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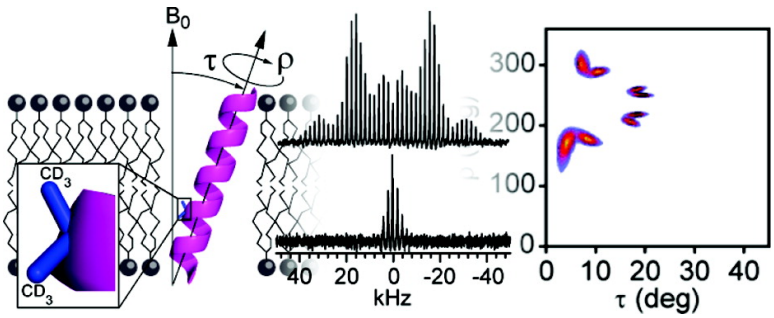
J. Am. Chem. Soc., **2007**, 129 (47), 14717-14723 • DOI: 10.1021/ja0749690



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Membrane-Bound Conformation of Peptaibols with Methyl-Deuterated α-Amino Isobutyric Acids by ^{2}H Magic Angle Spinning Solid-State NMR Spectroscopy

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Abstract: We present the use of ^{2}H magic-angle spinning (MAS) NMR on methyl-deuterated α-amino isobutyric acid (Aib) as a new method to obtain fast and accurate structural constraints on peptaibols in membrane-bound environments. Using nonoriented vesicle-reconstituted samples we avoid the delicate preparation of oriented samples, and the use of MAS ensures high sensitivity and thereby very fast acquisition of experimental spectra. Furthermore, given the high content (∼40%) of Aib in peptaibols and the fact that the amino acid Aib may be synthesized from cheap starting materials, even in the case of ^{2}H, ^{13}C, or ^{15}N labeling, this method is ideally suited for studies of the membrane-bound conformation of peptaibols.

Introduction

Peptaibols are fungal peptides containing 5−25 residues with a high content of the helix-inducing amino acid Aib (α-amino isobutyric acid). They have attracted considerable interest since they, despite their small size, form ion-selective voltage-gated channels across the cell membrane.[1] This property implies antimicrobial activity, and thereby peptaibols and synthetic analogues have acquired substantial interest as alternatives to small-molecule antibiotics. Despite these interesting properties, detailed insight into the mechanisms of their membrane disrupting and ion-transporting features at an atomic level is still sparse, and only few peptaibol structures have been reported. One of the most well-characterized peptaibols is the 20-mer alamethicin which contains eight Aib residues. Alamethicin has been structurally characterized at atomic resolution by X-ray diffraction (XRD)[2] and NMR spectroscopy.[3−6] Besides alamethicin, there exist a few XRD structures of other peptaibols, but the general difficulties in crystallizing membrane-bound peptides have also hampered the extensive use of XRD for structure determination of peptaibols, and most of the known peptaibol crystal structures have been achieved from crystals grown in the absence of lipids. Likewise, liquid-state NMR structure analysis of peptaibols in membrane-bound form is complicated by slow molecular reorientation of peptide−membrane complexes. In more general terms, this technique also suffers from difficulties in assignment of Aib residues which lack the "fingerprint" H^{α}-proton and generally display substantial overlap of the abundant H^{β}-methyl protons.

Solid-state NMR represents an attractive alternative for atomic-resolution structural analysis of peptaibols, as it overcomes many of the problems described above and simultaneously may provide structural *and* conformational information on the ionophores in their native environment, the lipid bilayer.[3−5] Such combined information may be obtained by measuring anisotropic dipolar coupling and chemical shielding interactions for the peptaibol in lipid bilayers oriented macroscopically between glass plates with the bilayer normal parallel to the magnetic field.[3,4] In this manner, it is possible to establish a direct measurement of the peptide conformation in the bilayer.[6] A drawback of this approach, however, is that it requires highly oriented samples to extract accurate structural information. This may be quite difficult to achieve for membrane-disrupting peptides such as alamethicin, in particular for the functionally most relevant cases with the peptide present in high concentrations in the lipid bilayers where a significant orientational disorder—mosaic spread—may be encountered.[7] The result is that oriented alamethicin samples often lead to NMR spectra with substantial linebroadening, which in turn implies that oriented-sample spectra for uniformly ^{15}N labeled samples obtained from fungal expression display significant spectral overlap.[4] This overlap is highly unfortunate since the oriented-sample solid-state NMR approach relies exclusively on the

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resonance positions to derive the structural restraints. As a consequence, the most accurate constraints have so far been derived from sets of singly ^{15}N-labeled samples,[3] although selective labeling entails the requirement for much more spectrometer time and is very expensive in terms of extensive use of isotope-labeled protected amino acids for multiple-sample solid-phase peptide synthesis.

The dilemma is that on one hand, oriented solid-state NMR is one of the best techniques to obtain accurate structural and conformational information on uniformly isotope-labeled samples, but on the other hand, it is very difficult to achieve perfectly oriented samples due to membrane mosaic spread and/or peptide-induced membrane disruption. To overcome this, we have to either improve significantly the sample preparation methods or improve the NMR methods used to extract the crucial structural information from the nuclear spin interactions. Adapting the latter approach, we here follow the line of recent work in several groups,[7−14] using nonoriented samples to provide the same kind of information typically achieved from oriented samples, provided the peptides display a sufficiently high degree of lateral diffusion around the lipid bilayer normal.[11] This is accomplished by measuring residual effects from anisotropic nuclear spin interactions being partially averaged by this uniaxial diffusion. By comparing the magnitudes of the averaged and nonaveraged interactions, it is possible to extract orientational information as known for oriented samples. Here, we extend this approach to accommodate the strength of the most powerful oriented sample approaches which simultaneously measure *two* anisotropic interactions to achieve the complementary structural restraints from two differently oriented nuclear spin interactions. This has been nicely exploited by the so-called PISEMA experiments measuring ^{1}H-^{15}N dipole−dipole and ^{15}N chemical shielding interactions.[15]

In this work, we will exploit, for the first time, measurement of the ^{2}H quadrupole couplings in the *two* differently oriented, deuterated methyl groups being present in the amino acid Aib. This is an ideal experiment for peptaibols, taking specific advantage of their high Aib content (∼40%)[1] which allows us to present a strategy for obtaining high-precision structural constraints from the ^{2}H quadrupole-coupling interactions measured for methyl-^{2}H labeled Aib residues (Aib-d_6) using ^{2}H magic-angle spinning (MAS) NMR. A ^{2}H NMR spectrum of a vesicle-reconstituted peptaibol with an Aib-d_6 residue will display resonances from two sites corresponding to the two methyl groups at the C^{β} positions in Aib. By measuring the reduced quadrupole couplings, a set of accurate structural restraints may be established because the quadrupole coupling tensor for the methyl groups are oriented along the $C^{\beta}-C^{\alpha}$ bonds. The advantages of Aib-deuteration is that the presence of two methyl groups leads to complementary, and thus much stronger, structural restraints than what is typically observed in ^{2}H NMR of Ala-d_3 labeling.[9,12,16,17] Furthermore, the deuterated Aib amino acid may be synthesized from inexpensive starting materials implying that the overall cost of Aib-d_6 labeling is significantly less expensive than, for example, Ala-d_3 labeling.

Materials and Methods

Synthesis of *N*-Fmoc-Aib-d_6. The amino acid Aib (2-amino-3,3,3-trideuterio-2-trideuteriomethyl propionic acid/α-amino isobutyric acid) was synthesized as previously described.[18] To a stirred solution of ammonium chloride (2.57 g, 48.00 mmol) and sodium cyanide (2.35 g, 48 mmol) in CH_2Cl_2/H_2O 1:2 (15 mL) was added acetone-d_6 (2.94 mL, 40 mmol, 99.9% deuterated, Cambridge Isotope Laboratories). The reaction vessel was sealed and left stirring for 48 h. The reaction mixture was extracted with CH_2Cl_2 three times and the organic phase was dried and concentrated under reduced pressure. The resulting residue was treated with 12 M aqueous hydrochloric acid (20 mL) at 100 °C for 2 h and concentrated under reduced pressure. The resulting colorless residue was suspended in absolute ethanol (20 mL) and filtered to remove ammonium chloride. Evaporation of the filtrate afforded 2-amino-3,3,3-trideuterio-2-trideuteriomethyl propionic acid (Aib-d_6) (2.98 g, 68%) as pale yellow crystals. ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) 174.0, 55.9, 23.5 (m, 2C).

The Fmoc protected amino acid (*N*-Fmoc-2-amino-3,3,3-trideuterio-2-trideuteriomethyl propionic acid) was synthesized from the crude Aib-d_6 above.[19] Aib-d_6 (1.64 g, 15.00 mmol), Na_2CO_3 (7.95 g, 75.00 mmol), and 9-fluorenyl chloroformate (4.66 g, 18.00 mmol) were suspended in 150 mL dioxane/water (1:1) and stirred overnight at room temperature. The reaction mixture was poured into 90 mL of water and washed with diethyl ether three times. The aqueous layer was acidified using concentrated aqueous HCl and extracted with ethyl acetate three times. The organic phase was dried with $MgSO_4$, filtered, and concentrated under reduced pressure to give pure *N*-Fmoc-Aib-d_6 (2.92 g, 59%) as a colorless solid. ^{1}H NMR (400 MHz, DMSO−D_6): δ (ppm) 12.27 (s, 1H, CO_2H), 7.89 (d, 2H, J = 7.6 Hz), 7.72 (d, 2H, J = 7.2 Hz), 7.58 (bs, 1H, NH), 7.42 (m, 2H), 7.33 (m, 2H), 4.26−4.18 (m, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) 176.0, 154.9, 143.9 (2C), 140.8 (2C), 127.7 (2C), 127.2 (2C), 125.3 (2C), 120.2 (2C), 65.3, 55.0, 46.8, 24.2 (m, 2C). HRMS (ES-TOF): $C_{19}H_{13}D_6NO_4$ [M + Na^+], 354.1582; found, 354.1578.

Synthesis of [Aib$_8$-d_6]-Alamethicin. The sequence of alamethicin employed in this work corresponds to alamethicin F30/3: Ac−UPUAUAQU*VUGLUPVUUEQ-Phol, where the amino acids are listed in one-letter code with the following additional abbreviations: Acetyl (Ac), Aib (U), Aib-d_6 (U*), and L-phenylalaninol (Phol). The peptide was synthesized by Fmoc-solid-phase peptide synthesis on 2-chlorotrityl chloride resin preloaded with phenylalaninol (0.10 mmol, 0.45 mmol/g loading).[20] Couplings were performed on a MultiSynTech semiautomatic peptide synthesizer under a nitrogen atmosphere using in situ generated Fmoc-amino acid fluorides made from the protected Fmoc-amino acid (0.40 mmol), TFFH (0.39 mmol), and DIPEA (0.80 mmol) in DMF (1 mL). Fmoc deprotection was achieved using 5 mL 20% piperidine in DMF for 5 min repeated three times for each coupling step. For Aib residue no. 8 *N*-Fmoc-Aib-d_6 was used. Coupling times were 35 min, except for U−U and P−U couplings where coupling

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Table 1. Typical Magnitudes and Orientations of the Nuclear Spin Interactions Considered in This Work

interaction (λ)		χ^λ	λ_{iso}	λ_{aniso}	η_l	geometry
^{2}H quadrupole coupling[a]	1	$\pm 3/4$[b]	0	54.1 kHz	0	$\Omega_{PA} = (0°, -55.2°, 0°)$
	2	$\pm 3/4$[b]	0	54.1 kHz	0	$\Omega_{PA} = (0°, 55.2°, 0°)$
^{15}N chemical shift[c]		1	119 ppm	99 ppm	0.19	$\Omega_{PE} = (90°, 17°, 90°)$
^{1}H-^{15}N dipolar coupling[d]		± 1	0	9.94 kHz	0	$\Omega_{PE} = (90°, 0°, 0°)$

[a] Tensor magnitude determined from the powder spectrum, and geometry determined from PDB structures of peptaibols (see text). [b] This value is the factor $3(m - {}^1/_2)/(2I(2I-1))$ for ^{2}H ($I = 1$) for the $m \leftrightarrow m-1$ transition ($m = 0,1$). [c] Typical value, see ref 23 for details. [d] Values taken from ref 24.

times were extended to 4 h. N-Terminal acetylation was performed using Ac_2O (0.70 mmol) and DIPEA (1.40 mmol) in DMF (1 mL). Cleavage and deprotection of the peptide was performed by treating the peptidyl resin with 4 mL TFA/DCM/H_2O/TIPS 47:47:4:2 (v/v) for 45 min, followed by immediate precipitation of the peptide in cold *tert*-butyl ethyl ether (80 mL). Repeated centrifugation, decantation, and trituration (four times) with cold *tert*-butyl ethyl ether gave the crude peptide (116 mg, 59%) as an off white powder. The crude peptide was dissolved in 40% MeCN in water (ca. 30 mg/mL) and semi-preparative HPLC purification was performed on an Agilent 1100 system with a Vydac 208TP510 column (C8, 10 mm ID × 250 mm, 5 μm particle diameter). Purification was achieved using a linear gradient from 40 to 60% eluant B in eluant A (eluant A being 0.1% TFA in water and eluant B being 0.1% TFA in MeCN) at a flow rate of 5 mL/min over 20 min, affording [Aib$_8$-d_6]-alamethicin F30/3 (22 mg, 11%, $t_R = 17$ min). The purity was confirmed by analytical HPLC to be >90%. MS (MALDI-TOF): $C_{92}H_{144}D_6N_{22}O_{25}$ [M + Na^+], 1992.1; found, 1992.7.

Vesicle Reconstitution of Alamethicin. The [Aib$_8$-d_6]-alamethicin vesicle-reconstituted sample was prepared by suspending 25.8 mg DMPC in 1 mL of water. The suspension was vortexed and centrifuged repeatedly until complete homogeneity was achieved. [Aib$_8$-d_6]-alamethicin (5 mg corresponding to a peptide/lipid molar ratio of 1:15) was dissolved in ~100 μL of methanol and added to the lipid suspension, which made the solution transparent. Immediately after addition of peptide and brief vortexing of the peptide/lipid sample, 10 mL of water was added, and the sample was lyophilized. The dry vesicles were resuspended in 30.8 μL of water (1:1 solid/liquid mass ratio) and transferred to a 4 mm Bruker MAS rotor.

Solid-State NMR Experiments. All ^{2}H MAS NMR experiments were performed on a 16.45-T (700 MHz) Bruker Avance-2 spectrometer with a ^{2}H Larmor frequency of 107.46 MHz. The experiments were performed on a standard 4 mm triple-resonance Bruker probe (running in double-resonance mode) using single-pulse excitation (2 μs, 50 kHz) with a repetition delay of 0.5 s, sampling of 1994 complex points with a dwell time of 10 μs (spectral width of 100 kHz), 8000 transients (corresponding to a total experiment time of 67 min), a spinning frequency of 2000 Hz, 4 mm O.D. rotors with sample volumes of 60 μL, and sample temperatures of 30 °C, which is well above the gel-to-liquid crystalline phase transition at 23 °C. The experimental spectra were processed (zero-filled to 8192 points, 50 Hz Lorentzian line-broadening) and corrected for baseline distortions by spline interpolation using the open-source software package SIMPSON.[21,22]

Data Analysis. For an *oriented bilayer sample* with the bilayer normal aligned along z in the laboratory frame, that is, collinear with B_0, the resonance frequency governed by a nuclear spin interaction (λ) is given by

$$\nu_\lambda = \lambda_{iso} + \lambda_{aniso}\chi^\lambda\kappa(\eta_\lambda,\Omega_{PM},\Omega_{MB}) \quad (1)$$

where the isotropic and anisotropic components and asymmetry parameter are represented by λ_{iso}, λ_{aniso}, and η_λ, respectively, and χ^λ is an interaction-related constant of proportionality listed in Table 1. The geometric function (κ) of the anisotropic part of the interaction is given by

$$\kappa(\eta_\lambda,\Omega_{PM},\Omega_{MB}) = \sum_{m=-2}^{2} D^{(2)}_{m,0}(\Omega_{MB}) \times \left(D^{(2)}_{0,m}(\Omega_{PM}) - \frac{\eta_\lambda}{\sqrt{6}}(D^{(2)}_{-2,m}(\Omega_{PM}) + D^{(2)}_{2,m}(\Omega_{PM}))\right) \quad (2)$$

Here, a set of Euler angles (relating the coordinate systems X and Y, defined as $\Omega_{XY} = (\alpha_{XY},\beta_{XY},\gamma_{XY})$) are introduced for the coordinate transformations from the principal axis system (P) to an molecular reference system (M) and finally to the bilayer frame (B) which for oriented samples is collinear with the laboratory frame (L). $D^{(2)}_{m,m'}$ denotes the m,m' element of a second-rank Wigner rotation matrix element. The molecular reference system may be either (i) the peptide-plane system (E), (ii) the Aib side chain system (A), or (iii) the α-helix system (H). The definition of the E and A coordinate systems is illustrated in Figure 1, while the latter α-helix system has origin in the center of the helix with the z^A axis being the helix axis and with x^A pointing from the center of the helix toward C^α. Typical Euler angles for the P $\leftrightarrow$ E coordinate transformation (Ω_{PE}) are known from numerous studies and are listed in Table 1 for the ^{15}N chemical shift ($\lambda_{iso} = \delta_{iso}$, $\lambda_{aniso} = \delta_{aniso}$, with the latter denoting the chemical shift anisotropy) and ^{1}H$-^{15}$N dipolar coupling ($\lambda_{aniso} = b_{IS}$, where b_{IS} is the dipolar coupling constant) interactions. While ^{15}N chemical shift and ^{15}N$-^1$H dipolar coupling measurements typically are interpreted by the relation of the tensors to the peptide plane (to which the amide NH spin-pair belongs), we will for the ^{2}H quadrupole coupling ($\lambda_{aniso} = C_Q$, where C_Q is the quadrupole coupling constant) relate the interaction to the Aib side-chain systems (A). Thus, we will use the Euler angles (Ω_{PA}) listed in Table 1 which are derived as averages from the conformations of all Aib's in the PDB taking the typical assumption that the principal component of the quadrupole coupling interaction (z^P) is aligned along the $C^\beta-C^\alpha$ bond vectors, which is the typical scenario for CD_3 groups.[9,16] The Euler angles relating to the helix frame (Ω_{PH}) are calculated from the molecular geometry using SIMMOL.[22,23]

From eq 2 it is evident that the resonance position is typically defined by six parameters, three define the size of the interaction tensor (isotropic value, anisotropy, and the asymmetry) and three Euler angles relate the molecule to the laboratory frame. Considering invariance toward rotations around the B_0 field axis, the latter dependency is expressed in terms of two angles, such as the τ and ρ angles. Since the magnitude of the tensor is typically known a priori, the task is to determine the two angles. Typically we have only one or two measurements per residue, and hence it is clear that the determination of these angles may be accounted for by several pairs of orientational angles as typically represented in terms of a restriction plot[25] showing which angles are compatible with the measurement. In our hands, the restriction plot (R_λ) for interaction λ for an oriented sample is calculated

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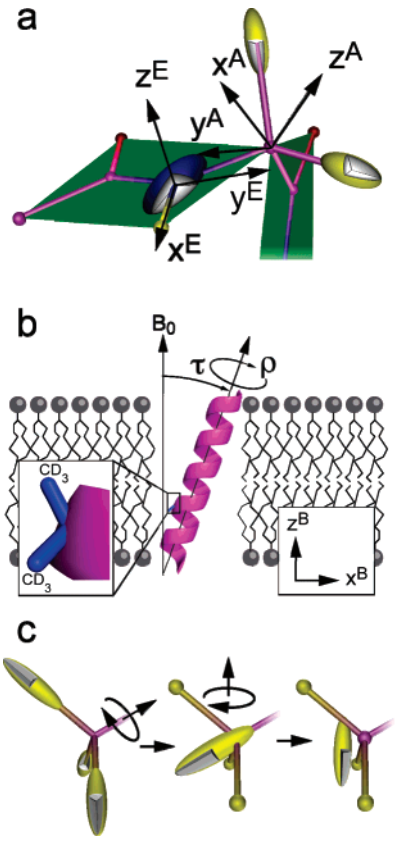

Figure 1. (a) Geometrical representation of a peptide plane and the side chain for an Aib residue, high-lighting the ^{15}N chemical shielding tensor in blue and the average ^{2}H tensors for each of the two CD_3 side-chain methyl groups in Aib. The peptide-plane coordinate system (E) is defined with x^E along the N−H bond, z^E perpendicular to the peptide plane, and y^E orthorgonal to these two axes. The Aib side-chain coordinate system (A) is defined with z^A intersecting the N−C$^\alpha$ and C′−C$^\alpha$ bonds, y^A perpendicular to the plane spanned by C$^\alpha$ and the two C$^\beta$′s, and with x^A orthogonal to these two axes. (b) Definition of the helix tilt (τ), rotational pitch (ρ) angles, and the bilayer frame (B). τ is the angle between the helix axis and the bilayer normal (z^B) for a rotation around y^B, while ρ is the rotation around the helix axis, with the zero point defined as the orientation where the C$^\alpha$ atom of the amino acid in question is located in the x^B,z^B plane. (c) Illustration of the effect of averaging of anisotropic interaction tensors by fast rotation. The leftmost figure shows the three ^{2}H quadrupole coupling tensors of the CD_3 group, the middle figure shows the averaged quadrupole coupling tensor resulting from fast rotation around the C$^\alpha$−C$^\beta$ bond, and the rightmost figure shows the averaged quadrupole coupling tensor resulting from averaging around the bilayer normal.

from comparison of the experimental resonance frequency and the calculated frequency for a particular conformation in terms of

$$R_\lambda(\Omega_{MB}) = \exp\left\{-4 \ln 2\left(\frac{\nu_\lambda^{exp} - \nu_\lambda^{sim}}{\Delta\nu_\lambda}\right)^2\right\} \quad (3)$$

where $\Delta\nu_\lambda$ represents the error limit for the experimental measurement, typically taken as the full-width-half-height line width. In this representation, the highest values of $R_\lambda(\Omega_{MB})$ reveal maximum compatibility of molecular conformations (Ω_{MB}) with the experimental data. For experiments with two restrictions, the overall restriction is achieved by multiplication of the individual restrictions, $R_{tot} = R_{\lambda 1}R_{\lambda 2}$.

For the *rotating nonoriented vesicle samples*, one should in principle consider an additional coordinate system defining the rotor (R) prior to transformation into the laboratory frame, as described extensively in the literature, for example, in refs 21 and 23. In this case, the transformation B ↔ R represents the powder averaging. Including this, the overall spinning sideband manifold may be reproduced by numerical simulations of experimental spectra as demonstrated in the subsequent section. For interpretation of the orientational information, it is sufficient that we are able to simulate the MAS spectra to provide information about the isotropic value, anisotropy, and asymmetry of the relevant nuclear spin interaction, and then compare these values with those obtained for a representative nonaveraged powder sample of the peptide in solid phase (not inserted into the hydrated membrane). In this representation, we exploit the fast rotational diffusion around the bilayer normal (z^B). This averaging is illustrated in Figure 1c which shows the 2-fold averaging of the quadrupole coupling tensor for a CD_3 group. First, the quadrupole coupling tensors for each of the three deuterons will be averaged by the fast rotation around the C−C bond. A consequence of this averaging is that we observe a partially averaged quadrupole coupling of $C_Q = 54$ kHz (represented by λ_{aniso} and listed in Table 1) while the quadrupole coupling constant for an isolated C−D is approximately three times larger[9] (aliphatic ^{2}H quadrupole couplings have been reported to $C_Q \approx 170$ kHz).[26] A second consequence is that the averaged quadrupole coupling tensor will be axially symmetric around the C−C bond. The second averaging around the bilayer normal will lead to a further reduced anisotropy of the nuclear spin interactions because of the motional averaging, such that

$$\lambda_{aniso}^{reduced} = \lambda_{aniso} \times \kappa(\eta_\lambda,\Omega_{PM},\Omega_{MB})$$
$$\eta_\lambda^{reduced} = 0 \quad (4)$$

Hence, by measuring the reduced anisotropic interaction in the nonoriented vesicle samples the geometric function may be determined as $\kappa(\eta_\lambda,\Omega_{PM},\Omega_{MB}) = \lambda_{aniso}^{reduced}/\lambda_{aniso}$ (with κ defined in eq 2), yielding exactly the same restriction plots for nonoriented samples as for oriented samples.[12,14]

Results and Discussion

With the aim of deriving accurate structural restraints from selectively labeled samples, we will consider both ^{15}N and ^{2}H labeling, as well as static and rotating samples in the following. This allows us to explore the capability of the various approaches and obtain a direct comparison for future reference. To illustrate the characteristics of the structural constraints achievable from static oriented-sample solid-state NMR, Figure 2 shows simulated ^{15}N and ^{2}H spectra for an uniaxially oriented ideal α-helical peptide (black lines in Figure 2 parts a, e, and g; black dots in 2c). This helical peptide has a single residue labeled with an ^{15}N amide atom and a single Aib residue isotope labeled with ^{2}H, respectively. The spectra act as input for the analysis, and by comparison with the corresponding powder patterns the so-called restriction plots showing which molecular conformations are in agreement with the input data are derived from eq 3. The most frequently used experimental approach relies on ^{15}N labeling of one (or more) backbone amide atom-(s) and recording a 1D ^{15}N spectrum (Figure 2a). This spectrum provides a continuum of possible conformations (Figure 2b) with helix tilt angles ranging from ∼5° to ∼30°. The 2D ^{1}H−^{15}N dipolar coupling/^{15}N chemical shift separated-local-field (SLF) experiment (Figure 2c) is more favorable since it leads to a much narrower conformational restriction (Figure 2d). This confined restriction plot is clearly advantageous since it leads to a more accurate determination of the molecular conformation, but this is achieved at the expense of needing to record a time-consuming 2D experiment, although methods to increase the speed of the 2D acquisition have recently been presented.[27] The

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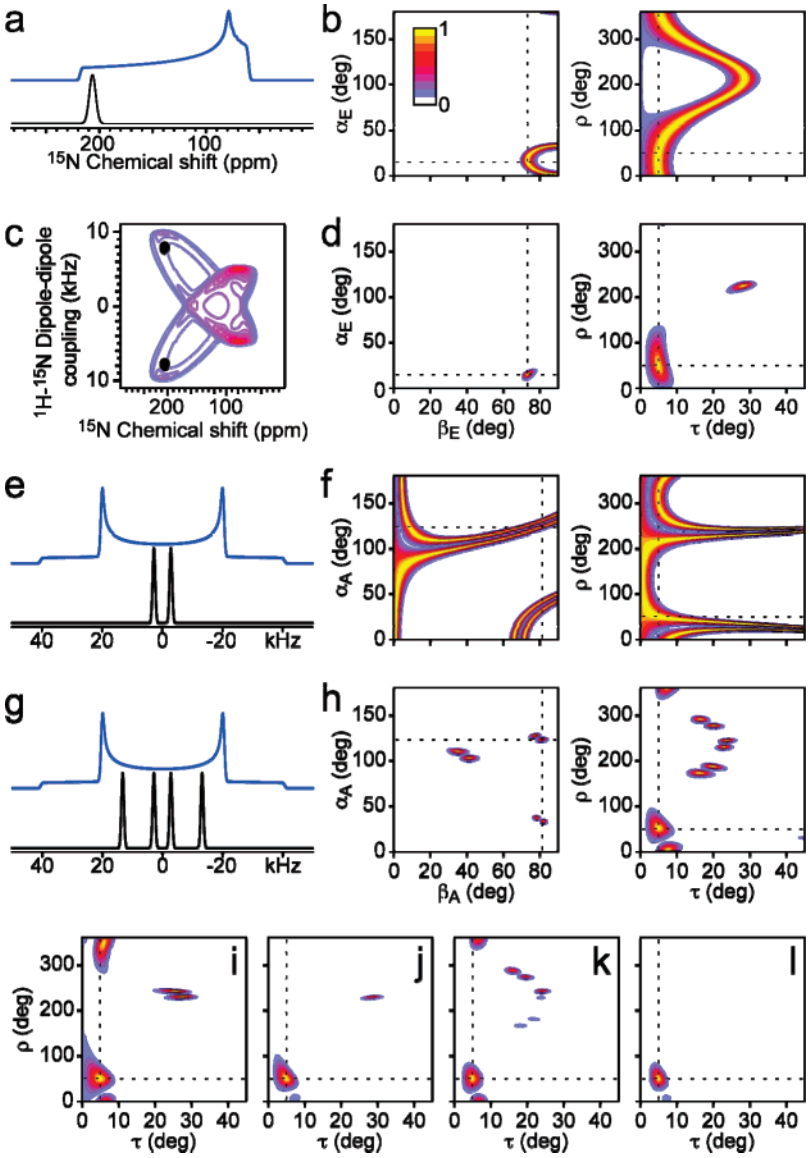

Figure 2. (a,c,e,g) Simulated ^{15}N (a,c) and ^{2}H (e,g) static-sample spectra for an ideal α-helical peptide (torsion angles $\phi,\psi = -65°, -40°$) with tilt and rotational pitch angles of $\tau,\rho = 5°,50°$ along with the corresponding powder spectra. (b,d,f,h) Restriction plots showing in color the conformations in terms of local orientations α_X,β_X (X represents either the peptide-plane (E) or Aib side-chain (A) coordinate system) (left) and helix conformatinons τ,ρ (right) being in agreement with the simulated spectra in panels a, c, e, g, respectively. (a) ^{15}N and (c) ^{1}H-^{15}N SLF spectra of the backbone amide. ^{2}H spectra of the methyl group in (e) an Ala and (g) an Aib residue. (i−l) Combined restrictions from ^{15}N and ^{2}H data (i,k) use the ^{15}N 1D spectrum in panel a, (j,l) use the SLF spectrum in panel c, (i,j) use the ^{2}H spectrum in panel e, and (k,l) use the ^{2}H spectrum in panel g. The intersection of the dashed lines in panels b, d, f, h, and i−l represents the input values. The inset in panel b shows the color contour levels.

third approach (Figure 2e) employs ^{2}H labeling of the methyl group of an Ala residue for which the orientation of the ^{2}H quadrupole coupling tensor is along the $C_\beta-C_\alpha$ bond. Using this knowledge, the quadrupolar splitting in the ^{2}H spectrum of the oriented peptide may be used to deduce orientational restraints as shown in Figure 2f.[7,10−12,16] As for the case of 1D ^{15}N spectra, this approach provides a continuum of possible conformations, and a combination of information from several labeled residues would be needed for determination of accurate molecular conformations. Addressing these typical experiments, our simulations support the known fact that the 2D SLF experiment provides much better structural restraints than the 1D experiments since it combines the complementary structural information from two different nuclear spin interactions.

Owing to the high content of Aib residues in peptaibols, they offer an alternative strategy, which is an inexpensive and fast method for obtaining structural restraints on peptaibols. The basis of this strategy is that Aib has two methyl groups in the β position (C^β), which upon deuteration will enable complementary structural restraints to be obtained from the ^{2}H spectra of each of the two deuterated methyl groups. A first investigation of this idea is illustrated by the simulated ^{2}H experiments in Figure 2g, and the restriction plots (Figure 2h) which clearly reveal a significant confinement of the restraints when comparing with the example of Ala-deuteration (Figure 2f). In fact, the use of methyl-deuterated Aib's (Aib-d_6) provides conformational restraints similar to those achieved from time-consuming 2D experiments (Figure 2c,d). However, while the 2D SLF experiment reduces the continuum of allowed conformations observed in the 1D experiment to only two possible configurations (here at τ,ρ ~5°,50° and ~28°,210°), the restriction plot from ^{2}H methyl-deuterated Aib shows a few more allowed

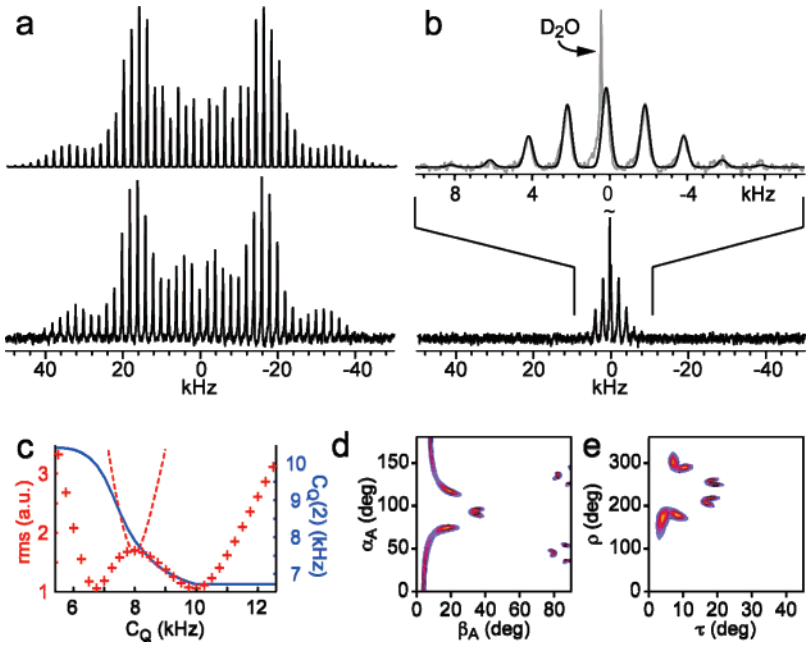

Figure 3. (a,b) Experimental (bottom) and simulated (top) ^{2}H MAS NMR spectra of (a) lyophilized and (b) vesicle-reconstituted [Aib$_8$-d_6]-alamethicin. In panel b an expansion of the experimental spectrum is shown in gray behind the simulated spectrum. Note the sharp line from the natural-abundance D_2O signal from water in the sample. (c) Plot of the root-mean-square (rms) deviation between experimental spectrum in panel b and simulated spectrum as a function of the quadrupole coupling constant for one of the two sites (red points) along with the quadrupole coupling constant for the other site (blue line). The red dashed line shows the rms curve when assuming only one site. Restriction plot showing conformations of (d) the Aib side-chain coordinate system and (e) the alamethicin molecule assuming it has an ideal α-helical structure. The color coding of the restriction plot is as in Figure 2.

conformations (in this particular case it has two large and six small allowed conformations). This is because it is not possible to unambiguously assign the two doublets in Figure 2g to each of the two CD_3-groups. Hence, we need to include this ambiguity in the restriction plot by, for each conformation (Ω_{MB}), evaluating which assignment gives the largest value for R_{tot} (cf. eq 4). Despite the few extra allowed conformations, the advantage of using Aib-d$_6$ labeling over Ala-d_3 labeling is indeed confirmed when comparing the restriction plots in Figure 2 parts f and h. Aisenbrey and Bechinger recently suggested the combined use of ^{15}N and ^{2}H labeling to provide the desired two complementary structural restraints.[7,11] To follow this idea, Figure 2 (i−l) shows the restriction plots obtained using combinations of the ^{15}N and ^{2}H restriction plots in Figure 2. Specifically, Figure 2 parts i and j represent restriction plots which could be achieved from ^{15}N and ^{2}H labeling of an Ala residue, while Figure 2 parts k and l show the corresponding plots for ^{15}N and ^{2}H labeling of an Aib residue. The difference from Figure 2i to 2j and 2k to 2l is that Figure parts 2i and 2k employ the restrictions from the 1D ^{15}N spectrum in Figure 2a, while Figure parts 2j and 2l employ restrictions from the 2D ^{1}H-^{15}N SLF experiment in Figure 2c. Interestingly, we observe in the present example, that only when data from four different nuclear spin interactions (^{15}N chemical shift, ^{1}H−^{15}N dipole−dipole coupling, and two ^{2}H quadrupole couplings), the restriction plot (Figure 2l) shows only one allowed conformation.

Given the delicate and time-consuming procedures for preparation of aligned samples we have chosen to measure the motionally averaged ^{2}H quadrupole coupling tensors of the CD_3 groups for alamethicin reconstituted in lipid vesicles from ^{2}H MAS NMR experiments of unoriented samples.[7,11−14] Our test sample may appear in different oligomeric channel-forming states in the membrane. Although some of the larger oligomers may be slightly too large to fulfill the requirement for fast rotational diffusion,[14] these assemblies would display significantly reduced signal intensity due to the increased exchange-induced T_2 relaxation, or simply display unaveraged powder spectra.[11]

The ^{2}H MAS NMR spectrum of [Aib$_8$-d_6]-alamethicin as a lyophilized powder is shown in Figure 3a. We will use this spectrum as the unaveraged spectrum. Alternatively, one could use the vesicle-reconstituted sample at low temperature, so the uniaxial averaging is frozen out, to achieve the same. We find the prior approach much simpler and significantly more sensitive. The only potential problem in using the lyophilized sample is that hydrogen-bonding patterns may often be different in lyophilized samples than in vesicle-reconstituted samples. However, this should not be problematic for the study of the deuterated methyl groups of Aib, which are not expected to participate in the normal hydrogen-bonding patterns. The unaveraged spectrum in Figure 3a may be modeled using SIMPSON[21,22] (Figure 3a) with one set of quadrupole coupling parameters ($C_Q = 53.1 \pm 2.1$ kHz, $\eta = 0$). Introducing a second site to the optimization yielded the same value for the two quadrupole coupling parameters, and hence the two methyl groups display the same value for the quadrupole coupling in this sample.

The ^{2}H MAS NMR spectrum of the peptide reconstituted into lipid vesicles at a peptide/lipid ratio of 1:15 gives the spectrum in Figure 3b. In this spectrum, we observe a significantly narrower spinning-sideband envelope indicating that the sample undergoes the assumed fast uniaxial diffusion. The spectrum may be modeled using two sites $C_Q(1) = 6.7 \pm 0.8$ kHz and $C_Q(2) = 10.0 \pm 1.5$ kHz. To demonstrate the reliability of this optimization, Figure 3c shows (with red points) the rms deviation from simulation to experimental spectrum as a function of one of the quadrupole coupling constants and the corresponding optimized value for the other quadrupole coupling

constant (blue line). The rms curve displays two clear-cut minima in agreement with the assumption of the presence of two sites, one for each of the methyl groups. Assuming only one site would disagree with our model and is strongly disfavored by its rms curve (dashed red line in Figure 3c). The scaled quadrupole couplings determined for the vesicle-reconstituted sample may be used to calculate the restriction plots in Figure 3 parts d and e by use of eqs 2−4. From Figure 3e we observe the allowed conformations $(\tau,\rho) = (6° \pm 2°, 175° \pm 5°)$; $(9° \pm 2°, 295° \pm 5°)$; $(17° \pm 1°, 215° \pm 5°)$; and $(17° \pm 1°, 255° \pm 5°)$ assuming an α-helical structure of alamethicin. Confining these to a single helix conformation would require measurements on samples with labeling of other amino acids, but we note that these four possible conformations represent significantly more accurate restrictions than could be achieved from other 1D experiments (cf. Figure 2). We further note that the two major restrictions corresponding to helix tilt angles of 6−9° agree well with previous work.[3]

The ^{2}H MAS spectrum of vesicle-reconstituted [Aib$_8$-d_6]-alamethicin in Figure 3b was achieved from a sample containing 5 mg of peptide in only 67 min. For comparison, we will typically need several hours for a 1D ^{15}N experiment and beyond 24 h to record a 2D SLF experiment of an oriented ^{15}N labeled peptide with the same peptide quantity. Combined with the ease of preparing the sample, we find that methyl-deuteration of Aib's provide an attractive alternative to conventional ^{15}N-based approaches for solid-state NMR structural characterization of peptaibols. In addition, the low cost of and simplicity of synthesizing deuterated Aib represent a great advantage of the present approach. Aib may be synthesized directly by a Strecker synthesis employing acetone, ammonium chloride, and sodium cyanide as starting materials.[18] Since the chemistry is well-established and the amino acid contains no chiral centers, the yield is generally good. Deuteration of the methyl groups of Aib is achieved using acetone-d_6 as starting material. Consequently, we have been able to keep the cost of synthesizing deuterated Fmoc-protected Aib low, almost as low as for unlabeled Fmoc-Aib from commercial sources.

Conclusion

In conclusion, we have demonstrated that ^{2}H MAS NMR experiments on vesicle-reconstituted peptaibols with Aib-d_6 labeling provide a fast and inexpensive path to accurate conformational constraints. In the present example of [Aib$_8$-d_6]-alamethicin we have found very narrow restrictions on the molecular conformation from 1D ^{2}H experiments, which are largely superior than the restrictions one could achieve in the same amount of time from a single ^{15}N label or a single deuterated methyl group in an alanine residue.

Acknowledgment. Support from the Danish National Research Foundation, the Danish Natural Science Research Council, Carlsbergfondet, and the Danish Biotechnology Instrument Centre is acknowledged.

JA0749690