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Secondary chemical shifts in immobilized peptides and proteins: A qualitative basis for structure refinement under Magic Angle Spinning

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Abstract

Resonance assignments recently obtained on immobilized polypeptides and a membrane protein aggregate under Magic Angle Spinning are compared to random coil values in the liquid state. The resulting chemical shift differences (secondary chemical shifts) are evaluated in light of the backbone torsion angle ψ previously reported using X-ray crystallography. In all cases, a remarkable correlation is found suggesting that the concept of secondary chemical shifts, well established in the liquid state, can be of similar importance in the context of multiple-labelled polypeptides studied under MAS conditions.

Introduction

The empirical relationship between the chemical shift detected in NMR and local structural motifs in peptides and proteins has been utilized in solution (Sternlicht and Wilson, 1967; Markley et al., 1967) and solid-phase systems (Pease et al., 1981; Saito, 1986) for a long time. In the absence of other spectroscopic methods, early studies on the backbone conformation of immobilized polypeptides required the accumulation of chemical shift data in systems with known secondary structures (Saito, 1986). The observed chemical shifts were classified for various structural motifs (Saito, 1986; Saito et al., 1998) providing an analytical basis for studies in fibrous proteins (Ishida et al., 1990; Heller et al., 1996; Kamihira et al., 2000; Balbach et al., 2000) and membrane proteins (Tuzi et al., 1992; Saito et al., 2000). Similar to many applications that measure distances and dihedral angles with high precision (for recent reviews see, e.g., Cross and Opella, 1994; Griffin, 1998; Tycko, 2000), the studies involved site- or residue-specific labeling schemes.

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In solution-state NMR, the corresponding secondary chemical shifts, i.e. the difference between the experimentally observed isotropic chemical shift and the random coil value (Spera and Bax, 1991; Wishart and Sykes, 1994) were combined with multiple-spin labeling and a suite of multi-dimensional correlation techniques (Wüthrich, 1986; Ernst, 1987; Cavanagh, 1996). As a result, these statistics have become of great value at an early stage of the structure determination process (Cornilescu et al., 1999) or they provide important information for the study of folding mechanisms (Dyson and Wright, 1998) in globular proteins.

Recent advancements in solid-state NMR methodology and hardware instrumentation have permitted to fully or at least partially assign resonances in multiple-labelled peptides (Hong and Griffin, 1998; Nomura et al., 1999; Rienstra et al., 2000), proteins (Straus et al., 1998; Hong, 1999; McDermott et al., 2000; Pauli et al., 2001) and membrane proteins (Egorova-Zachernyuk et al., 2001) under Magic Angle Spinning (Andrew, 1958) conditions. In principle, these results can be utilized to define secondary chemical shifts in the solid state. For several reasons however, care must

be exercised in directly comparing MAS-based correlation spectra of multi-labelled peptides and proteins to results obtained in isotropic solutions. Anisotropic chemical shielding and dipolar interactions may be comparable in size or even exceed MAS or radio frequency (r.f.) modulation rates. As a result, coherent recoupling effects (see, e.g., Vanderhart, 1986; Sodickson et al., 1993; Bennett et al., 1994; Baldus et al., 1998a) that may affect peak position and line width need to be minimized. Moreover, even if MAS frequencies can be employed that exceed all anisotropic interactions, conformational heterogeneity, bond-distortions by sample packing and variations in local susceptibility or motion may influence solid-state NMR spectra (Vanderhart, 1981; Saito et al., 1998).

With these aspects in mind it seems appropriate to investigate the concept of secondary chemical shifts in multiple-labelled peptides and proteins under MAS conditions in more detail. Obviously, such a study can currently only include a very limited number of systems. Nevertheless, we believe that our selection of three polypeptides of variable length and chemical environment gives a general indication on whether MAS-based structural studies in the aforementioned systems may profit from correlations regularly observed in solution-state NMR. For this purpose, we considered in the present analysis the tripeptide AlaGlyGly, a 62-residue SH3 domain from α-spectrin (Musacchio et al., 1992; Pauli et al., 2000) and the LH2 light-harvesting complex from the photosynthetic bacterium *Rhodopseudomonas acidophila* (McDermott et al., 1995). In each case fully labelled variants were studied, which allows for an analysis of a significant number of peptide residues and dihedral angles. For all three systems, we compare our experimental findings to structural data from X-ray crystallography. Unlike the liquid state, we relied solely on secondary chemical shifts obtained on ^{13}C signals since carbon spectroscopy is in general more sensitive than direct ^{15}N detection and it can usually be obtained at higher resolution than proton chemical shift information.

Materials and methods

The uniformly labeled trimer AGG was prepared (Pennington, 1994) using Boc-chemistry in solution starting from Boc-[*U*-^{13}C,^{15}N]-Gly-OMe. Stepwise elongation with Boc-[*U*-^{13}C,^{15}N]-Gly-OH and Boc-[*U*-^{13}C,^{15}N]-Ala-OH were accomplished using, respectively, BOP/HOBt and EDC/HOBt as coupling reagents. Semipermanent Boc protections were cleaved by 13% HCl in EtOAc. The protected tripeptide AGG (Boc-Ala-Gly-Gly-OMe) was subsequently purified by silica gel column chromatography. Final deprotection was effected by a consecutive treatment of AGG with 0.2 N NaOH (saponification of the methyl ester) and 95% aqueous TFA (cleavage of the Boc-group) to furnish labeled AGG in 7% overall yield. All isotope-labeled amino acid derivatives were purchased from Cambridge Isotope Laboratories, Inc. (USA). Three mg of fully labeled AGG were subsequently recrystallized using unlabeled AGG in 10-fold excess. For the preparation of uniformly (^{13}C,^{15}N) labeled variants of SH3 and LH2 we refer to recent publications (Pauli et al., 2000; Egorova-Zachernyuk et al., 2001). NMR assignments in AGG were obtained using a 4 mm (^{1}H,^{15}N,^{13}C) triple resonance probe in a 400 MHz wide bore instrument. Two-dimensional ^{15}N-^{13}C correlation experiments were conducted using previously described pulse schemes (Baldus et al., 1996) that contain a band-selective $^{15}N \rightarrow {}^{13}C$ transfer step (Baldus et al., 1998; Pauli et al., 2001). For homonuclear (^{13}C,^{13}C) polarization transfer a band-selective DQ scheme or proton-driven spin diffusion (Bloembergen, 1949) was used (vide infra). A detailed discussion of these pulse schemes is, e.g., given by Pauli et al. (2001).

Results and discussion

In the following analysis, we compare the experimentally observed (obs) carbon chemical shifts under MAS to standard, isotropic random coil (rc) values. For this purpose, we define

$$\begin{aligned}\Delta\delta = \delta_{C_\alpha} - \delta_{C_\beta} &= \{\delta_{C_\alpha}(\text{obs}) - \delta_{C_\alpha}(\text{rc})\} \\ &- \{\delta_{C_\beta}(\text{obs}) - \delta_{C_\beta}(\text{rc})\}. \qquad (1)\end{aligned}$$

With the exception of Gly residues (for which we assume $\delta_{C_\beta}(\text{obs}, \text{rc}) = 0$), $\Delta\delta$ represents the difference between C_α and C_β secondary shifts usually employed in solution-state NMR. Here, Wishart and Sykes (1994) have suggested the use of a (dimensionless) chemical shift index (CSI) that is closely related to $\Delta\delta$ given in Equation 1. In the solid state, the experimental line width of ^{13}C resonances under MAS is usually significantly larger than in liquids

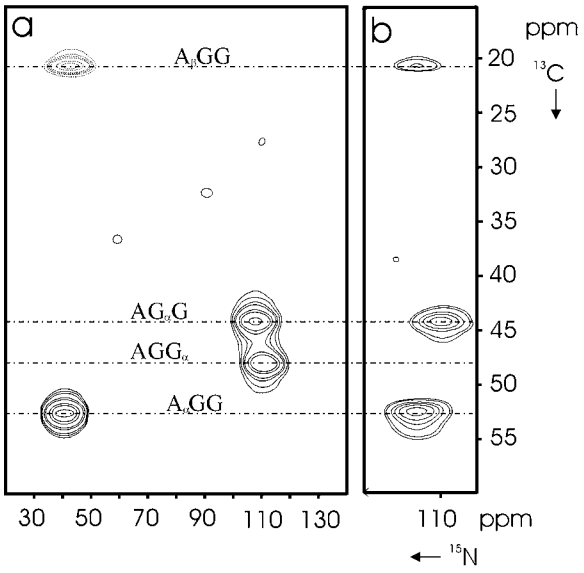

Figure 1. Heteronuclear ^{15}N-^{13}C correlation experiments on a uniformly (^{13}C,^{15}N) labeled variant of the tripeptide AlaGlyGly. In (a) results of an NCACB-type experiment are shown in which a band-selective cross polarization step (Baldus et al., 1998b; Pauli et al., 2001) is used to transfer polarization from amide nitrogens to C_α carbons. Subsequently, a band-selective double-quantum transfer (Verel et al., 1998; Pauli et al., 2001) is used to excite C_β (Ala) resonances in negative intensity (dotted contour levels in (a)). Results of an NCOCACB-type of experiment are shown in (b). Inter-residue NH-CO transfer was established by readjusting the band-selective ^{15}N→^{13}C transfer (Baldus et al., 1998b; Pauli et al., 2001). Subsequently, proton driven spin diffusion (Bloembergen, 1949; Pauli et al., 2001) was employed for a mixing time of 15 ms giving rise to positive (NH,CO), (NH,C_α) and (NH,C_β) correlations. Only the side chain regions of the resulting 2D spectra are shown. Horizontal lines identify the carbon backbone and side chain resonances (indicated by the subscript) of all NH-$C_{\alpha,\beta}$ sets in AGG. Both experiments were conducted at 400 MHz (^{1}H resonance frequency) using a triple resonance 4 mm MAS probe at 277 K and a MAS frequency of 11 kHz.

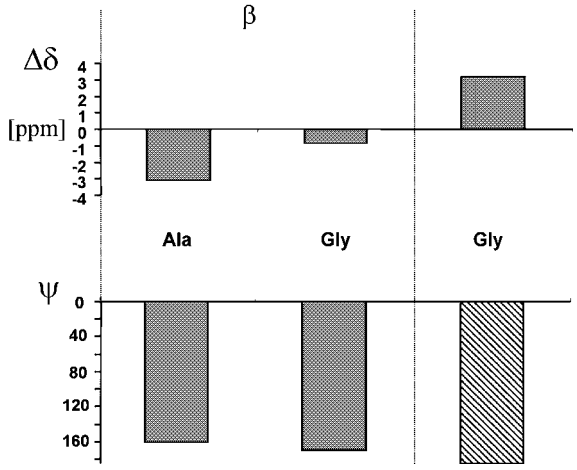

Figure 2. Upper row: Experimentally observed secondary chemical shift parameter Δδ (defined in Equation 1) compared to (lower row) the backbone dihedral angle ψ as obtained from X-ray crystallography in AlaGlyGly (Subramanian, 1983). Random coil values were obtained from statistical averages obtained in solution-state NMR (see, e.g., BioMagResBank: A repository for data from NMR spectroscopy on proteins, peptides and nucleic acids, http://www.bmrb.wisc.edu/index.html). The N-terminal Ala and the subsequent Gly residue are characterized by dihedral angles typically observed in β-strands. For the C-terminal Gly residue (where a backbone angle ψ cannot be defined), the angle given in the original X-ray study is shaded.

and we prefer the definition in Equation 1 that allows for a direct comparison of Δδ (given in ppm) to the experimentally detected line width.

In solution, experimentally observed random coil values are in very close agreement to the statistical averages of α-helical and β-strand chemical shifts (Wishart and Sykes, 1994). Hence we utilized for all amino acid residues under study statistical average chemical shift values as random coil references. Previous solution studies have revealed that C_α and C_β secondary shifts are affected by conformation dependent chemical shift changes in opposite ways (Spera and Bax, 1991). Any correlation between carbon chemical shifts and protein secondary structure should therefore be strongly reflected by the parameter Δδ that measures the difference between both quantities. Since the number of solid-phase systems that can be studied by Equation 1 is currently small we do not attempt to compare other statistical approaches e.g. containing an analysis of C_α and C_β resonances individually, the inclusion of additional ^{15}N or ^{1}H chemical shifts or any other spectroscopic parameters. A future analysis of a larger number of peptides and proteins might result in other statistical approaches that describe the empirical relation between chemical shift and protein structure more effectively in the solid state.

We begin with the tripeptide AlaGlyGly that has previously been studied by X-ray crystallography (Subramanian, 1983) and solid-state NMR (Bennett et al., 1998). For our purposes, we prepared a uniformly labelled variant of AGG and we employed heteronuclear NCACB- and NCOCACB-type of experiments leading to the results of Figure 1. The spectra were obtained using band-selective (^{15}N,^{13}C) transfers (Baldus et al., 1998b) followed by homonuclear (^{13}C,^{13}C) transfer units. In the NCACB case (Figure 1a), band-selective double-quantum transfer was employed (Verel et al., 1998; Pauli et al., 2001) that is characterized by 'up-down' single quantum intensities

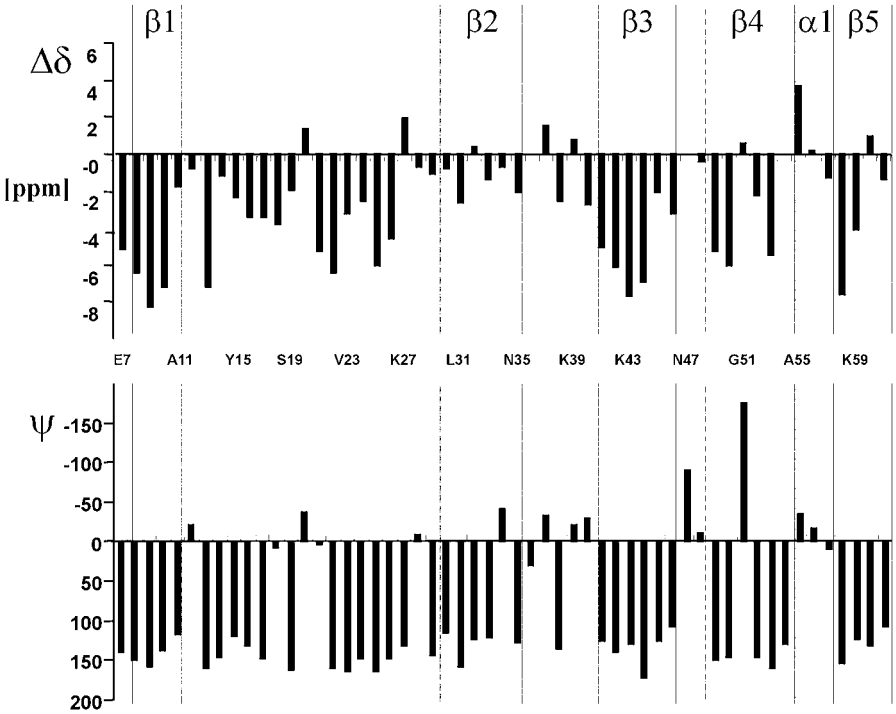

Figure 3. Upper row: Chemical shift parameter $\Delta\delta$ (see Equation 1) calculated from the SSNMR assignments obtained for the SH3 domain from α spectrin (Pauli et al., 2001) and random coil standards in liquid-state NMR. Lower row: Backbone angles as obtained from X-ray crystallography (PDB entry 1SHG). To facilitate the analysis, we have drawn vertical lines that indicate beta sheet or alpha helical regions of the protein. Residues of the peptide sequence are given in single letter notation.

(Baldus et al., 1994). In Figure 1b, proton driven spin diffusion was used to observe inter-residue NHCO, NHC_α and NHC_β correlations. Following Equation 1 the resulting resonance assignments are calculated in Figure 2 with respect to random coil predictions (upper row). For reference, Figure 2 (lower row) contains the backbone dihedral angle ψ as obtained from X-ray crystallography (Subramanian, 1983) and indicates a qualitative agreement for the AG segment for which a backbone dihedral angle ψ can be defined. Similar to the liquid state, we observe negative values of $\Delta\delta$ for large positive values of ψ. Furthermore, we determined the backbone dihedral angles ψ of the NMR sample using 2D multiple-quantum experiments (Luca et al., to be submitted) under MAS. These independent measurements are in close agreement to the values obtained in X-ray crystallography and to the qualitative observation in Figure 2.

Figure 3 contains experimental results for a 62-residue protein (SH3 domain from α spectrin, 7.2 kDa) of which spectral assignments were recently published (Pauli et al., 2001). Again, the carbon secondary chemical shift parameters $\Delta\delta$ are plotted against the backbone angle ψ derived from the X-ray structure (PDB entry 1SHG, Musacchio et al., 1992). To facilitate the analysis, we have drawn vertical lines that indicate beta sheet or alpha helical regions of the protein. A remarkable correlation between both parameters is observed throughout the polypeptide chain, in particular for the beta strands $\beta_1, \beta_3, \beta_4, \beta_5$ and the helical region α_1 (A55–Y57). Qualitative agreement is also detected for many of the connecting loop regions, such as L12–D29. Larger deviations are observed for the residues (I30–N35) that encompass the second beta strand of the molecule. Here, most residues exhibit negative $\Delta\delta$ parameters in qualitative agreement with the crystallographic data, but their absolute values are significantly smaller than those of other beta strands in the protein. In the case of SH3, the ^{13}C chemical shifts obtained in the solid state agree well with data obtained in solution (Oschkinat et al., to be published). It is thus unlikely that these differences are caused by macroscopic sample conditions. On the other hand, mobility effects or conformational heterogeneity could lead to a reduction of the observed $\Delta\delta$ values (Dyson and Wright, 1998).

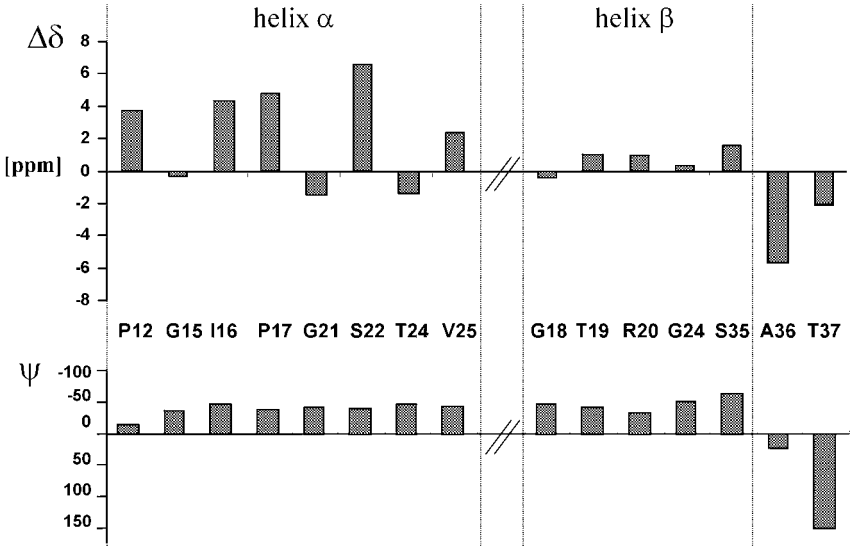

Figure 4. Comparison of experimentally observed secondary chemical shift parameter $\Delta\delta$ (upper row) (Egorova-Zachernyuk et al., 2001) to backbone dihedral angles as obtained from McDermott et al. (1995). The crystal structure (PDB entry 1KZU) contains three asymmetric units each including three protomer complexes. These complexes consist of α (53 amino acid residues) and β (41 amino acid residues) apo-proteins that are arranged in high symmetry. Helix α relates to residues found in subunit α whereas helix β contains residues found in the helical region of apo-protein β.

Finally, we plot in Figure 4 an analogous comparison using experimental results recently obtained on a [U-^{13}C,^{15}N] labeled version of the LH2 light-harvesting complex from the photosynthetic bacterium *Rhodopseudomonas acidophila*. The crystal structure (PDB entry 1KZU) contains three asymmetric units each including three protomer complexes. These complexes consist of α (53 amino acid residues) and β (41 amino acid residues) apo-proteins that are arranged in high symmetry in the 150 kDa membrane protein complex (McDermott et al., 1995). For LH2, a complete spectral assignment using MAS-based solid-state NMR methods is not yet available and we concentrate on the residues assigned by Egorova-Zachernyuk et al. (2001). In contrast to the polypeptides discussed so far, the X-ray structure of LH2 shows a mostly alpha helical arrangement that is well reproduced in the chemical shift statistics (i.e., $\Delta\delta > 0$) for protein subunit α (containing helix α in Figure 4). For the assigned Gly residues we find deviations for δ_{C_α} indicating that only the parameter defined in Equation 1 gives a qualitative measure for the secondary structure in the solid state. In apo-protein β only a small number of residues have so far been identified in the helical segment. Except for G18 (apo-protein β) the observed values of $\Delta\delta$ are in qualitative agreement with the X-ray results. Moreover, the helix-turn interface S35–T37 shows a significant change from positive to negative $\Delta\delta$ values in line with the crystallographic data and general observations in liquid-state NMR experiments on globular proteins.

Conclusions

Magic Angle Spinning represents one of the principal methods to obtain high resolution conditions in immobilized polypeptides. Recent advancements in the spectral assignment of fully labeled polypeptides of variable size and chemical environment here allowed for a comparison of the measured chemical shifts to random coil standards employed in the liquid state. In all cases, we find a remarkable correlation between the parameter $\Delta\delta$ that reflects the difference in C_α and C_β secondary chemical shifts under MAS and the backbone dihedral angle ψ obtained using X-ray crystallography. In close analogy to liquid-state NMR, we observe negative values of $\Delta\delta$ in beta-strand conformations and positive values for α-helical segments of an immobilized polypeptide. In the case of the SH3 domain from α spectrin, qualitative agreement is also found for many of the connecting loop regions of the protein. Deviations are predominantly observed for glycine residues for which *ab initio* quantum mechanical studies predict a reduced sensitivity of (isotropic

and anisotropic) chemical shift values to secondary structure (Havlin et al., 1997). A more detailed investigation could be attempted when additional NMR data in immobilized polypeptides under MAS are available.

Our analysis confirms that low-resolution structural models of immobilized polypeptides might be obtained from a simple analysis of a small number of characteristic chemical shift values that identify elements of secondary structure and a subsequent measurement of selected through-space interactions. Unlike previous approaches that relied on the study of selectively labelled peptides and proteins, our results indicate that fully labelled compounds can be utilized enhancing the general applicability and reliability of the method. Results in the tripeptide AlaGlyGly suggest that this concept can also be helpful in the study of small peptide fragments bound to larger (possibly membrane-spanning) complexes. In the current context, we have only relied on isotropic chemical shift information that can in principle be obtained from standard two- or higher-dimensional correlation spectroscopy. In cases where the observed resolution is insufficient to study entire proteins or for further refinement of the secondary structure, anisotropic chemical shift contributions can be included in the analysis (Luca et al., to be submitted). Moreover, coherent methods that, e.g., monitor the evolution of multiple-quantum coherence in the backbone or side chain sections of the protein can be employed. As shown by several research groups, in doubly labelled compounds (Schmidt-Rohr, 1996; Tycko et al., 1996; Feng et al., 2000) the accuracy of these methods can be very high.

Today, secondary chemical shifts are of great value in the structure determination process and for the rapid calculation of global protein folds (Bowers et al., 2000; Chou et al., 2000; Meiler et al., 2000) in liquid-state NMR. Our results suggest that a similar analysis can provide a qualitative basis for the complete and rapid (low-resolution) structural characterization of multiple-labelled membrane proteins or protein aggregates under MAS conditions. Since chemical shift assignments are sufficient, this method could be of particular interest for cases in which signal to noise considerations limit the application of more sophisticated NMR methods at present.

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