Solid-state NMR study of the SH3 domain of α-spectrin: application of $^{13}$C–$^{15}$N TEDOR and REDOR†

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ABSTRACT: A fully $^{13}$C–$^{15}$N-labeled and a selectively alanine-$^{13}$C$^0$ tryptophan-$^{15}$N$^{\text{Ing}}$-labeled sample of the Src homology region 3 (SH3) domain of α-spectrin (chicken), a 62 residue protein, were biosynthesized and studied by solid-state cross-polarization magic angle spinning (CP/MAS) NMR, $^{13}$C–$^{15}$N rotational echo double resonance (REDO) and $^{15}$N–$^{13}$C transferred echo double resonance (TEDOR) spectroscopy. In the first part of the study it is shown that spectral editing with the TEDOR sequence leads to a drastic simplification of the $^{13}$C MAS spectrum of the fully labeled sample, allowing the resolved spectroscopy of groups of $^{13}$C nuclei, according to their distance to neighboring $^{15}$N nuclei. In the second part of the study the inter-residual distance between the alanine residue Ala55 and the tryptophan residue Trp42 was determined by the measurement of the dipolar coupling between Ala-$^{13}$C$^0$ and Trp-$^{15}$N$^{\text{Ing}}$, yielding a dipolar coupling of 48 ± 8 Hz, which after correction for fast molecular vibrations gives a value of 53 ± 8 Hz, corresponding to a CN distance of 3.85 ± 0.25 Å. The result is compared to the CN distances obtained by x-ray diffraction and liquid-state NMR. Copyright © 2000 John Wiley & Sons, Ltd.

KEYWORDS: SH3 domain; α-spectrin; protein; TEDOR; REDOR; spectral editing; chemical editing; CN distances; dipolar solid-state NMR

INTRODUCTION

The biological function of a protein is mainly determined by two factors, the primary structure, i.e. its amino acid sequence, and the geometrical arrangement of the amino acid chain and the side groups, i.e. its secondary and higher structures.1 The primary structure of peptides and proteins of interest is known in general or can be determined by standard techniques. The determination of the higher structures is more elaborate. If suitably crystallized samples of the protein are available, it is possible to determine these higher structures by x-ray diffraction. An alternative approach is multi-dimensional liquid-state NMR spectroscopy of dissolved molecules.2,3 Both of these methods have their limitations: on the one hand, many proteins are not available in single-crystal form, for example owing to poorly crystallizing behavior. On the other hand, proteins often exceed the range of molecular weights suitable for liquid-state NMR studies, are only poorly soluble or tend to aggregate. Furthermore, the important group of membrane embedded proteins cannot be analyzed by x-ray diffraction or solution NMR.

Solid-state NMR spectroscopy4,5 can help in structural studies of these classes of proteins. Because of the special experimental requirements of this type of NMR, this approach is still under development. Two major problems have to be solved before a successful solid-state structural analysis of a protein is feasible. First, the spectral resolution has to be increased to achieve a resolution comparable to the resolution in liquid-state NMR. Second, techniques for monitoring neighbor relationships of different amino acid residues have to be developed, applied and combined with efficient techniques for distinguishing between the contributions from different types of interactions.

One approach for the study of proteins with solid-state NMR relies on orienting the sample with respect to the magnetic field $B_0$, e.g. by embedding the protein in a membrane. Orientation constraints are derived from dipolar couplings and chemical shift interactions, the values of which depend on the relative alignment of the molecule with respect to the external field.6,7 This technique is restricted to a limited class of proteins. Therefore, magic angle spinning (MAS)8,9 NMR techniques and in particular the cross-polarization (CP) MAS experiment10,11 have provided the major means to solve the resolution problem. The linewidths typically obtainable by MAS are in most cases still an order of magnitude worse than those typically found in liquid-state NMR. Only very recently with the development of high-speed $^{13}$C CP/MAS probes and high-field solid-state NMR spectrometers do linewidths suitable for real high resolution in the solid state seem to be achievable.

† This paper is dedicated to Professor Dr Harald Günther on the occasion of his 65th birthday.

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Two prerequisites are needed for a successful structure determination by MAS NMR spectroscopy: (i) a high spectral resolution to assign individual lines to particular atomic positions in the molecule by virtue of their isotropic chemical shift and (ii) a network of adjacency and distance information, which can be found by recording the couplings between different atoms in the molecule.

For the analysis of the distance information under high-resolution MAS conditions, several advanced NMR techniques are now available. A review of some of these techniques was given by Bennett et al. and the application of these techniques to biological samples was reviewed by Auger. The basic idea of these techniques is to reintroduce the dipolar coupling in the course of the MAS experiment by periodically disturbing the evolution of the spin system with the r.f. pulses, leading to a dipolar recoupling. For heteronuclear systems of two X-nuclei, the rotational echo double resonance (REDOR) experiment is of particular importance. This method allows the recoupling of dipolar interactions between different X-nuclei (e.g. 13N and 13C) by periodically inverting the sign of the dipolar interaction in the course of the experiment. The strength of the dipolar interaction can be determined by performing an echo experiment and measuring the signal decay caused by dipolar dephasing. This decay can be adjusted to a known master curve to determine the strength of the interaction or alternatively it can be transformed into the frequency domain. Examples of some recent applications of dipolar recoupling techniques to proteins and other biomolecules can be found in the literature.

The differences in the dipolar dephasing times in the 13C–15N REDOR experiment can be exploited as dipolar filters, which reduce the number of overlapping side-chain signals. However, the price for the simplification of the 13C spectrum is a considerable decrease in intensity. Moreover, these editing techniques remove the signal of the dephased nuclei and they cannot be used as building blocks of heteronuclear correlation experiments. For such tasks, active magnetization transfer experiments or coherence transfer techniques are more attractive. In the solid state, the active transfer of magnetization from one type of spin-1/2 nuclei to another type of spin-1/2 nuclei, e.g. from 13N to 13C, can be achieved via heteronuclear dipolar couplings. The TEDOR (transferred echo double resonance) technique is the solid-state NMR analog of the INEPT experiment in liquid-state NMR. After initial cross-polarization of the nuclei I, the evolution of the magnetization under the influence of the heteronuclear dipolar interaction during the time τ creates IS spin coherence, which is converted into S spin coherence by a simultaneous 90° pulse in both I and S channels and a second evolution for the time τ. The transfer efficiency is at a maximum if the two delays are equal, i.e. τ = τ. In single crystals or liquids, complete coherence transfer can be achieved by such a technique if the transfer time is chosen correctly. In non-oriented powder samples, however, only a partial transfer is possible, owing to the orientation dependence of the dipolar interactions.

The aim of this work was the evaluation of some solid-state NMR strategies for structural studies of proteins, employing a small protein with known structure as a ‘real world’ model of large solid proteins. In the first part a strategy for simplification of complex solid-state NMR spectra of fully 13C- and 15N-labeled samples with the help of the TEDOR sequence was assessed. The second strategy combined special chemical editing of the sample with dipolar solid-state NMR spectroscopy. In general, selective chemical editing of a protein sample is not an easy task. In our case we employed a relatively simple chemical labeling scheme by adding a single, selectively 13C-labeled amino acid to the medium for the biosynthesis. With this scheme a selective enrichment of this amino acid in the protein is achieved. This partial enrichment allows even on a non-high-field solid-state NMR spectrometer a faithful determination of non-trivial interresidual distances in a protein.

We choose the chicken (Gallus gallus) Src homology region 3 (SH3) domain (Fig. 1) of α-spectrin as a model system for our studies for several reasons. (i) With its 62 amino acids it is a relatively small protein including a great variety of 18 different amino acids. This results in a relatively wide distribution of 13C chemical shifts in the NMR spectrum and therefore a minimum signal overlap, which allows for a reasonable resolution even on a non-high-field solid-state NMR spectrometer. (ii) The samples used in the presented NMR experiments are stable against heat-induced molecular conformation changes. The solid-state 13C and 15N NMR spectra both displayed constant chemical shifts over a period of several months indicating a high structural stability of the SH3 domain. (iii) The structure of the SH3 domain has been analyzed by x-ray diffraction with a resolution of 1.8 Å. This allows control of the structural data obtained by solid-state NMR.

The first reported crystal structure of an SH3 domain is that of the α-spectrin SH3 domain (chicken). Spectrin is the major component of the cytoskeleton that underlies the cell membrane. It has an SH3 domain inserted into one structural repeat of the α-chain. This SH3 domain is a good model system for protein folding and stability.

Figure 1. X-ray structure of the backbone of the SH3 domain and the positions of tryptophan residue Trp42 and alanine residue Ala55. The arrow marks the distance from the 13C (Ala55) to the 15N ring (Trp42). The 13C–15N distance of the 13C (Ala55) and the 15N ring (Trp42) determined by x-ray diffraction and liquid-state NMR varies in the range 3.56–3.73 Å. Note the relatively large ‘free’ volume in the vicinity of the Trp42 residue.
studies and in particular for all β-structures of proteins. Its tertiary structure is that of a small scaffold composed of five antiparallel β-strands. The structure has been analyzed again by x-ray diffraction and also by liquid-state NMR spectroscopy. Structures of several other SH3 domains have also been determined. In all these studies, a similar common β-barrel motif was found in spite of the low degree of sequence similarity within the family. The function of the SH3 domain is still not clear, but the proteins, in which the SH3 domain can be found, are often involved in signal transduction pathways (see Musacchio et al. and references cited therein).

In principle, it is possible to study directly fully isotope-enriched SH3 protein samples by solid-state NMR. In practice, however, the strong signal overlap will prevent a sufficient resolution of the individual resonances, in particular with a 7 T spectrometer as used in this study. Therefore, the application of spectral or chemical editing techniques is necessary for enhancing the resolution in the NMR experiment.

Both of these techniques have their unique merits and drawbacks. Solid-state NMR spectral editing techniques are very expensive in the sense of signal losses for the following reasons: on the one hand, the $T_2$ relaxation times of coherences, which are involved in the TEDOR transfer, can be relatively short in the solid state, compared with liquid-state NMR, resulting in a shorter time window for the transfer. On the other hand, the orientation distribution of dipolar coupling in non-oriented powder samples prevents a simultaneous ‘resonance’ condition for all crystallites in the sample in the course of the TEDOR experiment, as compared with the equivalent INEPT sequence, where the ‘resonance condition’ is fulfilled for a transfer time of $\tau = 1/(2J)$.

On the other hand, chemical editing of a protein by selectively replacing only some amino acids can be difficult to achieve because of metabolic side reactions which may introduce additional isotope labels in unwanted molecular positions, because the microorganism employed for the biosynthesis digests part of the labeled amino acids and uses the labeled fragments for the biosynthesis of other amino acids.

Both editing techniques were employed in this study: in the first part, the heteronuclear $^{13}$C $^{15}$N dipolar interaction was employed for spectral editing and thus for simplification of the $^{13}$C NMR spectrum of the SH3 domain. In the second part, chemical editing of the SH3 domain was employed and an inter-residual distance between two nuclei of different β-strands was measured. From the known crystal structure of the SH3 domain, it is evident that the residue pair Ala55–Trp42 is a good candidate for such an experiment (Fig. 2).

The rest of this paper is organized as follows: the experimental part consists of an introduction into the REDOR technique for the measurement of heteronuclear dipolar couplings in the solid state, followed by a short overview of the sample preparation and a description of our experimental set-up. Then the experimental results are presented, discussed and finally summarized.

![Diagram](https://example.com/diagram.png)

**Figure 2.** Part of the SH3 domain amino acid sequence displaying the selectively $^{13}$C-labeled alanine residues Ala55 and Ala56 and the $^{15}$N-labeled tryptophan residue Trp42.

### EXPERIMENTAL

#### The REDOR experiment

The basic theory of the REDOR experiment is well known from the literature and will be only briefly summarized here. For a heteronuclear spin-1/2 pair, the solid-state NMR high-field MAS Hamiltonian is given as (J-coupling can be neglected)

$$
\hat{H}(t) = \omega_0(t)\hat{2}\hat{S}_z + \omega_1(t)\hat{S}_x + \omega_2(t)\hat{I}_z
$$

(1)

where $\omega_0(t)$ and $\omega_1(t)$ characterize the chemical shift values of the $S$ and $I$ spins, respectively, and $\omega_2(t)$ describes the dipolar coupling between $S$ and $I$ spins. In solid samples, all three frequencies in Eqn (1) are functions of the orientation of the molecule with respect to the external magnetic field, giving rise for non-oriented samples to the well known NMR powder pattern, i.e., broad lines caused by the distribution of resonance frequencies. These broad spectra can be transferred into narrow ‘liquid-like’ spectra by fast sample spinning around an axis inclined at an angle of 54.7° with respect to the direction of the magnetic field, the so-called magic angle. This sample spinning removes the distance information present in the dipolar part of the Hamiltonian by averaging the dipolar interaction to zero. This information is recovered by the REDOR sequence, an irradiation of two $\pi$-pulses during one rotor period $T_\rho$, the first at time $\tau$ and the second at the end of the rotor period.

The recoupled dipolar frequency of a specific spin packet depends on the orientation of the dipolar vector with respect to the external field, defined by the polar angles $\alpha$ and $\beta$

$$
\bar{\sigma}_0(\alpha, \beta, \tau) = \frac{1}{T_\rho} \int_0^{T_\rho} \int_0^{T_\rho} \omega_0(\alpha, \beta, t') \omega_0(\alpha, \beta, t') dt' - (\int_0^{T_\rho} \omega_0(\alpha, \beta, t') dt')^2
$$

(2)

with

$$
\omega_0(\alpha, \beta, t) = \pm \frac{1}{2} D [\sin^2 \beta \cos 2(\alpha + \omega_0 t) - \sqrt{2} \sin 2\beta \cos (\alpha + \omega_0 t)]
$$

(3)

The heteronuclear dipolar coupling $D$, which depends on the distance $r$ of the two nuclei $I$ and $S$ as

$$
D = \frac{\hbar}{4\pi} \frac{\gamma_s}{\gamma_i} \frac{1}{r^3}
$$

(4)

contains the structural information.
The measured REDOR signal $S_R$ can be expressed as a function of the number $N$ of applied REDOR cycles and the spinning frequency $1/T_r$:

$$S_R(\alpha, \beta) = T_i \rho(\alpha, \beta) S_i = \cos[\pi(\alpha, \beta, T_i/2)NT_i]$$  \hspace{1cm} (5)

For non-oriented powder samples the integral over the polar angles $\alpha$ and $\beta$ has to be calculated, yielding the REDOR signal:

$$S_R = \frac{1}{4\pi} \int_{\alpha,\beta} S_R(\alpha, \beta) \sin \beta d\beta d\alpha$$  \hspace{1cm} (6)

The most efficient dipolar recoupling is achieved if the first $\pi$-pulse is irradiated at the center of the rotor cycle, i.e. $\tau = T_i/2$. In this case there exists an analytical expression for the REDOR signal. \[ J_1(z), \text{cylindrical Bessel function of the first kind; } n, \text{ number of rotor cycles}: \]

$$S_R(n\nu_0 T_R) = \pi 2^1/4(n\sqrt{2}\nu_0 T_R) J_{n-1/4}(n\sqrt{2}\nu_0 T_R)$$  \hspace{1cm} (7)

and a direct transformation of the decay function from the time domain into the frequency domain, the so-called REDOR transform.

To take relaxation processes during the REDOR evolution periods ($nT_i$) into account, a reference experiment under the same conditions but without recoupling pulses is performed. With the reference signal $S_0$, the normalized dipolar dephasing is given as

$$\frac{\Delta S}{S_0} = \frac{S_0 - S_R}{S_0}$$  \hspace{1cm} (8)

**Sample preparation**

Two different types of isotope-labeled samples were employed, namely a fully $^{13}$C--$^{15}$N-labeled SH3 sample and a sample which was selectively $^{13}$C and $^{15}$N labeled: $^{13}$C was introduced in the $\beta$-position of the three alanine residues present in the SH3 domain (Ala11, Ala55, Ala56) and $^{15}$N in the ring of the two tryptophan residues (Trp41, Trp42) and in the side-chains of the two glutamine residues (Gln16, Gln50) and three asparagine residues (Asn35, Asn38, Asn47).

The fully labeled SH3 domain was prepared in the following way: a plasmid was employed as expression vector for the SH3 domain in the bacterium *Escherichia coli*. The bacteria were grown on nominally 100% $^{15}$NH$_4$Cl and nominally 100% $^{13}$C-labeled glucose. The aqueous protein solution was dried directly by lyophilization. 25

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The preparation of the selectively labeled SH3 sample is described in full detail elsewhere. 52 Because of metabolic side reactions in the course of the biosynthesis of the sample, part of the $^{13}$C and $^{15}$N went to other molecular positions (e.g. $^{15}$N into the backbone), resulting in a lower labeling of the target positions. From control measurements with liquid-state NMR, the following labeling factors were estimated: C$^\alpha$ (Ala55), 80% $^{13}$C enriched; N$^{\text{seg}}$ (Trp42), 65% $^{15}$N enriched. No $^{15}$N enrichment of the $^{13}$C-labeled alanine residues was found. The SH3 precipitated after changing the pH of the SH3 solution from 3.5 to 7.5. The dry mass of the precipitated sample was 12 mg.

The best $^{13}$C NMR linewidth of the selectively labeled SH3 domain was obtained by moistening the samples with doubly distilled water. 47 This mushy sample gave a well resolved spectrum of the alanine C$^\alpha$ lines even on our 7 T spectrometer.

**The spectrometer**

A detailed discussion of our laboratory-built three-channel solid-state NMR spectrometer (wide-bore 6.98 T magnet) has been given recently. 48 All MAS experiments were performed employing a 7 mm Bruker H-XY triple resonance probe. In the triple resonance experiments the typical 90° pulse length was 6.5 μs for all three channels, corresponding to 38 kHz $B_1$ field in frequency units. For $^1$H decoupling, $B_1$ was changed to 52 kHz to avoid unwanted cross-polarization by mismatching the Hartmann–Hahn condition and to improve the decoupling efficiency. For achieving the optimum decoupling efficiency, the TPPM sequence 49 was employed. This in conjunction with MAS was sufficient to remove $^1$H–X dipolar line broadening. The rotation frequency (normally 5 kHz) was actively controlled using a Doty spin rate controller. Deviations in the rotation frequency were typically below 2 Hz.

**RESULTS AND DISCUSSION**

**MAS NMR on the fully $^{13}$C--$^{15}$N-labeled SH3 domain**

In a first step, the conventional $^{13}$C (Fig. 3) and $^{15}$N (not shown) MAS and CP/MAS NMR spectra of the SH3 domain sample were recorded. The $^{13}$C spectrum of the SH3 domain exhibits severe line overlap due to the numerous nuclei with similar chemical shifts. The spectrum covers the typical chemical shift range of approximately 250 ppm with four distinct regions: (i) signals of the amide and carboxyl carbons in the range 170–175 ppm; (ii) signals of mainly the backbone $\alpha$-carbons in the range 596–603
48–65 ppm; (iii) signals of alkyl carbons in the side-chains in the range 15–25 ppm; and (iv) signals from aromatic side-chain carbons mainly in the range 110–130 ppm.

**TEDOR experiments on the fully ¹³C–¹⁵N-labeled SH3 domain**

The TEDOR experiment allows spectral editing of a complex spectrum. As an experimental example of such an application of the TEDOR technique, Fig. 4 compares the TEDOR edited ¹³C spectra of the fully ¹³C–¹⁵N-labeled SH3 domain sample for two different TEDOR transfer times (10 000 scans) to the normal CP/MAS spectrum (2060 scans). It is evident that, despite the five times higher number of scans, the signal-to-noise (S/N) ratio of the TEDOR spectra is much lower than the S/N ratio of the CP/MAS spectrum. The normalized S/N ratio can be estimated as 1 : 0.1 : 0.05. In the reference spectrum, lines of all ¹³C atoms are visible. The transfer times in the TEDOR experiment were chosen in such a way that only the couplings to one-bond and two-bond neighbors are strong enough to create substantial ¹³C signals. The TEDOR spectrum obtained after two rotor cycles (400 µs) displays only lines of the backbone α- and amide carbons. After four rotor cycles (800 µs) the side-chain β-carbons also become visible. These results show that such TEDOR techniques can be relatively easily applied to other ¹⁵N-labeled systems, where ¹³C positions at one or two bond distances are to be filtered from the background of ¹³C positions further away from the ¹⁵N. Moreover, it should be noted that the relaxation of the coherences in the TEDOR transfer is probably shortened by homonuclear couplings among the ¹³C spins. Therefore, one can expect that in diluted ¹³C systems also larger distances from the ¹⁵N label to the ¹³C nucleus can be filtered with reasonable S/N ratio.

Heteronuclear correlation techniques such as TEDOR can also be employed as building blocks of 2D or more generally multi-dimensional NMR experiments. As an example, Fig. 5 displays a two-dimensional ¹⁵N–¹³C HETCOR via the TEDOR experiment. The pulse sequence starts with CP to the ¹⁵N, then an evolution time ¹ is inserted, followed by the TEDOR transfer to the ¹³C and detection of the signal in the ¹³C ² period. The TEDOR transfer time was set to four rotor cycles. The 2D Fourier transformation produces a resolved MAS spectrum for the two interacting sets of heteronuclei. In the resulting 2D spectrum two types of carbons can be clearly distinguished, namely those which are coupled to backbone ¹⁵N and those which are coupled to lysine side-chain ¹⁵N nuclei.

**MAS and REDOR experiments on the selectively ¹³C–¹⁵N-labeled SH3 domain**

This section presents the ¹³C–¹⁵N REDOR results obtained on the selectively labeled SH3 domain sample. In a first step, the MAS spectra of the labeled sample were recorded. Figure 6 displays the ¹³C MAS spectrum of the sample. The three alanine methyl ¹³C (¹³C ¹) lines of the residues Ala11, Ala55 and Ala56 are clearly resolved. The unique assignment of the high-field line to the Ala55 residue was possible owing to the observed ¹³C–¹⁵N REDOR effect with the nearby tryptophan ring ¹⁵N (see below). The ¹⁵N MAS spectrum of the selectively labeled

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**Figure 4.** Spectral editing with the TEDOR sequence (νrot = 5 kHz). The ¹³C (observed) ¹⁵N spectra for n = 2 and 4 are compared with the unedited ¹³C CPMAS spectrum.

**Figure 5.** 2D heteronuclear correlation with the TEDOR sequence (νrot = 5 kHz). First the thermal ¹³C magnetization is destroyed by a saturation sequence, then the ¹⁵N coherence is transferred to the neighboring ¹³C. The lines mark which carbons are polarized from backbone nitrogen and lysine side-chain nitrogen, respectively.

**Figure 6.** Methyl region of the ¹³C spectrum of the alanine-¹³CH₃ enriched SH3 domain sample. Upper curve: normal CP/MAS spectrum. Lower curve: REDOR spectrum after a 16 ms dephasing time. The dephasing of the high-field line at 15 ppm allows the unique assignment of this line to the ¹³C ⁶ of residue Ala55.
SH3 domain sample (not shown) exhibits a strong line overlap in the central region of the spectrum. In the low-field range, lines are visible which can be attributed to the tryptophan ring $^{15}\text{N}$ of Trp41 and Trp42 by comparison with the liquid-state $^{15}\text{N}$ NMR spectrum.

In the next step, the $^{13}\text{C}$–$^{15}\text{N}$ REDOR decay curves of the selectively labeled sample were recorded employing XY-16 phase cycling at 5 kHz rotation frequency. Because of the better chemical shift dispersion in the $^{13}\text{C}$ MAS regime, a version of the REDOR experiment with observation of $^{13}\text{C}$ was chosen, where all $\pi$ pulses except the central echo pulse are irradiated in the $^{15}\text{N}$ channel. A strong REDOR effect on the $^{13}\text{C}$ of the tryptophan N ring is observed where all visible. The resulting REDOR data are shown in Fig. 7.

![Figure 7](image1)

**Figure 7.** $^{13}\text{C}$–$^{15}\text{N}$ REDOR data of $^{13}\text{C}^\text{a}$ (Ala55) of the selectively labeled SH3 domain sample. Owing to $T_2$ relaxation, only data with $\tau < 20\text{ ms}$ are used for the evaluation of the coupling. The dashed line at $\Delta S/S_0 = 0.65$ marks the final value expected from the $^{15}\text{N}$ isotope labeling factor of the tryptophan. The solid line is calculated from Eqn (7), employing a weighted superposition of 65% alanine-$^{13}\text{CH}_3$ tryptophan-$^{15}\text{N}$ and 35% alanine-$^{13}\text{CH}_3$ tryptophan-$^{14}\text{N}$, resulting in a dipolar coupling of $D = 48 \pm 8\text{ Hz}$, which after correction for fast molecular vibrations yields a CN distance of $3.85 \pm 0.25\text{ Å}$.

Because of the restricted sensitivity of the 7 T spectrometer, the short $T_2$ relaxation time of the alanine-$^{13}\text{C}^\text{a}$ signal and the small available amount of sample, it was not possible to follow the REDOR decay further than about 20 ms and the complete REDOR dephasing of the REDOR signal could not be observed. Therefore, we had to calculate the final value of the REDOR curve from the experimentally determined $^{15}\text{N}$ labeling factor, which has an uncertainty of ca $\pm 5\%$. A calculation with a $^{15}\text{N}$ labeling factor of 60% instead of 65% results in a motionally corrected $^{13}\text{C}$–$^{15}\text{N}$ dipolar coupling of $59 \pm 8\text{ Hz}$, corresponding to a CN distance of $3.75 \pm 0.20\text{ Å}$, which is in very good agreement with the CN distances obtained from liquid-state NMR spectroscopy. As will be shown below, this dependence of the REDOR evaluation on the isotope labeling can be avoided by the application of a combined TEDOR–REDOR experiment.

1. The dipolar coupling extracted from the REDOR data depends on the knowledge of the final value of the REDOR curve, which is determined by the isotope labeling factor of the $^{15}\text{N}$ nucleus. Owing to the restricted sensitivity of the 7 T spectrometer, the short $T_2$ relaxation, only data with $\tau < 20\text{ ms}$ are used for the evaluation of the coupling. The dashed line at $\Delta S/S_0 = 0.65$ marks the final value expected from the $^{15}\text{N}$ isotope labeling factor of the tryptophan. The solid line is calculated from Eqn (7), employing a weighted superposition of 65% alanine-$^{13}\text{CH}_3$ tryptophan-$^{15}\text{N}$ and 35% alanine-$^{13}\text{CH}_3$ tryptophan-$^{14}\text{N}$, resulting in a dipolar coupling of $D = 48 \pm 8\text{ Hz}$, which after correction for fast molecular vibrations yields a CN distance of $3.85 \pm 0.25\text{ Å}$.

2. The structure of the SH3 domain studied in this work is different from the structures studied by x-ray and liquid-state NMR spectroscopy, owing to the sample preparation. The spread of the distances observed by the other techniques already suggests that there is some structural freedom of the SH3 molecule concerning this distance. In particular, the x-ray structure suggest that the tryptophan side-chain (see Fig. 1) has a relatively large free volume for reorientations at its disposal, since the closest distances of the van der Waals spheres of the atoms of the tryptophan side-chain and the neighboring side-chains are between ca 0.5 and 1 Å. Therefore, it may be possible that the moistening of the sample, which was necessary to achieve the best resolution in the NMR spectra, causes a partial different structure of this region of the molecule. In particular, a change of the molecular conformation, either in the form of a rotation of the

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tryptophan ring or in the form of a slight rearrangement of the backbone chain, could have occurred, e.g. owing to water molecules which have diffused into the ‘free’ volume close to the tryptophan ring. To achieve a quantitative estimation of the structural changes, necessary for obtaining the found difference in the distance data, we studied the influence of the orientation of the tryptophan side-chain on the distance. Figure 8 defines the torsional angles $\psi_1$ and $\psi_2$. Calculations with a molecular modeling program show that $\psi_2$ has only a minor influence on the CN distance, but that the distance depends very strongly on $\psi_1$. In particular, a change of 4° in $\psi_1$ leads to a change of 0.3 Å in the CN distance, which corresponds to the difference between the REDOR distance of 3.85 Å and the shortest x-ray distance of 3.56 Å. Hence the assumption that torsional rotations of the tryptophan side-chain, caused for example by the moistening of the sample, are responsible for the variations in the x-ray data, and also the difference between x-ray and REDOR distance, is compatible with the structure of the protein in the vicinity of the tryptophan.

From the present data it is not possible to distinguish between these two alternatives, namely a systematic error in the REDOR curve, which is introduced by the isotope labeling factor, and a structural change of the molecule, and it is therefore not possible to draw a final conclusion about a structural change induced by the moistening of the sample.

It should be noted that the problem of accurately determining the labeling factor can be avoided by a combination of spectral and chemical editing. A possible example of such an experiment is a $^{13}$C–$^{15}$N TEDOR transfer followed by the $^{13}$C–$^{15}$N REDOR sequence. After the initial TEDOR sequence only those $^{13}$C spins that are polarized by $^{15}$N neighbors show a signal. This corresponds to an effective labeling factor of 100%. The problem with this approach is the loss in sensitivity in the TEDOR step, because only a partial coherence transfer from $^{15}$N to $^{13}$C is possible. To overcome this loss in sensitivity it is necessary to work at higher fields. Hence, for example, on a 14T solid-state NMR spectrometer such combined TEDOR–REDOR experiments should be feasible.

**CONCLUSION**

Whereas in previous work we studied only small molecules with REDOR spectroscopy, in this work a real protein, namely the SH3 domain of $\alpha$-spectrin (chicken), was studied as a model compound for the application of $^{15}$N–$^{13}$C TEDOR and $^{13}$C–$^{15}$N REDOR NMR spectroscopy. For these experiments, two different SH3 samples were synthesized and measured, namely a nominally 100% $^{13}$C- and $^{15}$N-labeled, lyophilized sample for the TEDOR experiment, and a selectively alanine $^{13}$C$^{\delta\delta}$- and tryptophan $^{15}$N$^{\delta\delta\delta\delta}$-labeled sample, precipitated from solution, for the REDOR experiment. In a first step it was shown that the TEDOR experiment can be employed as a very efficient dipolar filter for spectral editing of the $^{13}$C MAS spectrum. In a second step, the TEDOR sequence was employed as the contact sequence in the evolution period of a 2D solid-state heteronuclear correlation (HETCOR) experiment, allowing us to distinguish between those $^{13}$C coupled to backbone $^{15}$N and those $^{13}$C coupled to lysine side-chain $^{15}$N. Finally, the inter-residual distance between the alanine-C$^{\delta\delta}$ (Ala55) and the tryptophan (Trp42) $^{15}$N$^{\delta\delta\delta\delta}$ was measured by $^{13}$C–$^{15}$N REDOR spectroscopy. The REDOR measurements immediately allow one to attribute uniquely the dephasing $^{13}$C line to the Ala55 residue. From the REDOR data the dipolar $^{15}$N–$^{13}$C coupling is determined, and after correction for fast molecular vibrations and motions, converted into a CN distance of 3.85 ± 0.25 Å, which is very close to but slightly larger than the CN distances found by x-ray diffraction and liquid-state NMR (3.56–3.66 and 3.73 Å, respectively). This enlargement of the distance might be an indication of a conformational change of the SH3 domain e.g. due to the sample preparation, but it cannot be excluded that it is the result of the errors in the experimental determination of the $^{15}$N labeling factor. As a possible solution of this question a combined application of TEDOR and REDOR is discussed, which, however, is feasible only on a solid-state NMR spectrometer with higher field, i.e. higher sensitivity.

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