for the ^{15}N-glutamate labelled sample 5. This was

Table 1. Resolution-optimized combinatorial ^{15}N-labelling scheme

Res. Type	Sample					Freq. (%) [a]
	1	2	3	4	5	
Leu	X					9.9
Ala		X				8.3
Gly			X			6.9
Ser				X		6.8
Glu					X	6.3
Val				X	X	6.7
Ile			X	X		6.0
Lys		X	X			5.6
Arg	X	X				5.5
Thr			X		X	5.4
Asp	X				X	5.3
Pro						4.7
Asn	X		X			4.2
Phe		X			X	4.1
Gln		X		X		3.9
Tyr	X			X		3.1
Met		X		X	X	2.4
His	X		X	X		2.2
Cys	X	X			X	1.3
Trp		X	X	X		1.2

[a] Data taken from the NCBI database. (http://www.ncbi.nlm.nih.gov/Class/Structure/aa/aa_explorer.cgi)

sufficient to observe all ^{15}N-HSQC cross-peaks with recording times of 2.5 h per spectrum without concentrating the reaction mixture prior to measurement. The peak positions of residues labelled in more than one sample were extremely well reproduced in the spectra recorded of samples 1–4, but less so in sample 5. This was attributed to the fact that sample 5 had been dialyzed against a similar but not the same sample of NMR buffer as the first four preparations. The preparation of the five samples was achieved in a day, followed by overnight dialysis into NMR buffer.

The peak intensities from amino acids labelled in more than one of the samples were closely reproduced. Differences in peak intensities, however, were observed between different amino-acid types as a consequence of undesired side reactions that diluted the pool of ^{15}N-labelling for some but not all of the amino acids. The most pronounced effects were observed for glutamine residues for which the ^{15}N-HSQC cross-peaks were consistently weaker (about 4-fold) than those of the other residue types. Furthermore, a transaminase activity generated weak cross-peaks of glutamine in the samples prepared with ^{15}N-glutamate, as reported previously for wheat germ cell extracts (Morita et al., 2004).

Combinatorial labelling scheme

A minimum of five different samples is required to assign the ^{15}N-HSQC cross-peaks to the 19 different types of amino acids with backbone amide protons. Since five combinatorially labelled samples would allow the discrimination of $2^5 = 32$ different amino acid types, different combinatorial labelling schemes are possible. Our labelling scheme (Table 1) was designed to minimize the number of cross-peaks in the ^{15}N-HSQC spectra by labelling abundant amino acids only in a single one of the five samples. For general applicability, the scheme was based on the average amino-acid frequencies in proteins reported in the NCBI database. In addition, the scheme was designed to avoid any simultaneous ^{15}N-labelling of Gln and Asn, because those residues increase the chance of signal overlap due to the cross-peaks from side-chain amides. Since backbone amides of threonine residues can sometimes overlap with the cross-peaks from side-chain amides, in at least one of the five samples Thr was labelled when Asn and Gln were not labelled and *vice versa*. Finally, the scheme labelled only one of the samples with ^{15}N-Glu, since the protein yields were about 2-fold lower when the glutamate concentration, usually present at about 200 mM in the reaction buffer (Kigawa et al., 1999), was reduced to 1.5 mM. Given the amino-acid frequencies of the NCBI database, our scheme would label only about 31.5% of the residues in each of the five samples. For τ_C16, the scheme resulted in five samples with, respectively, 32, 38, 21, 29 and 29% of the amino-acid residues labelled with ^{15}N. The percentages of labelled residues were closely similar for τ_C14. Accordingly, the overlap observed in the ^{15}N-HSQC spectra of the five combinatorially labelled samples of τ_C16 and τ_C14 was greatly reduced compared to that expected for uniformly ^{15}N-labelled samples (Figure 1).

Residue-type identification

Figure 2 illustrates how cross-peaks that are overlapped in one of the samples are well

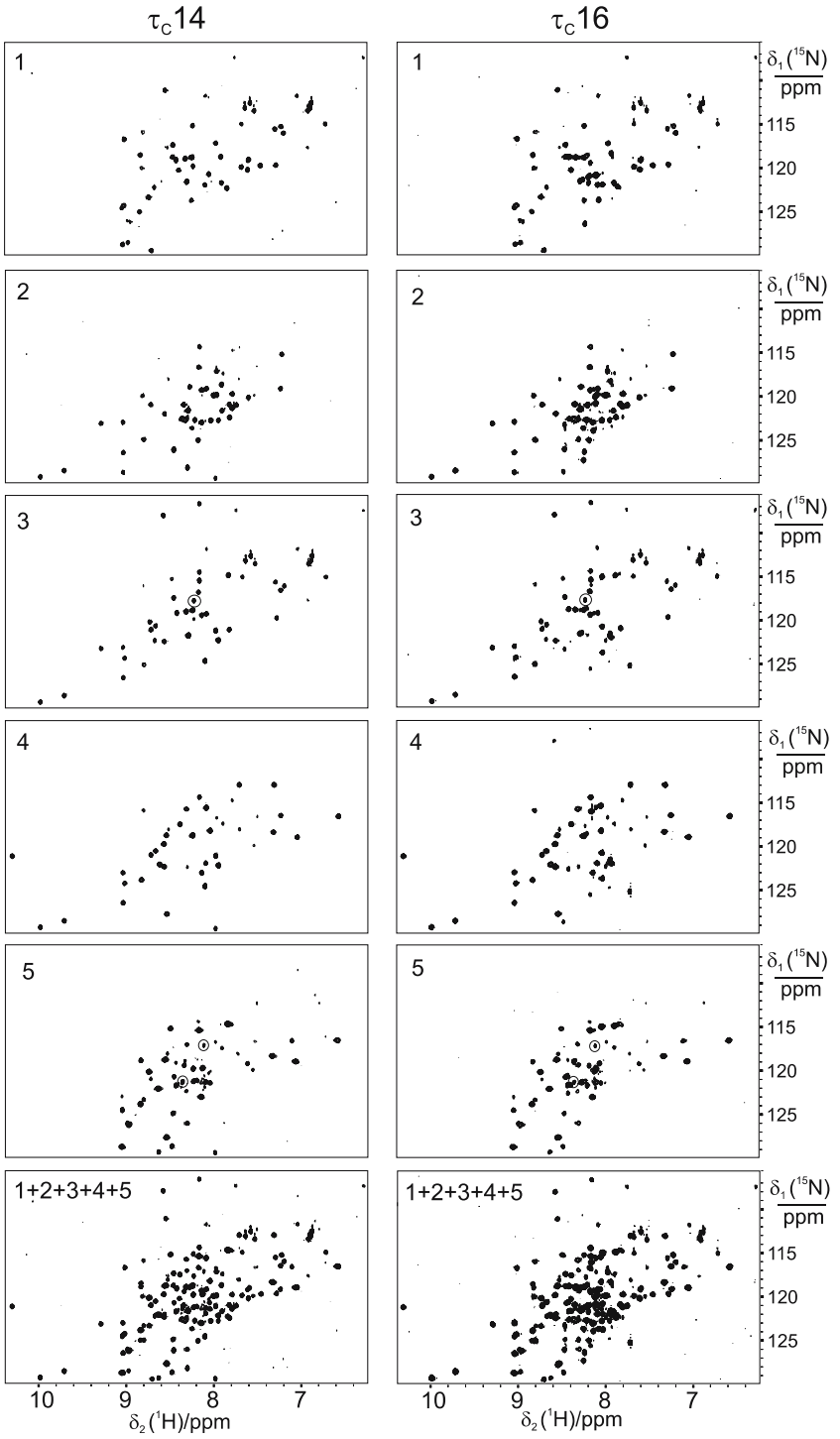

Figure 1. ^{15}N-HSQC spectra recorded of samples of τ_C14 and τ_C16 produced with combinatorial ^{15}N-labelling. The spectra were recorded at 25 °C and pH 6.9 at a ^{1}H-NMR frequency of 800 MHz. Numbers in the top left corner of each spectrum correspond to the five different labelling patterns of Table 1. Circles identify cross-peaks in samples 3 and 5 that did not belong to the proteins of interest. The bottom panel illustrates the 3-fold increased density of cross-peaks expected in uniformly labelled samples.

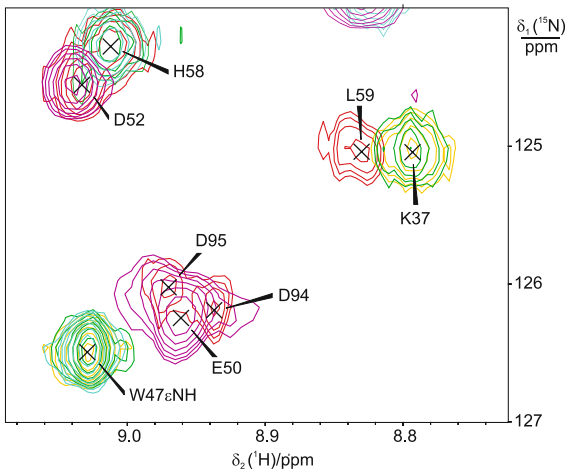

Figure 2. Spectral region selected from the ^{15}N-HSQC spectra of τ_C16 shown in Figure 1. All five spectra of τ_C16 were overlaid, with spectra 1–5 plotted with red, yellow, green, cyan and purple contour lines, respectively. The selective labelling scheme resolves some of the spectral overlap between the cross-peaks of Asp94, Asp95 (purple and red contours) and Glu50 (purple contours only).

resolved in other samples made with a different combination of ^{15}N-labelled amino acids. The cross-peaks of Asp94 and Asp95 (purple and red contours) can be distinguished from that of Glu50 (purple contours only). In the case of τ_C14 and τ_C16, no ^{15}N-HSQC cross-peaks were so badly overlapped that the assignments to amino-acid types would have been wrong or ambiguous. Only few signals were observed that did not seem to belong to the protein, including an apparent glycine peak in sample 3 and two glutamate peaks in sample 5 (circled in Figure 1). These additional peaks were identified as non-protein peaks by their narrower line shape and smaller intensities. The transaminase activity converting glutamate to glutamine did not lead to confusion between ^{15}N-Gln and ^{15}N-Met (Table 1), since all the ^{15}N-Met peaks consistently had much higher intensities in samples 2, 4 and 5 than any of the ^{15}N-Gln peaks.

The present combinatorially labelled samples of τ_C14 allowed us to correct an error in the amide assignment made from a uniformly ^{15}N/^{13}C-labelled sample (X.-C. Su and G. Otting, unpublished). The error involved a swap of the ^{15}N-frequencies of His6 and Glu7 (Figure 3a), where chemical shift degeneracies had resulted in ambiguities in the HNCACB/CBCA(CO)NH pair of 3D-NMR experiments.

Sequence-specific resonance assignments and structure of τ_C16

Availability of the residue-type identifications of the spectra allowed the confident evaluation of chemical shift changes of corresponding residues present in τ_C14 and τ_C16, even in those situations where cross-peaks moved to quite new positions in the NMR spectrum. Using the assumption that cross-peaks moved as little as possible between τ_C14 and τ_C16, the sequence-specific resonance assignments of τ_C14 were transferred to τ_C16. Figure 3b shows that the presence of the additional C-terminal segment in τ_C16 only affected the cross-peak positions of residues in the first and last helix of the structure of τ_C14. The amide chemical shifts of the 18 additional residues present in τ_C16 were characteristic of random-coil shifts and their signal intensities were significantly increased (Table 2), indicating increased mobility of the polypeptide chain towards the C-terminus (Figure 3c).

The combinatorially labelled protein samples were used to measure the $^3J(H^N, H^\alpha)$ coupling constants of τ_C16 with an experiment that encodes the coupling constant in different peak intensities observed in two ^{15}N-HMQC-type spectra (Ponstingl and Otting, 1998). Particularly accurate values could be measured for the intense peaks of the C-terminal residues. Most of the coupling constants measured for these residues were about 7 Hz, confirming the random-coil character of this peptide segment (Figure 3d). Increased coupling constants measured for Ile143 and Ile146 attested to the propensity of isoleucine for extended conformations. Notably, the $^3J(H^N, H^\alpha)$ couplings of the C-terminal segment were overestimated due to the multiplication factor of 1.1 used to compensate for differential relaxation between antiphase and in-phase magnetization (Ponstingl and Otting, 1998). This multiplication factor is appropriate for a rotational correlation time of more than 10 ns but not for highly mobile peptide segments.

In conclusion, all available data indicate that the C-terminal segment of τ_C16 is a random-coil peptide, and the small chemical shift changes observed for the N-terminal helix probably reflect non-specific transient interactions arising from the close proximity between the N- and C-terminal helices in the three-dimensional structure of τ_C14 (X.-C. Su and G. Otting, unpublished).

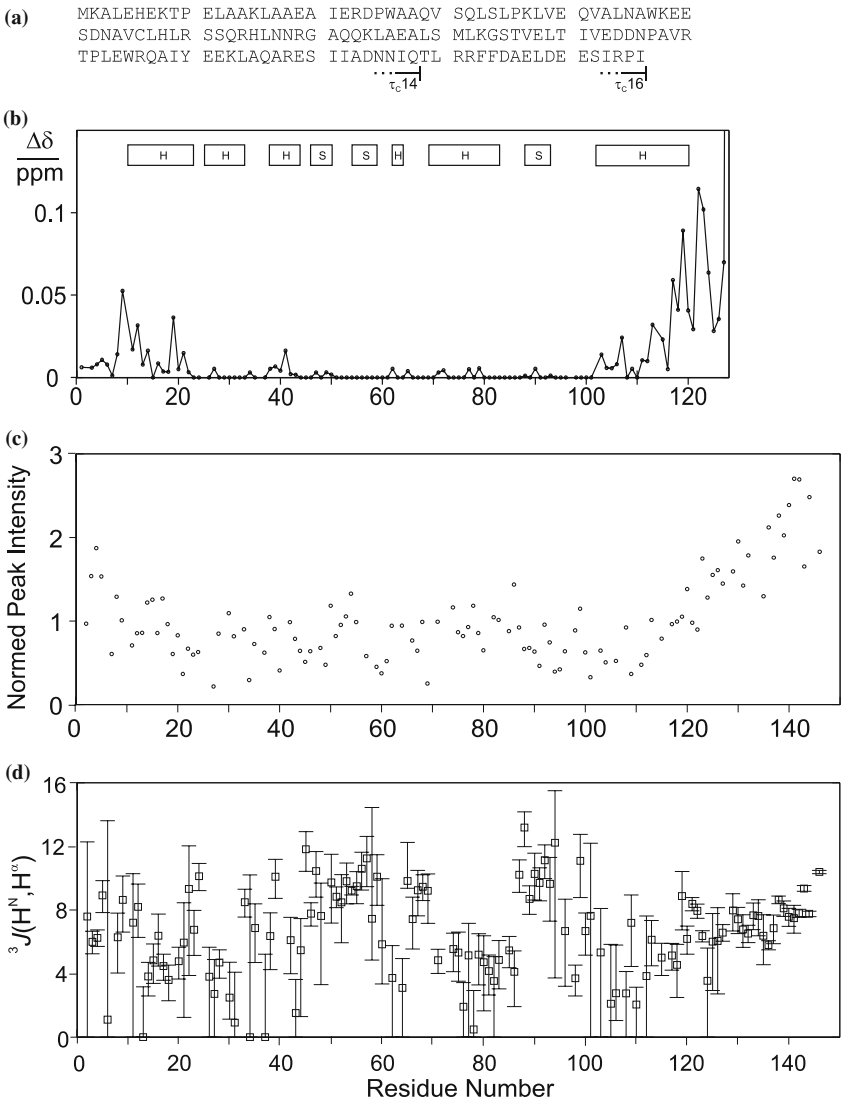

Figure 3. Amino-acid sequences of τ_C14 and τ_C16 and experimental structural information plotted against residue number. Lys2 of τ_C14 and τ_C16 corresponds to Lys499 in full-length τ. (a) Amino-acid sequences of τ_C14 and τ_C16. The two sequences differ only by the C-terminal 18 residues as indicated. In the present work, the residues were numbered Met1–Gln128 for τ_C14 and Met1–Ile146 for τ_C16. (b) Changes in chemical shifts of the N-terminal 128 residues of τ_C16 compared with the same residues in τ_C14. The vertical axis reports $[(\Delta\delta_H)^2 + (0.1\Delta\delta_N)^2]^{0.5}$. Bars indicate the location of helices (H) and β-strands (S) in the structure of τ_C14 (X.-C. Su and G. Otting, unpublished). (c) Intensities of ^{15}N-HSQC cross-peaks of the backbone amides of τ_C16. Different labelling efficiencies of different amino-acid types were accounted for by normalization of each of the peak intensities by the average for the corresponding amino-acid type. Data are shown only for residue types that occur at least five times in the amino-acid sequence and therefore allow adequate normalization. See Table 2 for the assignments of the C-terminal 18 residues used. (d) $^3J(H^N, H^\alpha)$ coupling constants measured from the CT-HMQC-HN experiment.

Discussion

Complete residue-type identification of the backbone amide cross-peaks of ^{15}N-HSQC spectra greatly facilitates sequence-specific resonance assignments. More important, it greatly enhances the assessment of chemical shift changes observed in crowded spectral regions upon titration with binding partners and in mutant or chemically modified proteins, as illustrated by the comparison between τ_C16 and τ_C14. Completeness of residue-type identification is of key importance for these applications.

Table 2. Assignment of C-terminal residues of τ_C16[a]

Residue	Chemical shifts (ppm)	
	$^1H^N$	^{15}N
Thr129	8.03	115.0
Leu130	8.07	123.7
Arg131	8.10	120.9
Arg132	8.19	121.0
Phe133	8.11	120.1
Phe134	8.09	119.8
Asp135	8.19	121.7
Ala136	8.13	124.0
Glu137	8.35	119.0
Leu138	8.03	121.9
Asp139	8.25	121.3
Glu140	8.40	121.6
Glu141	8.43	120.7
Ser142	8.15	116.0
Ile143	7.93	121.6
Arg144	8.23	126.4
Ile146	7.71	125.2

[a] Sequence-specific assignments for residues occurring more than once in the sequence are tentative and based on the assumption of increasing peak heights towards the C-terminus due to increased mobility (Figure 3c).

In vivo production, purification and measurement of at least 15 different samples, each containing just one of the common amino acids in ^{15}N-labelled form, has been achieved (Yamazaki et al., 1991), but it is both time-consuming and costly. Pulse sequences have been developed to achieve the selective observation of ^{15}N-HSQC-type cross-peaks from different amino-acid types in $^{15}N/^{13}C$-labelled proteins (Yamazaki et al., 1995; Schmieder et al., 1998; Schubert et al., 1999, 2001a, b, c, 2005). Other experiments were designed for the classification by groups of amino-acid types or by residue type of the preceding residue (Gehring and Guittet, 1995; Tashiro et al., 1995; Dötsch and Wagner, 1996). Most of these experiments suffer from poor sensitivity and are applicable only to small proteins for which backbone resonance assignments are anyway easy to obtain. With the advent of efficient cell-free synthesis systems that require only small amounts of ^{15}N-labelled amino acids and no chromatographic protein purification prior to NMR measurement (Guignard et al., 2002; Ozawa et al., 2004), the preparation of ^{15}N-labelled protein samples has become much more attractive. Combinatorial ^{15}N-labelling further shortens the sample preparation and analysis time. Although erroneous residue-type identifications could arise if two cross-peaks precisely overlap, not a single example of this occurred in the 146-residue protein τ_C16.

Previous combinatorial labelling schemes (Shortle 1994; Parker et al., 2004; Trbovic et al., 2005) did not attempt residue-type identifications for all 19 non-proline residues. In the most ambitious scheme (Parker et al., 2004), all residues are labelled with ^{15}N and pairs of sequential residues are identified using protein samples prepared with ^{15}N- and ^{13}C-labelled amino acids in different combinations and concentrations. As a drawback, spectral overlap is reduced only in the 2D-HNCO spectra required to identify the type of the previous residue and cross-peak intensities must be quantified in ^{15}N-HSQC spectra that are not simplified compared to spectra of uniformly labelled samples. Since multiple occurrences of amino-acid pairs are common in the amino-acid sequences of proteins, the scheme by Parker et al. (2004) is not sufficient for complete sequence-specific backbone assignments.

Although our combinatorial labelling scheme yields no information about the type of the preceding residue, it appears more attractive: (i) It minimizes the number of cross-peaks observed in each spectrum, thereby improving spectral resolution. (ii) It only relies on sensitive $^{15}N-^1H$ correlation spectra, making it more applicable for large proteins than schemes requiring 2D versions of 3D triple-resonance experiments (Parker et al., 2004; Shi et al., 2004; Trbovic et al., 2005). (iii) It uses the full cross-peak sensitivity since no partial labelling is required. (iv) It is highly cost-efficient, since it requires ^{15}N-labelled and unlabelled amino acids only. In the case of τ_C14, we obtained complete and correct sequence-specific assignments by combining the residue-type identification derived from combinatorial labelling with a single 3D-HNCA spectrum of a uniformly $^{15}N/^{13}C$-labelled sample (data not shown). Combinatorial labelling thus presents an efficient strategy to reduce the number of 3D NMR experiments usually recorded for backbone resonance assignments.

In order to minimize the number of cross-peaks observed in each sample, it may be worthwhile to optimize our labelling scheme for the amino-acid abundances in the individual protein of interest. This can be achieved by replacing the amino-acids in the left column of Table 1 with those in the

protein sorted in descending order of abundance, while maintaining the constraints discussed above (e.g. minimal overlap with side-chain NH_2 groups).

Using exclusively inexpensive ^{15}N-labelled amino-acids in the combinatorial labelling scheme and a total of 10 ^{15}N-HSQC spectra of τ_C14 and τ_C16, we could show in the present example that the C-terminal 18 residues of τ_C16 are highly mobile. This result is interesting, since these residues have been shown to be critical for the function of τ in binding to the α subunit of the *E. coli* DNA polymerase III (S. Jergic and N.E. Dixon, unpublished). The interaction with the α subunit may explain the observation that τ_C16 is highly cytotoxic towards *E. coli* cells, whereas τ_C14 exhibits no noticeable cytotoxicity (S. Jergic and N.E. Dixon, unpublished). In the cell-free protein production system, τ_C16 was produced with similar yields to τ_C14. The short measurement times of ^{15}N-HSQC spectra and the possibility to measure freshly synthesized samples were essential for the analysis of τ_C16, for which samples prepared *in vivo* had proven to be very sensitive to proteolysis.

The main remaining problems were associated with a transaminase activity that converted glutamate to glutamine, the observation of a couple of non-protein cross-peaks and the 2-fold reduced yield for samples prepared in the absence of a high concentration of glutamate in the reaction mixture. The possible confusion of glutamate with methionine residues can be avoided by modification of the labelling scheme of Table 1, e.g. adding ^{15}N-Met to sample 1 instead of sample 5. We have not previously observed the appearance of non-protein cross-peaks that were not completely removed by dialysis (Ozawa et al., 2004).

Even in the face of possible uncertainties due to side reactions or spectral overlap, the combination of cell-free protein synthesis with combinatorial ^{15}N-labelling presents a remarkably efficient strategy: only one day was required for sample preparation of τ_C14 and τ_C16, and one day for recording and analysis of the ^{15}N-HSQC spectra. In addition, the samples produced can be used for further measurements, for example of $^3J(H^N, H^\alpha)$ coupling constants.

In the absence of complete cross-peak overlap, classification of every ^{15}N-HSQC cross-peak by its residue type is equivalent to the spectral resolution achieved by 19 samples, where each is produced with a different ^{15}N-labelled non-proline amino acid. We anticipate that our combinatorial labelling scheme will be most useful for the identification of chemical shift changes induced in ^{15}N-HSQC spectra upon ligand binding (Emerson et al., 2003; Zartler et al., 2003) and for the analysis of modified proteins as demonstrated here.

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