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Dilute spin-exchange assignment of solid-state NMR spectra of oriented proteins: Acetylcholine M2 in bilayers

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Abstract

The assignment of amide resonances in the two-dimensional PISEMA (*Polarization Inversion with Spin Exchange at the Magic Angle*) spectrum of uniformly ¹⁵N labeled M2 peptide corresponding to the channel-lining segment of the acetylcholine receptor in oriented phospholipid bilayers is described. The majority of the resonances were assigned through comparisons with spectra from selectively ¹⁵N labeled recombinant peptides and specifically ¹⁵N labeled synthetic peptides. Some resonances were assigned to specific amino acid residues by means of homonuclear ¹⁵N spin-exchange spectroscopy. A modification to the conventional spin-exchange pulse sequence that significantly shortens the length of the experiments by combining the intervals for ¹⁵N spin-exchange and ¹H magnetization recovery is described.

Keywords

acetylcholine receptor; M2 channel peptide; membranes; PISEMA; resonance assignments; solid-state NMR spectroscopy; spin-exchange

Introduction

Solid-state NMR spectroscopy is capable of determining the three-dimensional structures of membrane proteins in phospholipid bilayers. The proteins are immobilized within the lipid bilayers and behave essentially like solids, enabling high resolution NMR spectra containing structural information to be obtained by magic angle sample spinning (Griffiths and Griffin, 1993; McDowell and Schaefer, 1996; Smith et al., 1996), or by uniaxially orienting the sample with respect to the direction of the applied magnetic field (Cross and Opella, 1994; Marassi and Opella, 1998). Solid-state NMR of oriented samples is unique in its ability to determine the complete topology, including three-dimensional structure and orientation, of a protein within the bilayer (Ketchum et al., 1993; Opella et al., 1999). Solid-state NMR spectra of oriented samples are characterized by single line resonances in all frequency dimensions, and since the observed resonance frequencies depend on the orientation of the

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molecular sites relative to the magnetic field, they provide the orientational constraints used in structure determination (Opella et al., 1987).

The recent development of a family of two-, three-, and four-dimensional solid-state NMR experiments (Wu et al., 1994; Ramamoorthy et al., 1995a,b) makes it feasible to obtain completely resolved spectra from uniformly ^{15}N labeled membrane proteins in oriented bilayers (Marassi et al., 1997). The solid-state NMR experiments for two-dimensional ^1H - ^{15}N dipolar coupling/ ^{15}N chemical shift correlation, and three-dimensional ^1H chemical shift/ ^1H - ^{15}N dipolar coupling/ ^{15}N chemical shift correlation, are based on the PISEMA (*Polarization Inversion with Spin Exchange at the Magic Angle*) pulse sequence (Wu et al., 1994; Ramamoorthy et al., 1995a). In the 3D spectra each resonance arises from a single amide site and is characterized by ^1H chemical shift, ^1H - ^{15}N dipolar coupling and ^{15}N chemical shift frequencies. These three frequency dimensions provide the means for resolving resonances in the spectra of uniformly labeled proteins as well as the simultaneous measurement of multiple orientationally dependent parameters.

Uniform ^{15}N labeling of proteins was first devised for solid-state NMR spectroscopy (Cross et al., 1982); however, in the past, nearly all studies of membrane proteins in bilayers have required samples labeled at one or only a few sites because of the limited resolution of the spectra. This made determination of complete structures laborious, and limited applications to peptides which could be prepared by solid-phase synthesis, small proteins with favorable distributions of amino acids, or selected regions of larger proteins. The ability to resolve resonances in the solid-state NMR spectra of uniformly labeled proteins extends the application of solid-state NMR spectroscopy to membrane proteins that can be expressed and isotopically labeled in bacteria. For example, we demonstrated experimentally that the membrane protein Vpu from HIV-1 has one transmembrane helix and two in-plane amphipathic helices in membrane bilayers (Marassi et al., 1996). In addition, we were able to show that the membrane-bound form of the 190-residue colicin E1 channel polypeptide has a 38-residue helical hairpin inserted into the bilayer, which is consistent with the 'umbrella' model for its interaction with the membrane (Kim et al., 1998).

We have recently determined the 3D structure of the 25-residue functional peptide corresponding to the predicted channel-lining M2 segment of the nicotinic, ligand-gated acetylcholine receptor (AChR) in phospholipid bilayers (Opella et al., 1999). The amino acid sequences of M2 segments are highly conserved, and both mutagenesis and affinity labeling studies identify them as the major polypeptide component of the pores responsible for the ion-channel activity of the AChR (Lena and Changeux, 1997; Colquhoun and Sakmann, 1998). Ion channel measurements on both recombinant and synthetic AChR M2 peptides indicate that the peptides used in the NMR experiments are functional, and form sequence-specific, discrete ion-channels in lipid bilayers (Opella et al., 1999). The AChR M2 peptide forms a straight transmembrane α -helix with no kinks. The helix inserts in the lipid bilayer at an angle of 12° relative to the bilayer normal, with a rotation about the helix long axis such that the polar residues face the N-terminal side of the membrane, which is assigned to be intracellular. A model of the channel based on the solid-state NMR structure of the M2 peptide, and assuming a symmetric pentameric arrangement in bilayers, results in a funnel-like architecture for the channel, with the wide opening on the N-terminal intracellular side (Opella et al., 1999).

The orientational constraints used for structure determination of AChR M2 peptide were obtained from the ^{15}N chemical shift and ^1H - ^{15}N dipolar coupling frequencies measured from a single 2D PISEMA spectrum of uniformly ^{15}N labeled M2 peptide in oriented bilayers. For some of the specifically ^{15}N labeled AChR M2 peptides, ^1H chemical shift

frequencies were measured from 2D ^{15}N chemical shift/ ^1H chemical shift HETCOR (HETeronuclear CORrelation) spectra (Ramamoorthy et al., 1995a).

In this article we describe the assignment of the solid-state NMR resonances from AChR M2 in oriented lipid bilayers. The resonances were assigned to specific residues with a combination of homonuclear ^{15}N spin-exchange spectroscopy and comparisons to spectra from selectively and specifically labeled samples. ^{15}N spin-exchange can be effected by spin diffusion or cross relaxation processes originating in the homonuclear dipole-dipole interactions (Jeener et al., 1979), and always discriminates strongly towards pairs of nuclei in close proximity. As a result, it offers a method for assigning resonances from sites in adjacent residues. Homonuclear spin-exchange experiments provide a general assignment strategy for solid-state NMR spectra of uniformly ^{15}N labeled proteins. Homonuclear spin-exchange has been demonstrated among abundant ^1H nuclei in model peptides (Ramamoorthy et al., 1996a), and among ^{15}N and ^{13}C sites in peptides, proteins, and nucleic acids (Cross et al., 1983; Frey and Opella, 1984; Morden and Opella, 1986; Ramamoorthy et al., 1995b).

Materials and methods

N-acetyl-L-valyl-L-leucine (NAVL)

The synthesis and crystallization of the dipeptide NAVL, labeled with ^{15}N in both amide sites has been described (Ramamoorthy et al., 1995b).

M2 peptides

Recombinant peptides were derived from fusion proteins expressed in *E. coli*. Amino acid sequences corresponding to the M2 segments were separately cloned into the glutathione S-transferase (GST) (Pharmacia Biotech, Uppsala, Sweden), or maltose binding protein (MBP) (New England Biolabs, Beverly, MA) fusion protein expression systems. For uniformly labeled samples, *E. coli* cells were grown in media containing ^{15}N enriched ammonium sulfate. For selectively labeled samples, the growth media contained all amino acids with only one type ^{15}N labeled. The fusion proteins were isolated by affinity or ion exchange chromatography, and cleaved enzymatically with FXa (for the MBP-AChR M2 fusion) or thrombin (for the GST-AChR M2 fusion). The M2 peptides were isolated by gel filtration or ion exchange chromatography and purified by HPLC. After cleavage from the carrier protein, the recombinant AChR M2 peptide has the sequence XS EKMST AISVL LAQAV FLLLT SQR where X is Gly in peptides derived from GST fusion proteins, or Ile in peptides derived from MBP fusion proteins. The underlined residues are introduced by the expression system. All isotopically labeled materials were from Cambridge Isotope Laboratories (Andover, MA). Synthetic specifically ^{15}N labeled peptides were prepared as described (Iwamoto et al., 1994).

Oriented bilayer samples

The samples were prepared at a protein/lipid molar ratio of 1.4/100. They contained 2 mg of ^{15}N labeled AChR M2 peptide and 40 mg of 1,2-dimyristoyl-sn-glycerophosphocholine (DMPC) (Avanti Polar Lipids, Alabaster, AL). DMPC was dissolved in trifluoroethanol (TFE) and added to the dry peptide, the solution was then bath sonicated for 5 min and allowed to stand overnight at -20°C . The solution was evenly distributed over the surface of 25 glass slides with dimensions $11 \times 11 \times 0.07$ mm (Marienfeld Glassware, Bad Margentheim, Germany). The TFE was evaporated under high vacuum and 2 μl of sterile filtered water was added to each plate. The plates were stacked and placed in a chamber containing a saturated solution of ammonium phosphate. Oriented bilayers formed upon hydration of the sample in this chamber at 30°C for 15 h. Before insertion into the RF coil

of the NMR probe, the sample was wrapped in a thin layer of parafilm and sealed in a thin film of poly-ethylene in order to maintain sample hydration during the experiments.

Solid-state NMR spectroscopy

The experiments were performed on a home-built spectrometer with a wide-bore Magnex 550/89 magnet and on a Chemagnetics-Otsuka Electronics (Ft. Collins, CO) spectrometer with a wide bore Oxford 400/89 magnet. The single coil probes were double tuned to the resonance frequencies of ^1H at 549.9 (400.4) MHz, and ^{15}N at 55.7 (40.6) MHz. In both probes the square, 4 turn coils had inner dimensions of $11 \times 11 \times 2$ mm. One-dimensional ^{15}N chemical shift spectra were obtained with single contact, 1 ms, CPMOIST (Cross-Polarization with Mismatch-Optimized IS Transfer) cross-polarization to generate ^{15}N magnetization that was acquired in the presence of continuous ^1H irradiation (Pines et al., 1973; Levitt et al., 1986). The 2D PISEMA experiments (Wu et al., 1994) are based on the principle of separated local field spectroscopy (Waugh, 1976), and utilize flip-flop, phase- and frequency-switched Lee-Goldburg homonuclear decoupling (Lee and Goldburg, 1965; Mehring and Waugh, 1972; Bielecki et al., 1990) to provide line-narrowing in the ^1H - ^{15}N dipolar coupling dimension.

For the samples of uniformly ^{15}N labeled M2 peptide in lipid bilayers, the Lee-Goldburg off-resonance condition for magic angle RF irradiation was satisfied by ^1H frequency jumps of 35.4 kHz, as dictated by a ^1H RF field strength of 1.2 mT. During the SEMA (Spin Exchange at the Magic Angle) period the ^{15}N RF field strength was increased to 1.4 mT in order to match the ^1H effective RF field strength. In all experiments the ^1H RF field strength was increased to 2.0 mT during data acquisition in order to decouple the heteronuclear ^1H - ^{15}N dipolar interaction. For some of the specifically ^{15}N labeled AChR M2 samples 2D ^1H chemical shift/ ^{15}N chemical shift HETCOR spectra were obtained by fixing the SEMA period, which is incremented in the 3D correlation pulse sequence (Ramamoorthy et al., 1995a). Other conditions for the 1D and 2D solid-state NMR experiments were as described (Opella et al., 1998). For the ^{15}N labeled NAVL single crystal the ^1H frequency jumps were 50.5 kHz, as dictated by a ^1H RF field strength of 1.7 mT. During the SEMA period, the ^{15}N RF field strength was increased to 2.0 mT in order to match the ^1H effective RF field strength. In all experiments, a ^1H RF field strength of 3.5 mT was used during data acquisition. The NMR data were processed using the program FELIX (Biosym Technology, San Diego, CA). The ^{15}N and ^1H chemical shifts were referenced to 0 ppm for liquid ammonia and tetramethylsilane, respectively.

Results and discussion

The pulse sequences for the 2D and 3D spin-exchange experiments are diagrammed in Figure 1. The principal difference between the homonuclear dilute spin-exchange experiment shown in Figure 1A (Szeverenyi et al., 1982) and the ^1H exchange experiment originally introduced for solution NMR spectroscopy (Jeener et al., 1979) is that cross polarization (CP) replaces the $\pi/2$ pulse in the preparation period. During t_1 the ^{15}N spin chemical shift frequencies evolve under ^1H decoupled conditions identical to those of the direct detection period, t_2 . A $\pi/2$ pulse rotates the ^{15}N magnetization to the z axis where homonuclear spin-exchange occurs among neighboring ^{15}N spins during the mixing time t_m . Finally, a $\pi/2$ pulse rotates the ^{15}N magnetization back to the transverse plane for detection with continuous ^1H frequency irradiation for heteronuclear ^1H - ^{15}N decoupling. For proteins in oriented lipid bilayers, recycle delays of at least 5 s are required for complete T_1 relaxation of the ^1H magnetization following the detection period. A flip-back $\pi/2$ pulse at the end of the detection period returns residual ^1H spin magnetization to the z axis, but has limited effectiveness in protein samples with short rotating frame relaxation times (Tegenfeldt and Haeberlen, 1979).

The 3D pulse sequence shown in Figure 1B combines PISEMA with dilute ^{15}N spin exchange. It yields spectra where ^{15}N chemical shift resonances, acquired in t_3 , are correlated by homonuclear ^{15}N spin-exchange in t_2 , and ^1H - ^{15}N dipolar coupling in t_1 . During the SEMA period in t_1 , ^1H - ^{15}N dipolar coupling frequencies evolve while the ^1H - ^1H homonuclear dipolar interactions are decoupled using flip-flop Lee–Goldburg irradiation. ^{15}N chemical shift frequencies evolve during t_2 and are ^1H decoupled, under conditions identical to those of the direct detection period in t_3 . Homonuclear ^{15}N spin-exchange occurs during the mixing time t_m .

The homonuclear ^{15}N - ^{15}N dipolar couplings in proteins are weak because the gyromagnetic ratio of ^{15}N is low and the relevant ^{15}N - ^{15}N internuclear distances are long. The distances from residue i to $i + 1$ vary between about 2.8 Å for α -helices, and 3.5 Å for β -sheets. This corresponds to couplings that vary between 0 and 30 Hz, depending on the orientation of the internuclear vector relative to the magnetic field. Such small dipolar couplings result in slow homonuclear spin exchange and mixing times of several seconds are required for magnetization transfer to occur. The mixing interval adds significantly to the length of the experiments.

The versions of 2D and 3D pulse sequences for ^{15}N spin-exchange experiments shown in Figures 1C and 1D are more efficient because they combine the functions of the dilute spin mixing interval and the recycle delay for recovery of ^1H magnetization. A $\pi/2$ flip-back pulse at the beginning of the mixing period rotates the ^1H magnetization to the z axis. This not only prevents it from being dephased during t_m , it also enables recovery due to T_1 relaxation. At the end of t_m the ^1H spins are rotated into the xy plane by another $\pi/2$ pulse, and spin locked along the y axis during the detection of ^{15}N magnetization. The ^1H spin-lock irradiation serves both to decouple the ^1H - ^{15}N dipolar interaction during data acquisition, and to preserve ^1H spin polarization. ^{15}N magnetization is generated by cross polarization immediately following data acquisition. In these pulse sequences the deletion of the recycle delay period allows 2D and 3D spectra to be obtained in a much shorter time than is permitted by the corresponding experiments shown in Figures 1A and 1B. The long mixing times required for ^{15}N spin-exchange maintain low RF duty cycles and prevent sample heating.

Since magnetization in the rotating frame decays with a time constant characterized by $T_{1\rho}$, the successful implementation of the experiments in Figures 1C and 1D requires samples with ^1H $T_{1\rho}$ values that are longer than the combine times for signal detection in t_2 or t_3 (5 ms), and cross-polarization (1 ms). Crystalline samples like NAVL have ^1H $T_{1\rho}$ values of several seconds and satisfy this condition easily. However, the ^1H $T_{1\rho}$ values of proteins incorporated in phospholipid bilayers are typically only a few milliseconds, and this precludes the use of the pulse sequences in Figures 1C and 1D at room temperature. The ^1H $T_{1\rho}$ values can be lengthened by performing the experiments at low temperatures, or by diluting the ^1H nuclei with ^2H nuclei through the use of uniformly ^2H labeled samples.

The two-dimensional ^{15}N spin-exchange spectrum of a single crystal of ^{15}N labeled NAVL is shown in Figure 2A. The four diagonal ^{15}N resonances result from the two amide nitrogens in each of the two magnetically inequivalent molecules of the crystal unit cell. The cross peaks indicate which pairs of diagonal resonances exchanged their magnetization via the weak homonuclear dipolar couplings during the mixing time interval. The two ^{15}N nuclei in each dipeptide molecule are separated by 3.47 Å (Carroll et al., 1990) and cross peaks identify those pairs of nitrogen sites. The closest intermolecular ^{15}N nuclei are separated by 4.99 Å and substantially longer mixing times (about 8 s) are required to effect spin exchange between these sites (Ramamoorthy et al., 1996b). Thus, the resonances labeled a and b arise from the two nitrogens in one molecule of the unit cell, while

resonances c and d are from the two nitrogens in the other molecule. The different cross peak intensities observed for the two molecules may be attributed to their different orientation with respect to the magnetic field.

In these experiments the resolution is limited by the ^{15}N chemical shift in both frequency dimensions, therefore the 2D ^{15}N spin-exchange spectrum of uniformly ^{15}N labeled M2 peptide in bilayers is highly overlapped (Figure 3A). Additional frequency axes are necessary for resolution in proteins that are uniformly ^{15}N labeled. The resolution available from 2D correlation PISEMA spectra is very high, as illustrated in Figures 2B and 3B for the dipeptide crystal and AChR M2, respectively. The 3D PISEMA experiment with homonuclear ^{15}N spin-exchange provides both sufficient resolution as well as a resonance assignment pathway. Spectral strips taken from the 3D spectra are shown in Figure 2C for the dipeptide crystal, and in Figure 3C for the AChR M2 in oriented lipid bi-layers. The strips were each taken at specific dipolar coupling frequencies. The cross peaks between neighboring ^{15}N nuclei were used to trace the backbone connectivity between residues 6 and 10, and the resonances from residues 6 and 8 in the PISEMA spectrum of the AChR M2 could be assigned in this way. In α -helices the internuclear ^{15}N - ^{15}N distances for non-neighboring residues i to $i+2$, $i+3$ and $i+4$ are long (4.2, 5.3 and 6.8 Å), and mixing times longer than the 3 s used in this experiment are required to effect ^{15}N spin exchange between these sites. At longer mixing times ^{15}N magnetization is lost through T_1 relaxation processes and the spectral intensity severely decreases. The cross peaks observed in Figure 3C are from ^{15}N spin exchange between residues i and $i+1$.

The other resonances in the PISEMA spectrum of uniformly ^{15}N labeled AChR M2 were assigned to specific residues through comparisons with spectra from selectively ^{15}N labeled recombinant peptides and specifically ^{15}N labeled synthetic peptides. The 2D PISEMA spectra shown in Figure 4 were obtained from synthetic AChR M2 peptides specifically ^{15}N labeled at Ala6 (Figure 4A), or Leu10, Leu11 and Ala12 (Figure 4B). The spectrum in Figure 4C was obtained from recombinant M2 selectively labeled with ^{15}N -Ile. There are two Ile residues in the M2 peptide derived from the MBP-AChR M2 fusion protein, one at the N-terminus and Ile⁻⁷. In the N-terminal Ile the amino nitrogen has a ^{15}N chemical shift frequency of 36 ppm and a dipolar coupling near 0 kHz and falls outside the amide ^{15}N chemical shift range. Thus, the resonance in Figure 4C is from Ile7.

The orientationally dependent ^{15}N chemical shift and ^1H - ^{15}N dipolar coupling frequencies measured from the PISEMA spectrum in Figure 3B provided orientational constraints for the structure determination of AChR M2 in lipid bilayers (Opella et al., 1998). The ^1H chemical shift frequencies measured from 2D $^1\text{H}/^{15}\text{N}$ HETCOR spectra, for some residues, provided an additional set of constraints. The HETCOR spectrum of synthetic M2, ^{15}N labeled at Leu10, Leu11 and Ala12 is shown in Figure 4D. All the frequencies are listed in Table 1. They were input to a program that calculates the allowed orientation of each peptide plane relative to the magnetic field (Tycko et al., 1986). Neighboring peptide planes of fixed orientation were then connected through their common C_α atom using a program that calculates the dihedral angles ϕ and ψ for those neighboring peptide combinations that satisfy a tetrahedral angle geometry at the α -carbon (Marassi and Opella, unpublished).

The ability to obtain completely resolved solid-state NMR spectra from uniformly ^{15}N labeled proteins in lipid bilayers necessitates the development of pulse sequences for resonance assignment. The data in Figure 3 demonstrate that it is possible to assign resonances in the solid-state NMR spectra of a uniformly ^{15}N labeled protein in oriented lipid bi-layers using homonuclear spin-exchange experiments. A complementary strategy involving triple resonance experiments on ^{13}C and ^{15}N labeled samples is also under development (Tan et al., 1999).

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Figure 1.

2D and 3D pulse sequences for resonance assignment using homonuclear ^{15}N spin-exchange spectroscopy. (A, C) 2D pulse sequences for ^{15}N chemical shift/ ^{15}N chemical shift correlation. (B, D) 3D pulse sequences for ^1H - ^{15}N dipolar coupling/ ^{15}N chemical shift/ ^{15}N chemical shift correlation. The sequences in (C) and (D) make more efficient use of the ^1H magnetization.

Figure 2.

Homonuclear ^{15}N spin-exchange resonance assignment of the spectrum from a ^{15}N labeled dipeptide N-acetyl-L-valyl-L-leucine single crystal. (A) 2D ^{15}N spin-exchange spectrum acquired using the pulse sequence shown in Figure 1C. Four transients were coadded for each of 128 t_1 values incremented by $40\ \mu\text{s}$. The mixing time was 4.0 s. (B) 2D PISEMA spectrum. Four transients were coadded for each of 64 t_1 values incremented by $28.6\ \mu\text{s}$. The recycle delay was 3 s. (C) Spectral strips from a 3D PISEMA with ^{15}N spin-exchange spectrum, acquired using the pulse sequence shown in Figure 1D. Experimental conditions were as described for the 2D experiments.

Figure 3.

Homonuclear ^{15}N spin-exchange resonance assignment of the spectrum from uniformly ^{15}N labeled AChR M2 peptide in oriented lipid bilayers. (A) 2D ^{15}N spin-exchange spectrum acquired using the pulse sequence shown in Figure 1A. One hundred and twenty eight transients were coadded for each of 128 t_1 values incremented by $40\ \mu\text{s}$. The mixing time was 3.0 s, and the recycle delay was 2 s. (B) 2D PISEMA spectrum. One hundred and twenty eight transients were coadded for each of 64 t_1 values incremented by $40.8\ \mu\text{s}$. The recycle delay was 4 s. (C) Spectral strips from a 3D PISEMA with ^{15}N spin-exchange spectrum, acquired using the pulse sequence shown in Figure 1B. Experimental conditions were as described for the 2D experiments.

Figure 4.

(A, B, C) 2D ^1H - ^{15}N dipolar coupling/ ^{15}N chemical shift correlation PISEMA spectra from ^{15}N labeled AChR M2 peptides in oriented lipid bilayers. (A) Synthetic AChR M2 specifically ^{15}N labeled at Ala6. (B) Synthetic AChR M2 specifically ^{15}N labeled at Leu10, Leu11 and Ala12. (C) Recombinant AChR M2 selectively ^{15}N labeled at Ile. In each spectrum 64 t_1 values were incremented by $40.8\ \mu\text{s}$, and the recycle delay was 4 s. (D) Two-dimensional ^1H chemical shift/ ^{15}N chemical shift HETCOR spectrum for synthetic AChR M2 specifically ^{15}N labeled at Leu10, Leu11 and Ala12. Sixty-four t_1 values were incremented by $26.1\ \mu\text{s}$, and the recycle delay was 4 s.

Table 1

Solid-state NMR frequencies for residues 6 to 19 of the AChR M2 peptide in oriented lipid bilayers

Residue	^{15}N chemical shift ^a (ppm)	^1H - ^{15}N dipolar coupling ^b (kHz)	^1H chemical shift ^c (ppm)
Ala6	197.6	12.4	
Ile7	198.8	12.8	
Ser8	185.5	15.2	
Val9	190.3	11.2	
Leu10	216.2	14.2	9.1
Leu11	202.4	16.8	9.3
Ala12	183.1	14.2	9.4
Gln13	178.9	9.4	
Ala14	215.6	15.2	8.0
Val15	175.9	13.8	
Phe16	193.3	13.2	
Leu17	215.6	13.0	8.0
Leu18	198.2	16.6	
Leu19	177.7	14.8	

^aReferenced to 0 ppm for liquid ammonia. Estimated error is ± 0.5 ppm.^bEstimated error is ± 0.25 kHz.^cReferenced to 0 ppm for liquid tetramethylsilane. Estimated error is ± 1.0 ppm.