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Sample Optimization and Identification of Signal Patterns of Amino Acid Side Chains in 2D RFDR Spectra of the α-Spectrin SH3 Domain

Jutta Pauli,* Barth van Rossum,*,† Hans Förster,‡ Huub J. M. de Groot,† and Hartmut Oschkinat*,[1]

*Forschungsinstitut für Molekulare Pharmakologie, Alfred-Kowalke-Strasse 4, D-10315 Berlin, Germany; †Gorlaeus Laboratories, Einsteinweg 55, P.O. Box 9502, 2300 RA Leiden, The Netherlands; and ‡Bruker Analytik GmbH, Am Silberstreifen, Rheinstetten, Germany

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Future structural investigations of proteins by solid-state CP-MAS NMR will rely on uniformly labeled protein samples showing spectra with an excellent resolution. NMR samples of the solid α-spectrin SH3 domain were generated in four different ways, and their ^{13}C CPMAS spectra were compared. The spectrum of a [u-^{13}C, ^{15}N]-labeled sample generated by precipitation shows very narrow ^{13}C signals and resolved scalar carbon–carbon couplings. Linewidths of 16–19 Hz were found for the three alanine C$^{\beta}$ signals of a selectively labeled [70% 3-^{13}C]alanine-enriched SH3 sample. The signal pattern of the isoleucine, of all prolines, valines, alanines, and serines, and of three of the four threonines were identified in 2D ^{13}C–^{13}C RFDR spectra of the [u-^{13}C,^{15}N]-labeled SH3 sample. A comparison of the ^{13}C chemical shifts of the found signal patterns with the ^{13}C assignment obtained in solution shows an intriguing match. © 2000 Academic Press

Key Words: **solid-state NMR spectroscopy; protein structure determination; resolution; RFDR spectroscopy; ^{13}C linewidth; high magnetic fields.**

INTRODUCTION

Currently, solid-state NMR is developing into a tool for determining the three-dimensional structures of proteins and, in particular, of membrane-integrated ones. Possibilities for structural investigations of the latter would be of great importance, as they regulate vital cellular processes. The feasibility of structural studies depends on the existence of appropriate pulse schemes, sophisticated hardware, and the availability of suitable protein preparations. Recently, a number of important steps toward protein structure determination concepts have been made. Pulse sequences for distance determination (*1, 2*), for assignment purposes (*3–6*), for the measurement of bond orientations with respect to the external magnetic field (*7, 8*), and for determination of intramolecular angles (*2, 9–12*) were developed. Furthermore, using flat coils (*13*), magic angle oriented sample spinning (*14*) or elevated spinning speeds improved protein solid-state NMR spectra. Another significant step forward was the accomplishment of complete ^{13}C and ^{1}H assignments of moderately large uniformly ^{13}C-enriched chlorophyll-based aggregates and the detection of intermolecular correlations in such systems. This was achieved by the radiofrequency-driven recoupling (RFDR) technique and frequency-switched Lee–Goldburg decoupled ^{1}H–^{13}C correlation spectroscopy (*15–17*). In particular, intermolecular heteronuclear correlations provided distance constraints which were used for the *de novo* structure determination of chlorophyll a/water aggregates (*18*). The latter are microscopically ordered systems without long-range translational symmetry and cannot be investigated by high-resolution diffraction techniques.

Structural studies depend also on the availability of protein preparations yielding spectra with narrow lines and hence maximum resolution and signal intensity. Therefore, several approaches for optimizing the preparation protocols of the samples toward a minimal linewidth have been proposed. Studelska *et al.* (*19*) have lyophilized the protein complex after adding so-called "cryoprotectants," such as polyethylene glycol (PEG-8000) and saccharides. Jakeman *et al.* (*20*) have applied modified versions of this approach to a protein–ligand complex and observed a phosphorous linewidth between 125 and 150 Hz. Furthermore, a linewidth around 100 Hz was found in ^{13}C spectra of a lyophylized [u-^{13}C,^{15}N]-labeled ubiquitin sample which was stored in a water atmosphere (*5*). Another option is to use crystalline samples, e.g., in the manner of Straus *et al.* (*21).* These methods need to be tested at the beginning of structural investigations on solid proteins.

In this Communication, we compare several sample preparation techniques for a specific protein, the α-spectrin SH3 domain (7.2 kDa, 62 amino acids, MDETGKELVLALYDYQEKSPREVTMKKGDILTLLNSTNKDWWKVEVNDRQGFVPAAYVKKLD), as a prerequisite for a structural study. The potential of the best SH3 sample type for structural investigations is demonstrated by the identification of signal patterns of several amino acid types in 2D ^{13}C–^{13}C RFDR spectra.

RESULTS

The effects of four different sample preparation methods on the spectra of the [u-^{13}C,^{15}N] α-spectrin SH3 domain were

[1] To whom correspondence should be addressed. Fax: +49 30 51551 235. E-mail: Oschkinat@fmp-berlin.de.

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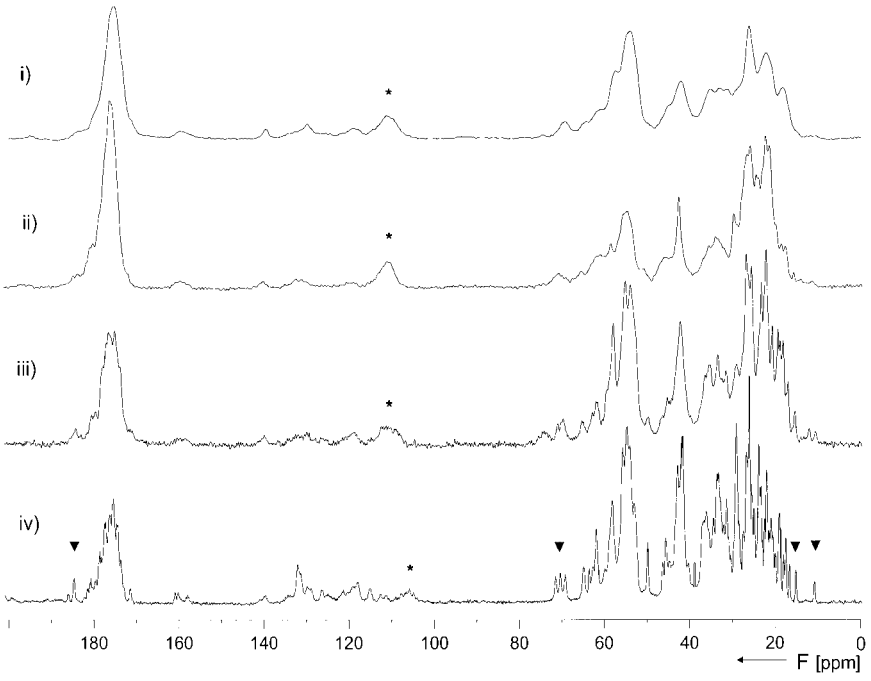

FIG. 1. 1D ^{13}C-RAMP CPMAS NMR spectra of differently prepared [u-^{13}C,^{15}N]-labeled α-spectrin SH3 samples at a spinning frequency $\omega_R/2\pi$ of 13 kHz and at field strengths of 18.8 T (i–iii) and 17.6 T (iv). Sample (i) was lyophilized from aqueous low-salt buffer. Sample (ii) was the same as (i), however, after adding water, but such that the protein remains solid. Sample (iii) was lyophilized from a solution also containing PEG-8000 and sucrose. Sample (iv) was precipitated from a $(NH_4)_2SO_4$-rich solution. The asterisks mark the sidebands of the carbonyls and the triangles highlight examples of well-resolved signals discussed in the text.

investigated using 1D ^{13}C CPMAS NMR. Sample (i) was lyophilized from an aqueous low-salt solution. Sample (ii) was prepared in a similar way as (i) and supplemented by a drop of water, resulting in a moist sample. Sample (iii) was lyophilized from a $(NH_4)_2SO_4$ solution, which additionally contained PEG-8000 and sucrose (which we will refer to as the lyoprotected sample). Sample (iv) was precipitated from a $(NH_4)_2SO_4$-rich solution by changing its pH, yielding a wet precipitate.

The 1D CPMAS ^{13}C spectra of these four preparations are shown in Fig. 1. The linewidths of the signals decrease from (i) to (iv). The signals of the precipitated sample (iv) are the best concerning resolution, hence a number of well-resolved signals are found. Among these are the signals at 11.4 and 15.9 ppm, at 69.5, 70.7, and 71.6 ppm, and the carbonyl signal at 183.3 ppm. These signals are marked by a triangle in Fig. 1iv. A linewidth of 70 Hz is estimated for the signal at 15.9 ppm in spectrum Fig. 1iv, while it is 130 Hz wide in spectrum Fig. 1iii and 190 Hz in spectrum Fig. 1ii.

In the next step, we explored the minimal linewidth that can be achieved by precipitating the protein from a $(NH_4)_2SO_4$-rich solution. The 1D ^{13}C CPMAS spectra of two differently labeled SH3 samples, the [u-^{13}C,^{15}N]-labeled SH3 and the [70% 3-^{13}C]alanine enriched SH3, are shown in Figs. 2a and 2b, respectively. The latter sample was prepared to minimize the line broadening caused by one- and higher bond ^{13}C–^{13}C or ^{13}C–^{15}N scalar couplings. For spectra 2a and 2b a spinning frequency $\omega_R/2\pi$ of 13 kHz and a decoupling field of 100 kHz were used. Linewidths between 16 and 19 Hz were measured for the C$^{\beta}$ signals of the three alanines in the [70% 3-^{13}C]alanine-enriched sample. The methyl signals of the [u-^{13}C,^{15}N] SH3 show some fine structure, which we attribute to the ^{1}J(^{13}C–^{13}C) coupling. Both samples were prepared in the same manner, therefore we can rule out structural heterogeneity as a cause for the observed signal splittings. These splittings are around 30 Hz, which is in the order of the ^{1}J (^{13}C–^{13}C) couplings present in aliphatic side chains.

The resolution observed in the spectra of the precipitated and uniformly labeled α-spectrin SH3 domain is promising, and it is anticipated that such samples are suitable for the generation of carbon assignments. RFDR (*15*, *25*) is a robust technique for collecting homonuclear ^{13}C–^{13}C correlation data. An RFDR spectrum recorded from the precipitated sample (iv) is shown in Fig. 3. The experiment was performed at a moderate spinning speed of 8 kHz. The applied RFDR mixing time of 3 ms is long enough to facilitate the occurence of cross peaks due to relayed coherence transfer. Such signals may help to identify the signal patterns of the different amino acid types. Relayed cross peaks were distinguished from correlation signals resulting from direct transfer by comparison of RFDR spectra recorded with different mixing times. Signal patterns of certain amino acid types in the RFDR spectra can be identified by their pattern topology and/or due to the fact that some of their

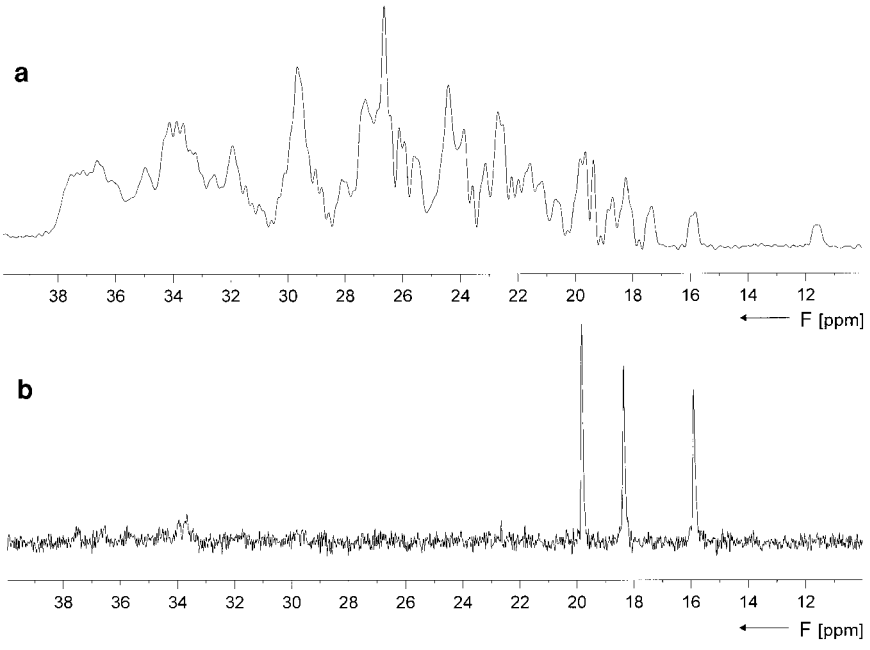

FIG. 2. Comparison of the 1D ^{13}C RAMP CPMAS spectra of the [u-^{13}C,^{15}N]-labeled α-spectrin SH3 sample (a) and of the [70% 3-^{13}C]alanine-enriched sample (b), both in precipitated form at a spinning frequency $\omega_R/2\pi$ of 13 kHz and at a field of 17.6 T.

carbon signals show characteristic chemical shifts determined by electronic effects. Earlier studies (*21, 27*) have pointed out that the observed ^{13}C chemical shifts of solid peptides and proteins correspond roughly to those observed in solution, probably because of similar structure. In crystalline Antamanide, for example, most of the observed chemical shift differences between solid and solution are in the range of 0.2–2.5 ppm. Hence, we expect the carbon signals to occur in similar chemical shift ranges in solid and in solution. This enables the identification of signal networks of certain amino acid types which are shown in Figs. 3 and 4 and Table 1.

The RFDR spectrum of the precipitated α-spectrin SH3 sample shows well-resolved and characteristic signal patterns for isoleucines, prolines, valines, threonines, alanines, and serines. For instance, the cross peak network of the single isoleucine in the α-spectrin SH3 domain can be identified using the diagonal peak of the δ-methyl carbon at 11.4 ppm as a starting point. All cross peaks involving directly coupled carbons ($C^{\delta}C^{\gamma}$, $C^{\gamma}C^{\beta}$, $C^{\gamma\prime}C^{\beta}$, $C^{\beta}C^{\alpha}$) and those indicating relayed transfer ($C^{\delta}C^{\beta}$, $C^{\delta}C^{\alpha}$, $C^{\gamma}C^{\alpha}$, $C^{\gamma\prime}C^{\alpha}$, $C^{\gamma}C^{\gamma\prime}$) are found. For the two prolines, all cross peaks due to direct transfer ($C^{\delta}C^{\gamma}$, $C^{\gamma}C^{\beta}$, $C^{\beta}C^{\alpha}$) and all relayed cross peaks ($C^{\delta}C^{\beta}$, $C^{\delta}C^{\alpha}$, $C^{\gamma}C^{\alpha}$) are found. Similarly, there are also six distinct cross peak patterns due to the six valines (Fig. 4) present in the protein. The C^{α}, C^{β}, and the methyl carbons (C^{γ} and $C^{\gamma\prime}$) resonate around 58–62 ppm, between 33 and 38 ppm, and between 17 and 24 ppm, respectively, giving rise to unambiguous patterns. Three threonine residues show well-resolved correlations between their respective α- and β-carbons in the area between 60–66 and 70–75 ppm and between their β- and methyl carbons (C^{γ}) in the chemical shift range of 70–75 and 20–25 ppm. There are also relayed cross peaks ($C^{\gamma}C^{\alpha}$). Three cross peaks are observed between alanine α- and β-carbons at chemical shifts of 50–55 and 15–20 ppm, respectively. There are also two distinct cross peaks involving correlations between the α- and β-carbon signals of the two serines in the area between 55–60 and 60–65 ppm.

DISCUSSION

The protein sequence of SH3 shows only one isoleucine, two prolines, six valines, three alanines, two serines, and four threonine residues. For all of these amino acids except threonines, the same number of signal patterns were found in the well-resolved RFDR spectra. One of the threonine patterns, most likely the one of T4, is obviously missing. T4 is located at the N-terminus, which is known to be flexible in solution. This flexibility can result in a multitude of long-living conformers upon precipitating the sample. Such a multitude of conformers can lead to broad, nondetectable NMR signals in solid state. This may account for the apparent absence of a fourth signal pattern in the spectrum, which is then the pattern of T4. This is supported by NCA, NCO, and H–C Lee–Goldburg experiments (data not shown) which show only signals of three of the four threonines and two of the three glycines. No T–G connectivity is found in the NCO spectrum as expected for T4 and G5, hence we conclude that the signals of the N-terminal residues are not observed in our spectra.

Carbon and hydrogen signals of amino acids in a folded protein show characteristic solution chemical shifts which

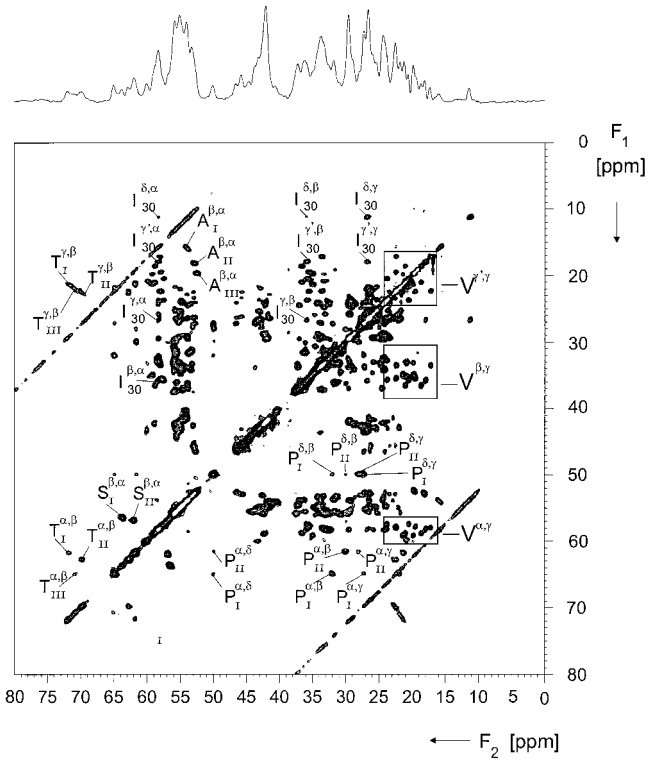

FIG. 3. 2D ^{13}C–^{13}C RFDR spectrum of the precipitated [u-^{13}C,^{15}N]-labeled α-spectrin SH3 sample in precipitated form with a mixing time of 3 ms and at a spinning frequency $\omega_R/2\pi$ of 8 kHz. The data were collected at a field of 17.6 T. The appropriate 1D ^{13}C RAMP CPMAS spectrum is shown on the top of the 2D spectrum.

slightly differ from the random coil values. These differences are connected to the secondary and tertiary structures of the protein. Characteristic chemical shifts are also found in our spectra for the alanines, the prolines, and the threonines (Table 1). The individual chemical shifts of the alanine methyl groups (Fig. 2) show a dispersion of 3.9 ppm. A similar situation is found for the C^α chemical shifts of the prolines, which differ by 3.4 ppm. The C^α values of the threonines show a dispersion of 3.2 ppm. Setting the up-field shifted alanine methyl group to 15.9 ppm, which is its value in solution (Table 1), the chemical shifts of the methyl groups of the two other alanines agree with those in solution with deviations of around 0.3 ppm. Using this calibration of the solid-state ^{13}C NMR spectra the chemical shifts of the analyzed signal patterns of the isoleucine, the prolines, the alanines, and the threonines are confronted with the assignment of those residue types obtained in solution (*28*) (Table 1). From this it seems rather likely that an unambiguous sequential assignment procedure through triple-resonance experiments will reveal for example that A_I is A55, P_I is P20, and P_{II} is P54. An exact sequence-specific assignment on the basis of well-resolved NCOCA and NCACB spectra is in progress.

In summary, this study demonstrates the feasibility of structural studies on immobilized and [u-^{13}C,^{15}N]-labeled protein samples by CPMAS NMR, provided that appropriate sample preparations are available. 1D and 2D ^{13}C spectra with an excellent resolution were recorded from a precipitated protein sample, the [u-^{13}C,^{15}N] α-spectrin SH3 domain. The presented spectra raise the expectation that an appropriate combination of heteronuclear 2D and 3D solid-state spectra may lead to full resonance assignments for proteins up to 100 amino acids.

MATERIALS AND METHODS

pET3d plasmid coding for chicken brain α-spectrin SH3 domain was a generous gift of Dr. Saraste, EMBL. The SH3 domain was expressed in the *Escherichia coli*, using a minimal medium based on M9 salts. A total of 1.5 g [u-^{13}C]glucose and 1.0 g $^{15}NH_4Cl$ per liter of medium were added in the case of the [u-^{13}C,^{15}N]-labeled SH3 domain. A total of 0.5 g [u-^{13}C]glucose, 0.4 g $^{15}NH_4Cl$, 0.2 g [3-^{13}C]Ala, 0.16 g Glu, respectively,

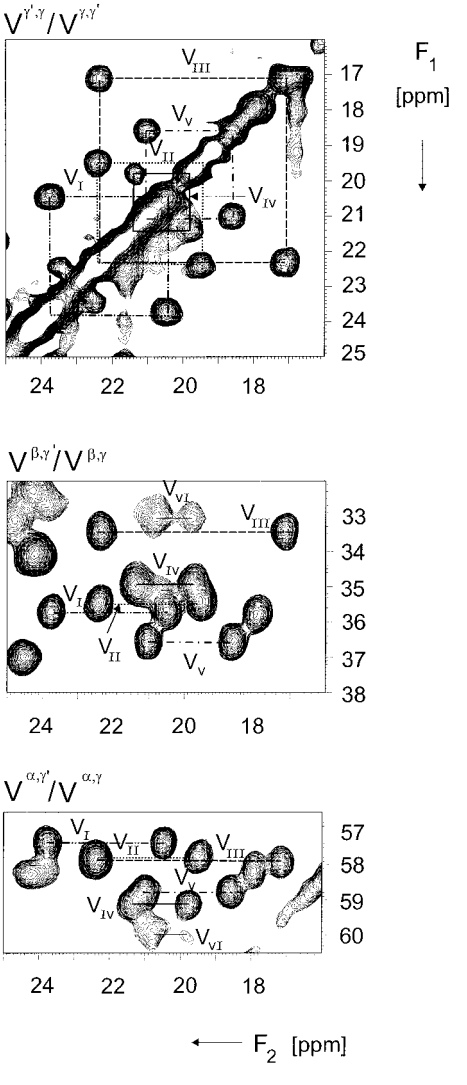

FIG. 4. Valine signal patterns extracted from the 2D ^{13}C–^{13}C RFDR spectrum in Fig. 3.

TABLE 1

^{13}C Solid-State Chemical Shifts (I, P_I, P_{II}, V_I–V_{VI}, A_I–A_{III}, S_I, S_{II}, T_I–T_{III}) and Solution Chemical Shifts of Certain Amino Acids of the α-Spectrin SH3 Domain

Residue	C^α [ppm]		C^β [ppm]		C^γ [ppm]		$C^{\gamma'}$ [ppm]		C^δ [ppm]	
I	58.3		35.9		26.8		18.1		11.4	
I30		60.2		38.4		27.7		18.9		12.4
P_I	65.0		32.1		27.3				50.1	
P20		64.7		32.2		27.6				51.2
P_{II}	61.6		30.1		28.3				50.0	
P54		61.9		30.4		28.0				50.1
V_I	57.3		35.7		23.7		20.4			
V_{II}	57.8		35.5		22.4		19.5			
V_{III}	57.9		33.5		22.3		17.0			
V_{IV}	59.1		35.0		21.3		19.8			
V_V	59.3		36.4		21.0		18.6			
V_{VI}	59.9		33.1		20.8		19.8			
A_I	54.0		15.9							
A55		54.6		15.9						
A_{II}	52.8		18.2							
A56		53.0		18.0						
A_{III}	52.4		19.8							
A11		52.6		19.5						
S_I	56.5		63.7							
S_{II}	56.8		62.0							
T_I	61.8		71.8		21.3					
T24		61.8		71.1		22.1				
T_{II}	62.7		69.8		22.5					
T32		63.3		69.8		22.1				
T_{III}	65.0		70.9		21.6					
T37		65.6		69.8		22.1				
T4		62.7		69.7		21.6				

0.04 g Ser and Thr, respectively, 0.1g Asn, Asp, Arg, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, and Tyr, respectively, per liter of medium were added in the case of the [70% 3-^{13}C]-enriched SH3 domain. The proteins were purified by anion exchange chromatography (Q-Sepharose FF, Amersham Pharmacia Biotech), gel filtration (Superdex 75 pg, Amersham Pharmacia Biotech), and dialysis.

Preparation of the Lyophylized Sample

A 0.32 mM [u-^{13}C,^{15}N] SH3 solution (0.02% NaN_3, pH 3.5) was frozen in liquid nitrogen before it was lyophylized. Dry protein (20 mg) was then introduced into a 4-mm ZrO_2-CRAMPS rotor (Bruker, Karlsruhe, Germany).

Preparation of the Lyoprotected Sample

A 0.32 mM [u-^{13}C,^{15}N] SH3 solution, also containing 1% $(NH_4)_2SO_4$, 1% PEG-8000 (Sigma), 0.7% sucrose (Sigma), was frozen at a temperature of -18°C. The sample was then kept 24 h at -80°C before the lyophylization was started. The dry mixture (16 mg) (containing 1.5 mg protein) was put into a 4-mm CRAMPS rotor.

Preparation of the Precipitated Sample

A 200 mM $(NH_4)_2SO_4$ solution (pH 3.5, 0.04% NaN_3) was added to a 3.3 mM SH3 solution (pH 3.5) at the volume ratio of 1 to 1. The same amount of $(NH_4)_2SO_4$ was used for crystallization of the protein (*22*). SH3 was precipitated by changing the pH of the mixture to a value of 7.5 in NH_3 atmosphere. The solution was kept in a refrigerator (4°C) for 3 days before the precipitate was separated by centrifugation (20 min, 15000 U/min). Approximately 10 mg of protein was then transferred into a 4-mm CRAMPS rotor.

NMR Spectroscopy

The NMR measurements were recorded on DMX800 and DMX750 spectrometers, using a MAS probehead (Bruker). The rotation frequency of the 4-mm CRAMPS rotors was stabilized to ± 2 Hz. The 1D ^{13}C NMR spectra were recorded at 201.21 MHz (i)–(iii) and at 188.59 MHz by using a spinning frequency $\omega_R/2\pi$ of 13 kHz. A standard RAMP CP pulse sequence was applied (*24*) using ^{13}C B_1 fields of 60125 Hz (i)–(iii) or 51020 Hz and a 100%/50% ramp on the ^{1}H during a contact time of 1 ms. Recycle delays of 2s and 512 scans were employed. The 2D spectra were acquired with the RFDR technique (*25*) at a radiofrequency of 188.59 MHz and at a spinning frequency $\omega_R/2\pi$ of 8 kHz, using the pulse sequence published by Boender *et al.* (*15*). The 90° proton pulse was set to 2.8 μs. ^{13}C B_1 fields of 43103 Hz were used during the RAMP CP sequence with a contact time of 0.5 ms. A 100%/50% ramp was applied on the proton frequency band. Rotor-synchronised π-pulses with a length of 23.3 μs were applied during the RFDR mixing times of 1 or 3 ms.

TPPM (*26*) was applied in all experiments to decouple protons in t_1, the RFDR mixing time, and during the aquisition time. Pulse lengths of 4.6 μs (1D spectra) or 5.2 μs (RFDR spectra) and a phase modulation angle of 25° were used for the TPPM decoupling.

The solution ^{13}C chemical shifts were obtained by analyzing the TOWNY (^{1}H^{1}H TOCSY–^{1}H^{13}C HSQC) spectrum of 10% ^{13}C, 100% ^{15}N-labeled chicken α-spectrin SH3 domain, measured at 297 K (3.3 mM, pH 3.5, H_2O/D_2O = 9:1, 0.02% NaN_3). The previously published ^{1}H proton chemical shifts were the starting point thereby (*23*). ^{13}C^{1}H HSQC spectra have provided evidence that the influences of the pH and salt content on the ^{13}C carbon chemical shifts are only minor. The HSQC spectrum of a 0.7 mM SH3 solution (pH 3.5, no salts) shows few differences from the HSQC spectrum of a 0.16 mM SH3 solution (pH 7.5, 100 mM $(NH_4)_2SO_4$) concerning their ^{13}C chemical shift values.

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