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Stereospecific assignments of the isopropyl methyl groups of the membrane protein OmpX in DHPC micelles

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Abstract

In NMR studies of large molecular structures, the number of conformational constraints based on NOE measurements is typically limited due to the need for partial deuteration. As a consequence, when using selective protonation of peripheral methyl groups on a perdeuterated background, stereospecific assignments of the diastereotopic methyl groups of Val and Leu can have a particularly large impact on the quality of the NMR structure determination. For example, 3D ^{15}N- and ^{13}C-resolved [^{1}H,^{1}H]-NOESY spectra of the *E. Coli* membrane protein OmpX in mixed micelles with DHPC, which have an overall molecular weight of about 60 kDa, showed that about 50% of all obtainable NOEs involve the diastereotopic methyl groups of Val and Leu. In this paper, we used biosynthetically-directed fractional ^{13}C labeling of OmpX and [^{13}C,^{1}H]-HSQC spectroscopy to obtain stereospecific methyl assignments of Val and Leu in OmpX/DHPC. For practical purposes it is of interest that this data could be obtained without use of a deuterated background, and that combinations of NMR experiments have been found for obtaining the desired information either at a ^{1}H frequency of 500 MHz, or with significantly reduced measuring time on a high-frequency instrument.

Abbreviations: 2D – two-dimensional; 3D – three-dimensional; *ct* – constant time; DHPC – dihexanoylphosphatidylcholine (1,2-dihexanoyl-*sn*-glycero-3-phosphocholine); HSQC – heteronuclear single quantum coherence; NOE – nuclear Overhauser enhancement; NOESY – NOE spectroscopy; OmpX – outer membrane protein X from *Escherichia coli*; TROSY – transverse relaxation-optimized spectroscopy.

Introduction

NMR studies with large molecules often require extensive deuteration to decrease transverse relaxation rates (Gardner and Kay, 1998; LeMaster, 1994; Wider and Wüthrich, 1999), which then limits the number of accessible ^{1}H–^{1}H-NOE distance constraints. In recent investigations of the membrane protein OmpX reconstituted in DHPC micelles with a total molecular weight of about 60 kDa, the number of ^{1}H–^{1}H-NOEs was increased by selective protonation of the Val-$\gamma^{1,2}$, Leu-$\delta^{1,2}$ and Ile-δ^{1} methyl groups in the otherwise uniformly ^{2}H,^{15}N,^{13}C-labeled protein ([u-^{2}H,^{13}C,^{15}N/L,V,Iδ^{1}-^{13}CH$_3$]-OmpX). Sequence-specific assignments of all Val-$\gamma^{1,2}$, Leu-$\delta^{1,2}$ and Ile-δ^{1} isopropyl groups have previously been obtained using H(C)(CC)-TOCSY-(CO)-[^{15}N,^{1}H]-TROSY experiments (Hilty et al., 2002). Different chemical shifts were observed for the pairs of diastereotopic methyls (Wüthrich, 1986), but because of the overall scarcity of NOE constraints, their stereospecific assignment could not be determined during the structure calculation.

This paper describes the determination of stereospecific assignments for the Val and Leu methyl groups in OmpX/DHPC with the use of biosynthetically-directed fractional ^{13}C-labeling (Neri et al., 1989; Senn et al., 1989). The protein was labeled

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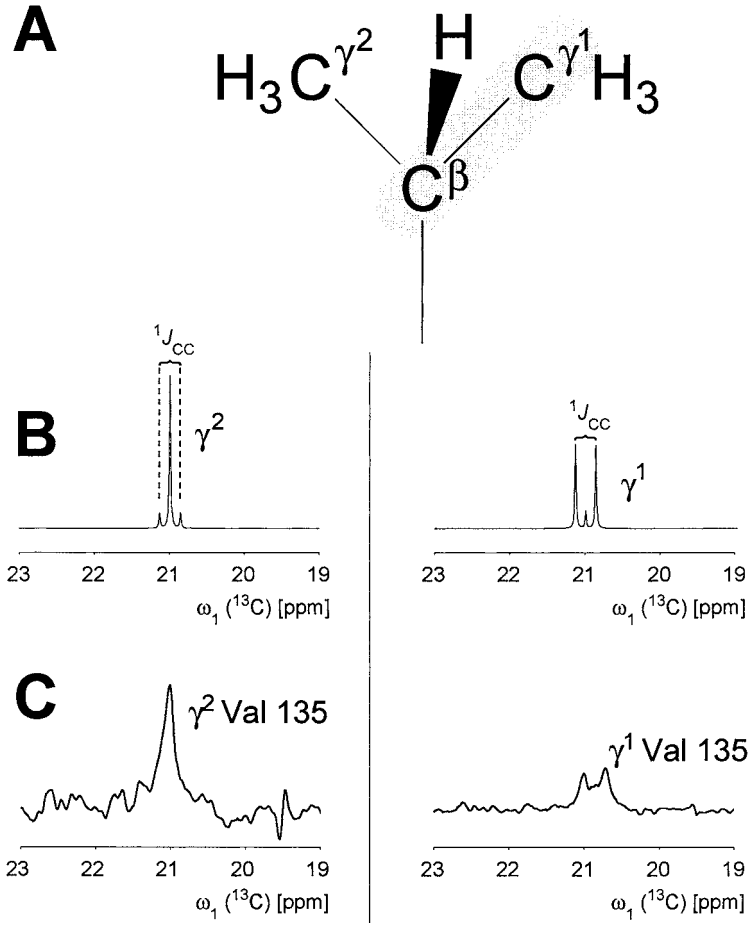

Figure 1. (A) Chemical structure of the valine side-chain. The shaded C–C fragment originates from the same glucose source molecule, i.e., in most product molecules these carbon atoms are either both ^{12}C or both ^{13}C. (B) Simulated cross sections along the $\omega_1(^{13}C)$ dimension of a [^{13}C,^{1}H]-HSQC spectrum for the valine γ^1 and γ^2 isopropyl methyl groups, assuming 10% ^{13}C labeling. The minor components have an intensity of approximately 10%, which accounts for contributions from non-standard biosynthetic pathways in addition to the intensity expected from the fractional labeling (Szyperski et al., 1992). A ^{1}H frequency of 500 MHz and a ^{13}C–^{13}C coupling constant, $^{1}J_{CC}$, of 35 Hz were used. (C) Experimental cross-sections through the resonances of the γ^1 and γ^2 isopropyl methyls of Val 135 in OmpX/DHPC, taken from the [^{13}C,^{1}H]-HSQC spectrum of Figure 2. The trace for the γ^2 methyl group has higher apparent noise because of its proximity to a t_1 noise band (see Figure 2B).

to a level of approximately 10% with ^{13}C, and 2D [^{13}C,^{1}H]-HSQC spectroscopy was used to achieve the stereospecific assignments of the diastereotopic methyl groups (Bodenhausen and Ruben, 1980). The metabolic pathways in the bacteria used to overexpress the protein determine which atoms in the final product originate from the same source molecule (for example, the shaded area in Figure 1A), and the resulting labeling patterns enable a distinction of the pro-*S* and pro-*R* chiral methyl groups from the ^{13}C–^{13}C couplings (Figures 1B and C).

Materials and methods

An OmpX/DHPC sample labeled uniformly with ^{15}N and to the extent of ~10% with ^{13}C, [u-^{15}N,10%-^{13}C]-OmpX/DHPC, was produced by overexpression in *E. coli* BL21(DE3)pLysS cells harboring the plasmid pET3b-OmpX (Fernández et al., 2001). The cells were grown in one liter of minimal medium containing 0.4 g [$^{13}C_6$]-D-glucose (>98% ^{13}C) and 3.6 g [$^{12}C_6$]-D-glucose (98.9% ^{12}C; natural abundance) as the carbon source, and 1 g of $^{15}NH_4Cl$ (>98% ^{15}N) as the nitrogen source. Cells were harvested by centrifugation once they reached an optical density at 600 nm of 0.7, and the protein was purified and re-

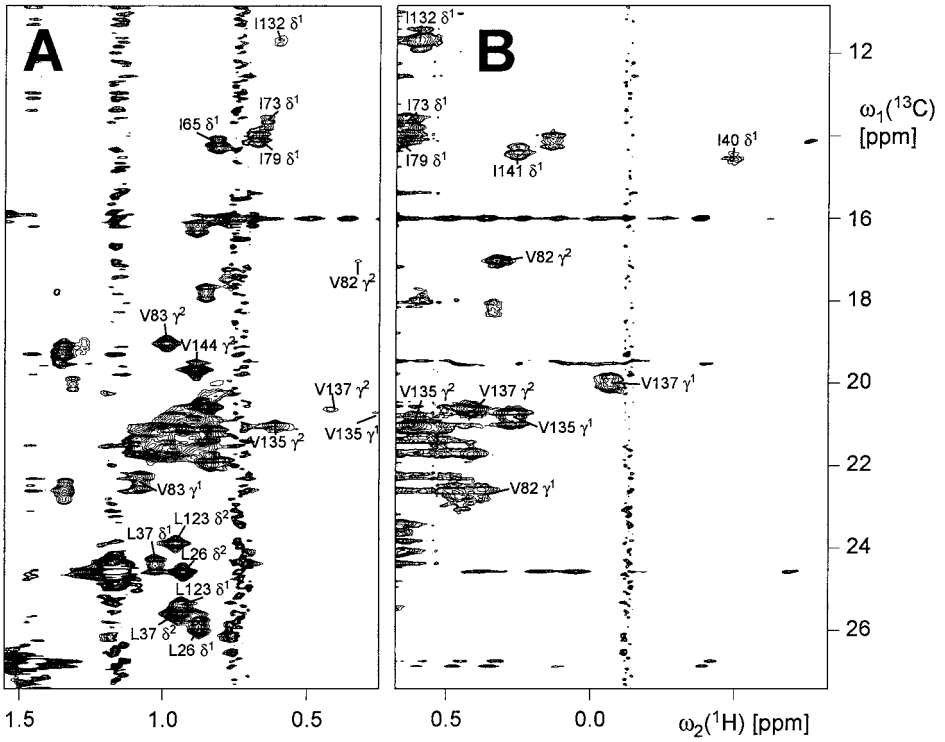

Figure 2. (A, B) Two spectral regions from the [^{13}C,^{1}H]-HSQC spectrum of [u-^{15}N,10%-^{13}C]-OmpX/DHPC. To optimally view all the methyl resonances of Val, Leu and Ile, the spectrum in B has been plotted with lower contour levels. The experiment was recorded on a Bruker Avance 500 MHz spectrometer equipped with a cryoprobe, with 64 transients per increment, $t_{1\,\max}(^{13}\mathrm{C}) = 104$ ms, $t_{2\,\max}(^{1}\mathrm{H}) = 150$ ms, time domain data size 1050 × 1024 complex points. The methyl resonances are labeled with the one-letter amino acid code, the sequence position and the methyl group identifier.

folded from inclusion bodies (Fernández et al., 2001). The NMR sample conditions were: protein concentration ∼2 mM, solvent 95% H_2O/5% D_2O, 20 mM phosphate buffer, 100 mM NaCl, ∼200 mM DHPC, pH 6.5. All the spectra were measured at 30 °C. The correct folding of the protein was verified from a 2D [^{15}N,^{1}H]-TROSY spectrum (Pervushin et al., 1998).

A 2D [^{13}C,^{1}H]-HSQC spectrum was recorded at a proton resonance frequency of 500 MHz, with sufficiently large resolution along $\omega_1(^{13}\mathrm{C})$ to observe the line splittings due to the 35 Hz $^{1}J_{CC}$ scalar couplings (Figure 2) (Szyperski et al., 1992). Additionally, two 500 MHz constant time (*ct*) [^{13}C,^{1}H]-HSQC spectra (Vuister and Bax, 1992; Wider, 1998) were measured, with constant time evolution delays of 26.6 ms and 53.2 ms, respectively (Figures 3A and B), and a 2D [^{13}C,^{1}H]-HSQC spectrum was recorded at a ^{1}H resonance frequency of 900 MHz (Figure 4). In all these experiments the presence of 200 mM protonated DHPC gave rise to t_1-noise bands in the spectral regions of interest. To alleviate this problem, spin-lock pulses of 1 ms duration at the ^{1}H carrier frequency were used prior to acquisition (Messerle et al., 1989).

Results

Figure 2 shows that good quality [^{13}C,^{1}H]-HSQC spectra of the 60 kDa [u-^{15}N,10%-^{13}C]-OmpX/DHPC have been obtained, and that the singlet and doublet splittings along $\omega_1(^{13}\mathrm{C})$ can readily be observed. In Figure 2A the spectrum has been plotted with higher contour levels than in Figure 2B, so as to make the spectrum readable despite the t_1-noise from the DHPC signals. As an illustration, cross sections along $\omega_1(^{13}\mathrm{C})$ through the γ^1 and γ^2 resonances of Val 135 are shown in Figure 1C. Although the minor components of the expected three-peak-pattern (Figure 1B) are not visible due to the inherently low sensitivity, the singlet and doublet peak patterns are clearly distinguishable.

In the spectra of OmpX/DHPC one observes more extensive resonance overlap than with smaller proteins

due to both faster relaxation and the resulting line broadening, and the inherent small dispersion of the large number of signals from solvent-exposed methyl groups. For example, in Figure 2A the singlets and doublets near [$\omega_1(^{13}C) = 21$ ppm / $\omega_2(^1H) = 1.0$ ppm] cannot all be resolved. To overcome these limitations, we recorded two *ct*-[^{13}C,^{1}H]-HSQC spectra with *ct*-evolution times of 26.6 ms and 53.2 ms (Figure 3). As expected, the signal-to-noise ratio in these spectra is reduced when compared to the corresponding [^{13}C,^{1}H]-HSQC spectrum (Figure 2), but nonetheless all methyl peaks in the crowded regions were detected. With regard to the ease of spectral interpretation, the intensity loss is compensated by the fact that all resonance lines become narrow singlet peaks in the ^{13}C dimension, and that with a ^{13}C *ct*-evolution time of 26.6 ms there are negative peaks in the place of the doublets in the [^{13}C,^{1}H]-HSQC spectrum (Klaus et al., 1997). The *ct*-[^{13}C,^{1}H]-HSQC spectrum with a ^{13}C *ct*-evolution time of 53.2 ms shows only positive peaks and thus does not provide the information needed for the stereospecific assignments, but because of the further improved resolution in the $\omega_1(^{13}C)$-dimension (Figure 3) it was very useful in supporting resonance identifications. With the combination of a [^{13}C,^{1}H]-HSQC and two *ct*-[^{13}C,^{1}H]-HSQC spectra at 500 MHz, it was thus possible to stereospecifically assign the methyl resonances of 9 out of the 13 Val and Leu residues with certainty, and the resonances of three additional residues with high probability (marked with parentheses in Figures 3 and 4). The uncertainty in the latter assignments is primarily due to the fact that the methyl groups Ala-β, Ile-γ^2 and Thr-γ^2 have not been assigned and could therefore be mistaken for Val or Leu resonances.

In a second approach toward overcoming limitations imposed by spectral overlap in the 500 MHz [^{13}C,^{1}H]-HSQC spectrum (Figure 2), we explored the use of the highest field available to us. In a [^{13}C,^{1}H]-HSQC spectrum recorded at a proton resonance frequency of 900 MHz (Figure 4), most of the peaks in the crowded spectral regions could readily be identified, and nearly all stereospecific assignments of Val and Leu isopropyl methyl groups could be obtained from this single [^{13}C,^{1}H]-HSQC spectrum.

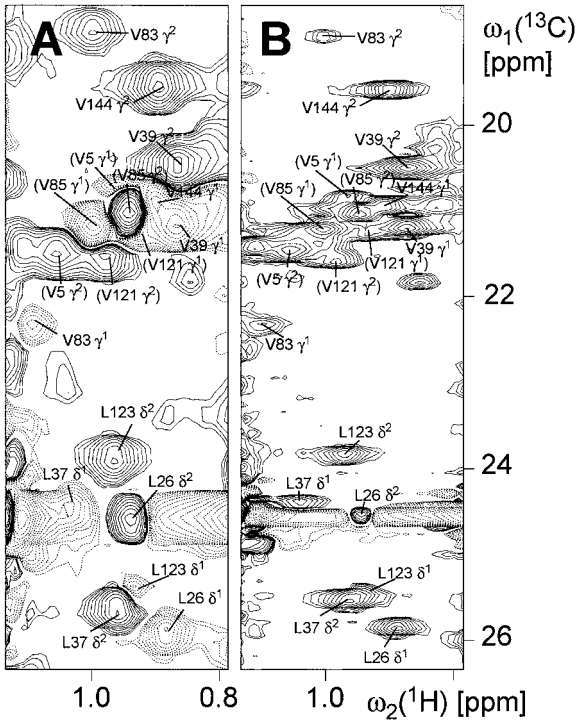

Figure 3. Spectral region [$\omega_1(^{13}C) = 19 - 26$ ppm, $\omega_2(^1H) = 0.8 - 1.1$ ppm] from two *ct*-[^{13}C,^{1}H]-HSQC spectra recorded with the same sample and the same equipment as the spectrum in Figure 2, using $t_{2\,max}(^1H) = 150$ ms and either $t_{1\,max}(^{13}C) = 26.6$ ms (A), or $t_{1\,max}(^{13}C) = 53.2$ ms (B). Spectrum A was recorded with 96 transients per increment and a time domain data size of 260 × 1024 complex points. Negative contour levels, corresponding to doublet peaks in Figure 2, are drawn with broken lines. Spectrum B was recorded with 48 transients per increment and a time domain data size of 520 × 1024 complex points. All peaks are positive, and compared to A, the spectrum has twice the resolution along $\omega_1(^{13}C)$. Assigned resonances are labeled as in Figure 2, where parentheses designate tentative assignments (see text).

Discussion

The experimental determination of stereospecific assignments becomes more important in structural studies of large, partially deuterated molecular structures. On the one hand, stereospecific assignments of methyl groups can often not be determined during the structure calculation due to the relatively low density of NOE constraints, and on the other hand, for the same reason, individual assignments of diastereotopic methyl groups have a great impact on the quality of the structure determination. For example, with a sample of [u-^{2}H,^{13}C,^{15}N/L,V,Iδ^1-^{13}CH$_3$]-OmpX/DHPC (Hilty et al., 2002), 258 out of a total of 526 NOE constraints involved methyl groups of Val and Leu residues (Figure 5) (Fernández, C., Hilty, C., Wider, G., Güntert, P. and Wüthrich, K.; to be published).

The study of the OmpX/DHPC system shows that even for a molecular complex of this size no deutera-

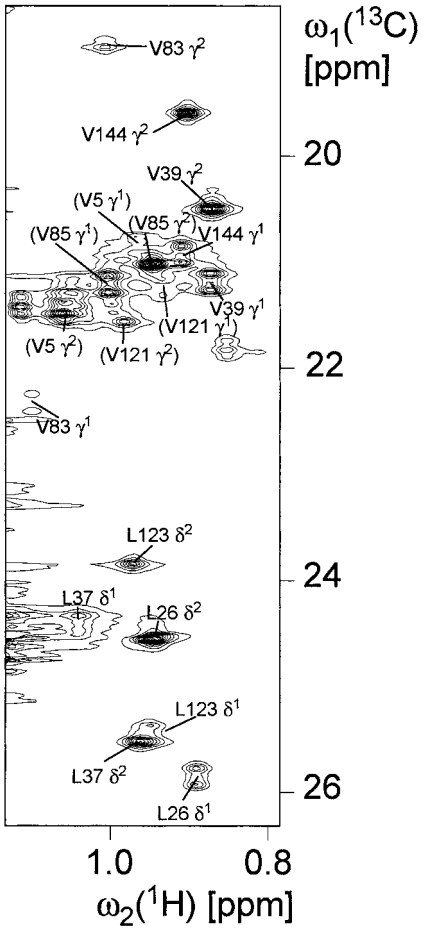

Figure 4. Same spectral region as in Figure 3 from a [^{13}C,^{1}H]-HSQC spectrum recorded on a 900 MHz Bruker Avance spectrometer equipped with a triple resonance TXI probehead, using the same sample as in Figure 2, with 20 transients per increment, $t_{1\,\max}(^{13}\mathrm{C})$ = 97 ms, $t_{2\,\max}(^{1}\mathrm{H})$ = 81 ms, time domain data size 1750 × 1024 complex points. Assigned resonances are labeled as in Figure 3.

tion is necessary to obtain stereospecific assignments with a nonrandomly ∼10% ^{13}C-labeled protein, which greatly reduces the cost of sample production. Based on the sequence-specific assignments of the methyl groups and the combination of a [^{13}C,^{1}H]-HSQC and two *ct*-[^{13}C,^{1}H]-HSQC spectra, stereospecific assignments could be obtained even at 500 MHz proton resonance frequency, whereby the experiments of Figures 2 and 3 nicely complement each other: The *ct*-[^{13}C,^{1}H]-HSQC spectra yield higher resolution, which is needed for the analysis of the crowded regions, whereas the regular [^{13}C,^{1}H]-HSQC spectrum gives a better signal-to-noise ratio, which is desirable for the analysis of the well dispersed peaks, which usu-

Figure 5. Mapping of the NOE distance constraints that involve isopropyl methyl groups of Val and Leu onto the NMR structure of OmpX (Fernández, C., Hilty, C., Wider, G., Güntert, P. and Wüthrich, K.; to be published). The 258 constraints were collected from 3D ^{15}N-resolved [^{1}H,^{1}H]-NOESY and 3D ^{13}C-resolved [^{1}H,^{1}H]-NOESY spectra recorded with a [u-^{2}H,^{13}C,^{15}N/L,V,Iδ^{1}-^{13}CH$_3$]-OmpX/DHPC sample (Fernández et al., 2002).

ally originate from the protein core and therefore have weaker intensities. Alternatively, a single [^{13}C,^{1}H]-HSQC spectrum recorded at a ^{1}H frequency of 900 MHz yielded practically the same amount of information as the aforementioned combination of three 500 MHz spectra.

For the OmpX/DHPC mixed micelles studied in this work, the previously prepared [u-^{2}H,^{13}C,^{15}N/L,V,Iδ^{1}-^{13}CH$_3$]-OmpX sample offers an independent control for the success of the fractional labeling. A [^{13}C,^{1}H]-HSQC spectrum of this sample contains also the signals of the δ^{1} methyls of isoleucine residues (Goto et al., 1999; Hilty et al., 2002). A comparison with a [^{13}C,^{1}H]-HSQC spectrum of [u-^{15}N,10%-^{13}C]-OmpX/DHPC (Figure 2) confirms that all Ile-δ^{1} resonances are singlets in the latter spectrum, as expected for successful fractional labeling (Szyperski et al., 1992).

In cases where the procedure described above does not permit to resolve all the overlapping methyl resonances originating from different amino acid types, it may be possible to use amino-acid selective fractional labeling (Atreya and Chary, 2001). For OmpX/DHPC, it would thus be possible to eliminate the signals arising from the Ala-β, Ile-γ^{2} and Thr-γ^{2} methyl groups, for which the assignments are not known and which therefore give rise to uncertainty in the stereospecific assignment for some valine residues in OmpX/DHPC (shown in parentheses in Figure 3). Alternatively, it would also be possible to selectively label the valine residues with ^{12}C on an otherwise 10% ^{13}C-labeled background. The disappearance of the corresponding peaks could be used as a confirmation that the tentatively assigned peaks do indeed originate from valine, and thus the assignment for these valine residues could be verified.

In conclusion, we have shown that stereospecific assignments of diastereotopic methyl groups can be obtained with a nonrandomly $\sim$10% ^{13}C-labeled membrane protein solubilized in DHPC without using deuteration. The experimental determination of stereospecific assignments becomes more important in structural studies of larger molecular structures due to the relatively small number of NOEs in partially deuterated proteins. In future applications for structure determinations of α-helical membrane proteins, side chain – side chain NOEs are expected to have a key role in defining the positions of multiple transmembrane helices relative to each other.

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