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ARTICLE

Selective backbone labelling of ILV methyl labelled proteins

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Abstract Adding the ^{13}C labelled 2-keto-isovalerate and 2-oxobutanoate precursors to a minimal medium composed of ^{12}C labelled glucose instead of the commonly used (^{2}D, ^{13}C) glucose leads not only to the ^{13}C labelling of (I, L, V) methyls but also to the selective ^{13}C labelling of the backbone C_α and CO carbons of the Ile and Val residues. As a result, the backbone (^{1}H, ^{15}N) correlations of the Ile and Val residues and their next neighbours in the $(i + 1)$ position can be selectively identified in HN(CA) and HN(CO) planes. The availability of a selective HSQC spectrum corresponding to the sole amide resonances of the Ile and Val residues allows connecting them to their corresponding methyls by the intra-residue NOE effect, and should therefore be applicable to larger systems.

Keywords NMR spectroscopy · Assignment · Selective labelling · Methyl trosy

Abbreviations

TauF1	Residues 163-441 of the Tau protein
HMQC	Heteronuclear multiple quantum coherence
HSQC	Heteronuclear single quantum coherence
NMR	Nuclear magnetic resonance
NOESY	Nuclear Overhauser Effect spectroscopy
TOCSY	Total correlation spectroscopy

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Introduction

Because of their good sensitivity and long relaxation times, methyl groups are ideally suited NMR probes for the investigation of large protein complexes. Selective methyl protonation and transverse relaxation optimized spectroscopy methods for methyl groups have extended the applicability of NMR from amide-based TROSY (Pervushin et al. 1997) to large monomeric proteins such as 723 residues enzyme malate synthase G (MSG) (Tugarinov and Kay 2003) and even complexes as large as the 26S proteasome (Sprangers et al. 2007). However, whereas optimized NMR experiments have been developed to connect methyls to the backbone moiety for proteins in the 100 kDa range (Yang et al. 2004), this method is expected to be problematic for larger complexes characterized by at best incomplete backbone assignments and by important relaxation losses during the scalar transfer. Most recent applications (Sprangers and Kay 2007; Gelis et al. 2007) have avoided this problem by first assigning the methyls in the subunits of the complex and then transferring these assignments to the subunit embedded in the larger complex. Assuming the resonance position of the methyl does not substantially change between the isolated subunit and the subunit in the complex, the assignment can be transferred between both forms in a straightforward manner to the closest corresponding resonance. Then, inter-methyl NOE data can be used to confirm these preliminary assignments. A shortcoming of this approach can be expected when the structure is not conserved for the subunit between the isolated and complexed state. Such is particularly the case when one deals with natively unfolded proteins, where the bound conformation is adopted upon binding.

Faced by this problem in our study of the neuronal Tau protein, that participates in the tubulin assembly and binds

tightly to the stabilized microtubules (Butner and Kirschner 1991; Mandelkow et al. 1995; Goode et al. 1997; Chau et al. 1998; Sillen et al. 2007), we have explored the different possible labelling schemes that are mentioned in the literature. We thereby found that adding the ^{13}C labelled 2-keto-isovalerate and 2-oxobutanoate precursors (irrespective of their deuteration level) to a minimal medium composed of ^{12}C labelled glucose instead of the commonly used (^{2}D, ^{13}C) glucose leads to a selective ^{13}C labelling of the C_α and CO carbons of only the Ile and Val residues. As a result, the HN[CA] plane of such a sample contains only the backbone (^{1}H, ^{15}N) correlations of the Ile and Val residues and their next neighbours in the $(i + 1)$ position, whereas the HN[CO] plane only has the latter correlations. A similar labelling scheme was obtained by adding the individual ^{15}N, ^{13}C labelled Ile, Val and Leu amino-acids to the culture medium (Tugarinov et al. 2003), but deuteration of these amino-acids themselves would have required their prior synthesis. Our labelling scheme leads to full or partial deuteration of the Ile or Val, depending on the choice of the deuteration pattern of the precursor. However, it introduces ^{13}C at the C_β position of only the Val residues, thereby distinguishing between both residue types. The availability of a reduced HSQC spectrum corresponding to the sole resonances of the Ile and Val residues led us to investigate whether this could help to connect the methyls towards their backbone amide by intra-residue NOEs between the methyl and its amide proton resonance. A crucial requirement evidently is that the amide spectrum still can be acquired by some method, excluding thereby too large complexes devoid of residual mobility. At this condition, the Nuclear Overhauser Effect (NOE) being more efficient with increasing protein size, the experiment is expected to be complementary to existing approaches based on scalar couplings (Tugarinov et al. 2003) for a large complex such as Tau bound to tubulin. As a preliminary, we queried a database of high-resolution protein structures to evaluate the intra-residue methyl-amide proton distances, and compared them with the same inter-residue distances. Although the intra-residue distance indeed is the shortest for many of the ILV residues, an unambiguous connection of methyl and proton resonances on this sole basis is dangerous. Combined with the unambiguous assignment of the Ile and Val amide correlations through the labelling scheme proposed, however, possible ambiguities are relieved, and methyl and amide moieties of the same residue can be connected through the NOE effect. Because the methyl degeneracy in Tau free in solution is complete, and because the NOE is very weak in this very flexible protein, we experimentally demonstrate the feasibility of the assignment protocol on the well-folded Cyclophilin B protein (CypB) produced with the same selective labelling scheme.

Materials and methods

Sample preparation

Specifically [ILV-$^{1}H_3$-^{13}C, ^{2}H, U-^{15}N] labelled TauF1 and CypB samples

Proteins with specific isoleucine, valine, leucine residues which are $^{13}CH_3$-labelled at only one single methyl position, with the other methyl group $^{12}CD_3$-labelled, were obtained by adding 2-keto-isovalerate [2-Keto-3-(methyl-d3)-butyric acid-1,2,3,4-$^{13}C_4$,3-d_1, sodium salt] and 2-oxobutanoate [2-Ketobutyric acid-$^{13}C_4$,3,3-d_2, sodium salt hydrate] precursors to a uniformly ^{15}N labelled M9 medium into D_2O with non ^{13}C labelled D-Glucose-d_7. Recombinant fragment F1 of human Tau, constituted of residues 163 to 441 of Tau441 (named TauF1; Tau441 for 441 residues) TauF1 and Cyclophilin B of 184 residues (CypB) were expressed and purified as described previously (Lippens et al. 2004; Hanoulle et al. 2007).

NMR spectroscopy

NMR samples were prepared in 25 mM Tris-d_{11} buffer at pH 6.8, 50 mM NaCl, 2.5 mM EDTA, 5 mM DTT, 1 mM D_4-TMSP (TriMethyl Silyl Propionate), 5% D_2O. All NMR spectra were recorded at 25°C on a Bruker Avance 600 spectrometer equipped with a triple resonance cryogenic probehead and on a Bruker AvanceII 800 with a regular triple resonance probehead (Bruker, Karlsruhe, Germany).

Measurements with TauF1 were done on a 50 μM specifically [ILV-$^{1}H_3$-^{13}C, ^{2}H, U-^{15}N] labelled TauF1 sample. 1D carbon spectra were acquired with the UDEFT sequence with NOE enhancement (Piotto et al. 2006) with 1,024 scans, a 3 s recycling delay and a spectral width of 300 ppm. The amide assignment of ILV residues and their preceding residues were deduced from 2D HN[CA] and HN[CO] type spectra (Grzesiek and Bax 1992) acquired with FID resolutions of 4.1 Hz ($F_3 = H^N$), 21.7 Hz ($F_1 = {}^{15}N$), 18.1 Hz ($F_2 = C_\alpha$) and 11.8 Hz ($F_2 = CO$). The H[N]CACB spectrum was recorded with a minimal window of 17 ppm centred at 56.1 ppm in the ^{13}C F_1 direction, leading to a folded-in Val C_β region completely separated from the C_α resonances of both Val and Ile. ^{13}C FID resolution was 18.8 Hz, whereas ^{1}H and ^{15}N parameters were as for the previous experiments.

Measurements on specifically [ILV-$^{1}H_3$-^{13}C, ^{2}H, U-^{15}N] labelled CypB sample

For the methyl assignment, 400 μM of CypB was used with the same type of labelling described for TauF1.

^{1}H-^{13}C heteronuclear correlation spectra (^{1}H-^{13}C HMQC) were acquired using sensitivity improvement (Palmer et al. 1991). NMR spectra were recorded with 8 scans per increment, and 64 complex points in the ^{13}C dimension. The 3D $^{1}H^{methyl}$ and $^{13}C^{methyl}$ resolved HSQC-NOESY-^{1}H-^{15}N HSQC spectra (HNH-NOESY and CNH-NOESY) (Diercks et al. 1999) were acquired at 600 MHz with a mixing time of 400 ms. The $^{1}H^{methyl}$ edited and $^{13}C^{methyl}$ edited spectra were recorded with [64, 64, 2048] complex points in the [$F_1 = {}^{13}C^{methyl}$, $F_2 = {}^{15}N$, $F_3 = {}^{1}H_N$] dimensions and with [32, 64, 2048] complex points in the [$F_1 = {}^{1}H^{methyl}$, $F_2 = {}^{15}N$, $F_3 = {}^{1}H_N$] dimensions. Experiments were recorded without constant-time period during t_1 to avoid signal loss. Although the ^{13}C labelling of the carbon adjacent to the ^{13}C labelled methyl resonance leads to a splitting of this resonance due to the ^{13}C–^{13}C scalar coupling in the case of a mid-size protein as CypB, such would not be the case for larger proteins, for which we expect significant sensitivity losses when applying constant-time evolution.

Data processing

Spectra were processed using Bruker TOPSPIN 1.3. 3D spectra with 2,048 points in the ^{1}H dimension and a 45° shifted sine squared bell window function, and 512 complex points in both indirect dimensions with a cosine squared bell window function and a forward-backward linear prediction was used in order to double the size of the time domain in the ^{15}N F2 dimension. 2D spectra were processed with 2,048 complex points in the ^{1}H dimension and 1,024 complex points in the ^{13}C dimension.

Statistical analysis

Starting from the 78 structures in the Talos database (Cornilescu et al. 1999), we selected for every Ile residue (total = 653 Ile residues) all amide protons in a sphere of radius 8 Å around its C_δ methyl carbon, and calculated the distance from these amide protons to the methyl C_δ. If the shortest distance corresponded to the amide proton of the Ile itself, the value of the "shortest intra-residue distance" category was incremented by 1. The same was done individually for the $C_{\gamma 1}$ and $C_{\gamma 2}$ carbons of the 808 Val residues, and for the $C_{\delta 1}$ and $C_{\delta 2}$ of the 955 Leu residues in the database.

Results and discussion

For larger proteins, uniform deuteration efficiently reduces transverse relaxation (Goto and Kay 2000; LeMaster 1988), but the need to extract structural information to reconstruct the tridimensional structure of a large protein has led to the idea of selectively reintroducing protons into the core of high-level deuterated proteins. Methyl containing side chains, often participating in the hydrophobic core of proteins and being NMR sensitive due to their three attached protons, were found to be good candidates for this approach (Gardner and Kay 1998; Goto et al. 1999; Rosen et al. 1996; Tugarinov et al. 2005). Whereas early attempts used the labelled amino-acids as precursors, the scrambling of the isotope label into other amino-acids through metabolic pathways within the expression host organism generally restricts this method. The methodology with 2-oxobutanoate or 2-keto-isovalerate as precursors (Goto et al. 1999) only leads to Leu/Val or Ile labelling without any scrambling (Figs. 1, 2), and has therefore found wide spread. The resulting methyl-methyl NOEs (Van Melckebeke et al. 2004) or proton-proton dipolar couplings (Sibille et al. 2002) were shown to lead to useful structural information.

^{2}D, ^{13}C labelling of the ILV residues in a ^{12}C background

The use of deuterated U-^{13}C labelled precursors with protons on one single methyl (Fig. 1) together with (^{2}D, ^{13}C) glucose and $^{15}NH_4Cl$ as the sole carbon and nitrogen sources leads to protein samples that are devoid of protons except for the amide positions (after back-exchange against

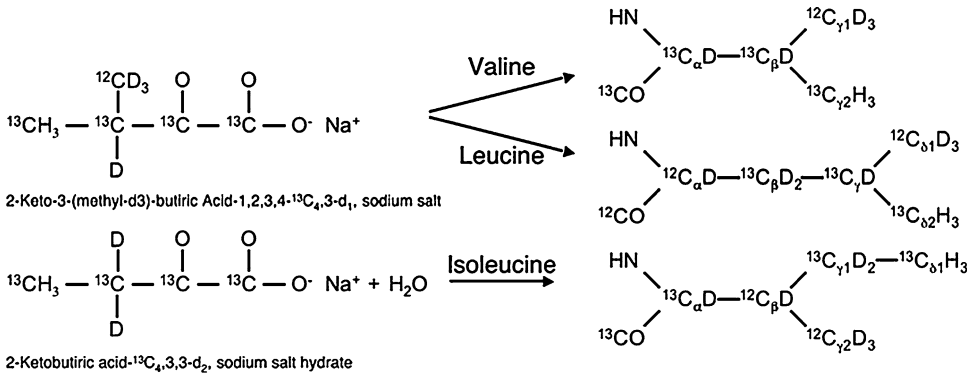

Fig. 1 Labelling profile obtained with (^{2}D, ^{12}C) labelled glucose and the 2-keto-isovalerate and 2-oxobutanoate precursors. The first precursor leads to a single ^{13}C protonated methyl in Leu and Val residues (statistically distributed over the two methyl positions), whereas the second one yields 100% of isoleucine $^{13}C_{\delta 1}$

an aqueous buffer) and for one single methyl for each Ile, Val or Leu residue. As both the specific precursors and the other prevailing carbon source (most commonly glucose) are totally enriched in the ^{13}C stable isotope, this procedure very efficiently leads to a uniformly ^{13}C labelled protein, although the ^{13}C label has different origins for different residues and even for different atoms in a given residue. Indeed, when considering the biosynthesis of valine starting from the 2-keto-isovalerate (Neidhardt et al. 1996), the precursor is in one single reaction step transformed by the branched chain amino-acid aminotransferase ilvE (E.C. 2.6.1.42) into the valine amino-acid, thereby conserving the complete labelling pattern of the precursor. For the biosynthesis of leucine, however, the 2-keto-isovalerate precursor is first transformed by the 2-isopropylmalate synthase leuA (E.C. 2.3.3.13) into 2-isopropylmalate. In this additional step, the acetyl CoA will contribute two carbons at the C_α and CO positions of the final leucine, implying that the labelling pattern of the precursor will not conserved. Because the acetyl-CoA is synthesized starting from the glucose as carbon source, the leucine residues of a protein produced in a (^2D, ^{12}C) supplemented minimal medium in D_2O will carry the labelling pattern of the 2-keto-isovalerate in their side chain, but will not be ^{13}C labelled as for their backbone carbons (Fig. 1). The isoleucine biosynthesis form 2-oxobutanoate is even more complex, as both its final C_β and $C_{\gamma 1}$ carbon come from the pyruvate synthesized by the bacteria, whereas both the backbone C_α and CO do come from the precursor, and thus carry its labelling signature. As a result, Ile residues will be $^{13}C_\alpha$ and ^{13}CO labelled but $^{12}C_\beta$ when grown in the ^{12}C glucose supplemented minimal medium (Fig. 1). With the fully ^{13}C labelled precursors developed for use with ^{13}C labelled glucose, though, one should also consider that the carbon atom adjacent to the targeted methyl will always be ^{13}C labelled, even in our proposed labelling scheme with (^2D, ^{12}C) glucose as alternative source of carbon.

Experimentally, we first prepared a Tau fragment (TauF1, residues 165-441) by over-expression in a minimal medium composed of $^{15}NH_4Cl$ and (^2D, ^{12}C) labelled glucose in D_2O as main nitrogen and carbon sources, respectively. The medium was supplemented with uniformly ^{13}C labelled 2-ketoisovalerate and 2-oxobutanoate precursors (Goto et al. 1999), whereby the latter were only protonated at their methyl positions (see Materials and methods). The absence of methyl correlations at proton frequencies other than those of Ile, Leu and Val in the ^1H-^{13}C HMQC spectrum (data not shown) confirmed the absence of leakage of the precursors in other methyl containing residues. In order to analyze the deuterium pattern, we recorded a 1D-^{13}C NMR spectra while decoupling ^1H and ^2H nuclei (Fig. 2a). When the {^1H} decoupling was off (Fig. 2b), the signal of carbons that are attached to a proton

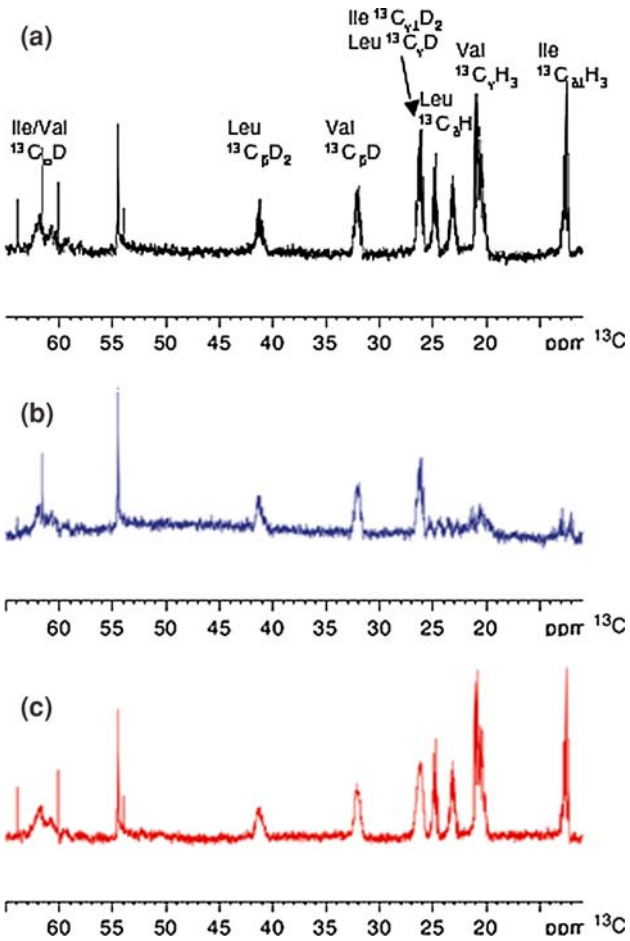

Fig. 2 **a** TauF1 1D-^{13}C spectrum {^1H, ^2H} decoupled **b** {^2H} decoupled and **c** {^1H} decoupled. C_α (55.1 ppm) of leucines, C_β (38.8 ppm) and $C_{\gamma 2}$ (17.4 ppm) of isoleucines are absent of this spectrum because they are not ^{13}C labelled

are expected to split due to the proton coupling. The experimental observation of this splitting confirmed the protonation state of the methyls of leucine and valine, and of the $C_{\delta 1}$ of isoleucine. On the contrary, when the {^2H} decoupling is off (Fig. 2c), the signals of carbons that are attached to a deuteron broaden, although the effect is lesser due to the reduced $^1J(^2D, ^{13}C)$ coupling constant compared to the $^1J(^1H, ^{13}C)$ one. C_γ and C_β carbons of leucine, C_β and C_α of valine and $C_{\gamma 1}$ and C_α of isoleucine were all broadened, confirming their deuterium labelling. No signals corresponding to the C_α of leucine or the C_β and $C_{\gamma 2}$ of isoleucine were detected, in agreement with the observation that these carbons come from the glucose source and not from the ^{13}C labelled precursors (Fig. 1).

^1H, ^{15}N subspectra of the ILV ^{13}C labelled protein

In order to obtain a selectively labelled Tau sample for the assignment of the protein free in solution, we decided to grow the overexpressing bacteria in a D_2O buffer with

(^{2}D, ^{12}C) glucose and (^{2}D, ^{13}C) labelled 2-keto-isovalerate and 2-oxobutanoate precursors (Goto et al. 1999), as described before. Because the proteins were produced in bacteria grown on a minimal medium with $^{15}NH_4Cl$ as sole nitrogen medium, the ^{1}H-^{15}N HSQC spectrum is identical to that of a ^{15}N only labelled protein. However, as no leakage of the ^{13}C labelled precursors into residues other than ILV occurs, and as the leucine biosynthetic pathway is such that its C_α and CO position do not find their origin in these precursors, the resulting sample has only backbone ^{13}C labelling for the Val and Ile residues. The HN[CA] plane therefore contains the backbone resonances of those two residue types, but equally the amide correlation peaks of those residues that follow a Val or Ile. Whereas "intra-residue-only" HNCA experiments have been developed to distinguish those intra- and inter-residue correlations (Brutscher 2002), a simpler experiment for our selectively labelled sample is to compare the HN[CA] plane with the corresponding HN[CO] plane. This latter plane contains only the residues that follow an Ile or Val residue, and allows therefore by a simple comparison to distinguish both kind of peaks in an (I, V) specific HSQC. Val and Ile residues can further be distinguished either by giving a single precursor during the overexpression, or by the absence of a ^{13}C labelled C_β carbon in the latter. Therefore, an H[N]CACB experiment (Fig. 3a) immediately indicates those amide resonances that lack a C_β correlation, and therefore are necessarily Ile residues. The limited spectral spread of the methyl and carbonyl carbons in the natively unfolded Tau (Lippens et al. 2004) allows for a very high resolution with a small number of points in the latter experiment, allowing to connect peaks (blue only and red peaks, Fig. 3b) as (Val, X) or (Ile, Y) pairs. Identification of one single residue of these pairs based on a uniformly (^{15}N, ^{13}C) labelled sample directly thereby gives the two assignments. Such a pairwise assigned spectrum for a fragment of Tau is shown in Fig. 3b. We found that interaction studies such as with heparin (Sibille et al. 2006) greatly benefited from this pairwise assignment, as the neighbouring residue provides an internal control on a putative chemical shift variation.

Side chain assignment methods using NOE based experiments

In our labelling scheme, magnetization transfer via scalar couplings from the methyl to the backbone nuclei can only work for Val, as the Ile and Leu residues end up without

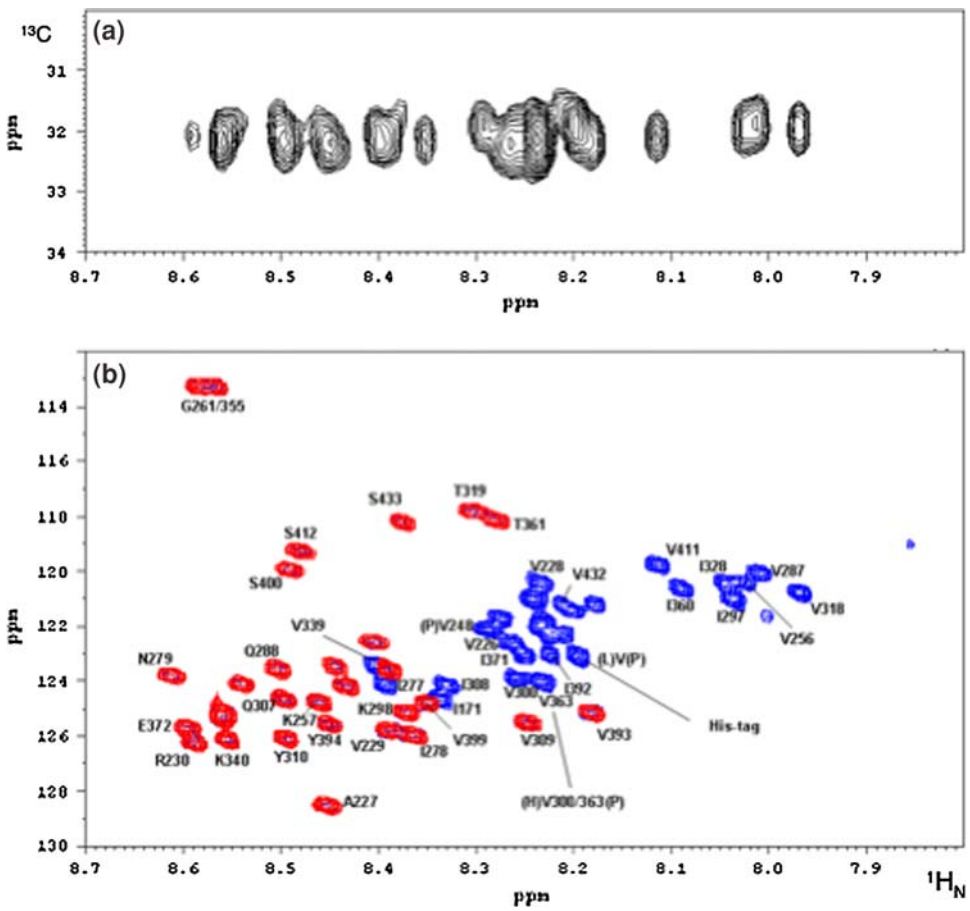

Fig. 3 Assignment of (I, V) residues and their next neighbours of the (I, L, V) labelled TauF1. **a** The H[NCA]CB plane around the C_β of valine allows to distinguish Val from Ile **b** HN[CA] plane (*blue*) and HN[CO] (*red*) of ILV labelled TauF1. The HN[CA] plane contains correlations of all Ile and Val residues, but equally of all residues that follow an Ile or Val. The HN[CO] plane only contains the latter

^{13}C label in their respective C_β and C_α positions. NOESY experiments with shorter mixing time in order to select the stronger intra-residue cross-peaks have previously been proposed to connect methyls and their corresponding amide resonances (Mueller et al. 2003), but a sample labelled with a mixture of uniformly deuterated amino-acids and uniformly $^{13}C/^{15}N$ amino-acids was necessary to interpret the NOESY peak intensities. Proton NOE based transfer from the methyl protons towards the amide proton as an alternative transfer method indeed is hampered by the possibility that a given methyl can be close to more than one amide proton. Although knowledge of the subset of Ile/Val amide resonances from the intersection of the HN[CA] and HN[CO] planes on the selectively labelled sample alleviates this problem, such is not the case for the Leu residues, and dipeptides composed of a combination of only Ile or Val residues equally might be misinterpreted as resonances of residues following an Ile or Val. Still, because especially for larger complexes, this method should complement the side chain to amide resonance connection by scalar coupling, we investigated further its applicability in the framework of the proposed selective labelling.

We first performed a statistical test on a representative set of high-resolution protein structures to validate the assumption that the intra-residue (H_N, C^{methyl}) distance indeed is the shortest one and gives the strongest NOE effect. Then, we tested experimentally the NOE based assignment on a folded protein, in this case CypB, because TauF1 as a largely unstructured protein does not only exhibit a very weak NOE effect, but moreover has all its methyl resonances perfectly superimposed in the ^1H-^{13}C HSQC spectrum.

Statistics

On the basis of the CypB crystal structure, we could see that the majority of the intra-residue (H_N, C^{methyl}) distances are shorter than any inter-residue counterpart. To confirm this on a larger data set, we extracted the same distances from 78 tridimensional protein structures coming from the Talos database (Cornilescu et al. 1999). When we report the histogram of intra-residue or inter-residue (H_N, C^{methyl}) distances as a function of distance (Fig. 4), the distribution of intra-residue distances samples generally shorter values than the inter-residue distances. A more detailed analysis shows that this translates into a 77.5% probability for an Ile C_δ to have its own amide proton closer than any other amide proton. For valine residues, taking into account the fact that the two C_γ carbons are labelled with an equal probability, the same calculus led to values of only 32.9% for the Val $C_{\gamma 1}$ but 92.1% for the Val $C_{\gamma 2}$. As a consequence, for a valine residue, the strongest NOE between its

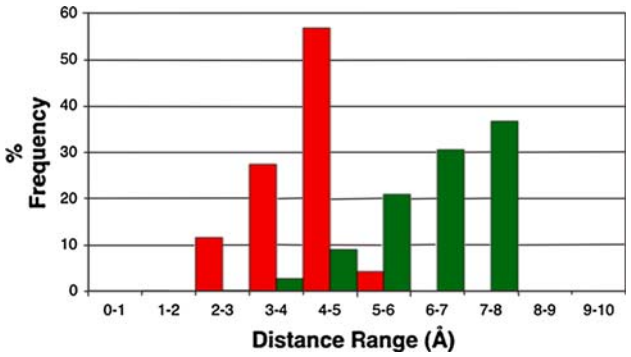

Fig. 4 Histogram of intra (*red*) and extra (*green*) distances between an amide proton and a methyl carbon. For the inter-residue distances, only the shortest of all inter-residue distances for a given methyl is taken into account

methyl (without stereospecific assignment) and an amide resonance only indicates with high certainty that both belong to the same residue if the $C_{\gamma 2}$ was chosen. For leucine residues, the probability for both $C_{\delta 1}$ and $C_{\delta 2}$ to have their intra-residue H_N proton as the closest amide is similar and close to 66%.

Our statistical analysis hence confirms that the shortest (^1H_N, $^1H^{methyl}$) distance or the most intense NOE peak in an (^1H_N, $^1H^{methyl}$)-NOESY experiment comes in a majority of the cases from the intra-residue correlation between an amide and methyl of the same residue, but equally cautions against the general use of the NOE effect to connect methyl and amide protons by NOE. As will be shown below, however, the fact that we do know the Val and Ile amide resonances in the ILV labelled samples as described here, will mean that we will only have to select the strongest NOE within this subset of amide correlations, and will thereby greatly enhance the predictive power of the observed NOEs.

Experimental

In order to experimentally connect the limited number of ILV methyl cross peaks to their corresponding amide resonances by NOE, we recorded two 3D experiments, a $^1H^{methyl}$-NOESY-NH_N and $^{13}C^{methyl}$-NOESY -NH_N on the ILV labelled CypB sample. Evolution in the first period involves the $^{13}C^{methyl}$ or $^1H^{methyl}$ magnetization, respectively, although it always is the latter proton magnetization that is transferred via dipolar coupling to the other protons during the 400 ms mixing time. The resulting spectra can be analysed in two different manners. First, one can start from the amide resonances previously assigned to Ile or Val residues, and extract the strips from both the $^1H^{methyl}$-NOESY-NH_N and $^{13}C^{methyl}$-NOESY-NH_N experiments. An example is shown with the strips extracted at the (H_N, N) chemical shift of Ile70. Three pairs of (^1H, $^{13}C)^{methyl}$

chemical shifts can be detected, but the relative intensity in both strips is a good indicator for the peak matching, as they all stem from the same NOE transfer. ^{1}H and ^{13}C chemical shifts are matched on the corresponding ^{1}H-^{13}C HMQC spectrum to see whether the two frequencies combine into an existing methyl peak. Alternatively, in a 4D $^{1}H^{methyl}$ and $^{13}C^{methyl}$ resolved HSQC-NOESY-^{1}H-^{15}N HSQC experiment, one could directly identify methyls by their combined ^{1}H and ^{13}C chemical shift. When we extract the 3 shortest distances for the Ile70 methyl from the X-ray structure of CypB (Mikol et al. 1994), PDB code 1CYN), we find: Ile70 d($^{1}H_N$, $^{13}C_{\delta 1}$) = 4.29 Å; Val147 d($^{1}H_N$, $^{13}C_{\gamma 1}$) = 5.69 Å; Ile122 d($^{1}H_N$, $^{13}C_{\delta 1}$) = 5.70 Å. Based on the assumption that the intra-residue NOE between the methyl and backbone protons is the strongest, we can assign (Fig. 5a) in both strips the most intense peak to the proton and carbon frequencies of the Ile70 methyl. Combining these two ^{1}H and ^{13}C values on the HMQC spectrum of CypB confirms the existence of a methyl with both values, and assigns it to Ile70. For the weaker peaks, we equally verified that they could be combined into an existing peak on the HMQC spectrum, but further assignment without structural knowledge was not possible at this moment. Starting from the backbone assignment of CypB (Hanoulle et al. 2007), and immediately limiting us to the amide peaks of (Ile, Val) pairs from the backbone selective labelling, this NOE based assignment allowed us to connect seven out of 10 isoleucines and 16 out of 19 valine methyls to their corresponding amide peak. For leucine, though, as we do not have prior knowledge on its backbone resonances, we were dependent on the prior assignment of leucine amide correlations. For this residue type, we identified five out of seven leucines, keeping in mind that for leucine and valine there are two methyls and they were not stereospecifically assigned. Indeed, the ^{1}H-^{13}C HMQC (Fig. 5) cross peak intensities confirmed the absence of stereospecific integration of ^{13}C labelled methyls at the valine C_γ and leucine C_δ positions.

An alternative manner to connect methyls and amide resonances is to start from the ^{1}H-^{13}C HMQC spectrum rather than from the ^{1}H-^{15}N HSQC. A given peak thereby defines the two values of the ^{1}H and ^{13}C chemical shift that

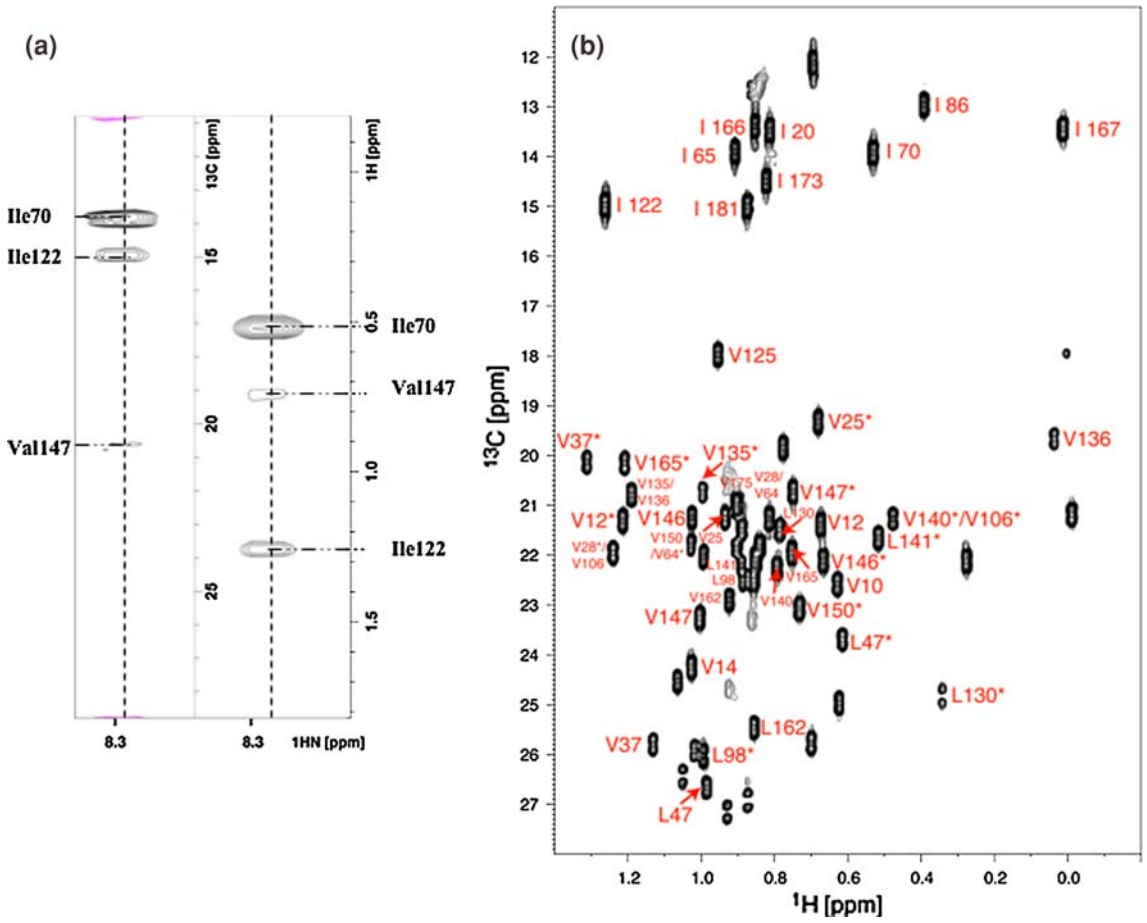

Fig. 5 **a** Strips of $^{13}C^{methyl}$ HSQC-NOESY-^{1}H-^{15}N HSQC and $^{1}H^{methyl}$ HSQC-NOESY-^{1}H-^{15}N HSQC spectra at the ($^{1}H_N$,^{15}N) of Ile70, and **b** annotated ^{1}H-^{13}C HMQC of the specifically [ILV-$^{1}H_3$-^{13}C, ^{2}H, U-^{15}N] labelled CypB sample. One of the two methyls of Leu or Val is (arbitrarily) labelled with an *asterisk*

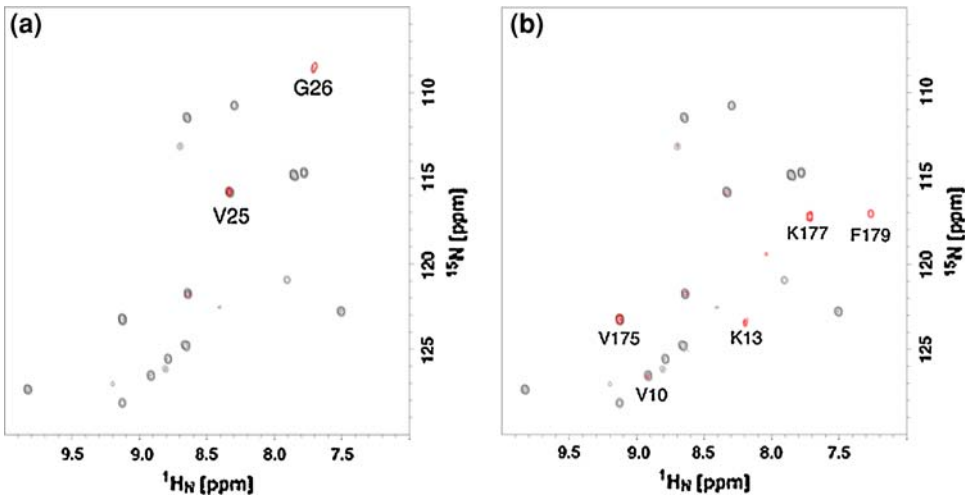

Fig. 6 Superimposition of the product (H_N, N) plane resulting from the product of the planes at the $^{13}C^{methyl}$ and $^{1}H^{methyl}$ chemical shift of **a** one of the Val25 methyl resonances (*red*) with the (Ile, Val) specific HSQC spectrum (*black*), or **b** the Val175 $C_{\gamma 1}$ methyl with the same (Ile, Val) specific HSQC spectrum (*black*)

characterize a given methyl. Extracting the two (H_N, N) planes from respectively the $^{1}H^{methyl}$ resolved HSQC-NOESY-^{1}H-^{15}N HSQC and the $^{13}C^{methyl}$ resolved HSQC-NOESY-^{1}H-^{15}N HSQC and multiplying both planes in a point-per-point manner, we obtain a resulting spectrum that contains only (H_N, N) cross peaks at those positions where the two extracted planes simultaneously had intensity (Fig. 6). We previously described this method of product lanes for the complete backbone assignment of both folded and unfolded proteins (Verdegem et al. 2008), but found here that it is equally efficient for connecting methyls and amide resonances. For the Ile and Val methyls, moreover, we can superimpose the calculated product spectrum onto the (I, V) selective plane rather than the regular HSQC, limiting ourselves thereby to intra-residue type cross peaks. As an example, we extract the (H_N, N) planes of the $^{13}C^{methyl}$ HSQC-NOESY-^{1}H-^{15}N HSQC spectrum at the $^{13}C^{methyl}$ chemical shift of one of the Val25 methyls, and equally the (H_N, N) planes of the $^{1}H^{methyl}$ HSQC-NOESY-^{1}H-^{15}N HSQC spectrum at the $^{1}H^{methyl}$ chemical shift of the same methyl. Point-by-point multiplication of both planes leads to the product plane with residue intensity at only two positions (Fig. 6, red spectrum). Based on the prevalence of intra-residue contacts over inter-residue ones, the most intense cross peak at (8.33, 115.7 ppm) is the better candidate for the amide corresponding to this methyl. Superposing the product plane on the (I, V) selective HSQC from the comparison of HN[CA] and HN[CO] spectra (Fig. 6, black spectrum), immediately confirms that indeed only the (8.33, 115.7 ppm) corresponds to an Ile or Val residue, excluding the other correlation peak at (7.70, 108.6 ppm) as a potential candidate for the amide peak corresponding to the chosen methyl. As a second example, we show the product plane of both planes extracted at the (^{1}H, ^{13}C)^{methyl} frequencies of the $C_{\gamma 1}$ methyl of Val175. In this case, this methyl is closer to the Lys177 amide proton, and the resulting NOE accordingly shows up as a stronger correlation in the resulting NOE product plane. Nevertheless, the criterion of NOE intensity only applies to those amide peaks previously identified as (I, V) amide correlations from the selective backbone labelling; the intra-residue cross-peak will be the strongest NOE towards an (I, V) amide peak. Only with a leucine methyl as starting peak, no selective amide correlation is expected to come out. This, however, can be used as an indication that indeed the methyl does not belong to an Ile or Val residue.

The labelling scheme proposed here can thus be used successfully to produce selectively backbone labelled samples independent of the size of the protein. The NOE based scheme connecting the amide resonance to its methyl was here demonstrated on a medium sized protein, and we are currently investigating how well it will perform in the context of a larger complex.

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