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Orientation of spin labels in *de novo* peptides†

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A series of *de novo* synthesised peptides including the artificial rigid paramagnetic amino acid TOAC at two positions with different distances from two to seven in the primary structure have been investigated by 9- and 94-GHz EPR spectroscopy under solid-state conditions. From simulations of the spectra of such two-spin systems, the distance and relative orientation of the paramagnetic centres can be deduced. This yields structural information on the peptides. A quantitative analysis of the spectra of individual peptides in different solvents as well as a qualitative analysis of the spectra of the peptide series shows that the peptides do not assume conformations corresponding to any of the common helical structures in proteins.
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KEYWORDS: EPR; multi-frequency; *de novo* peptides; spin labels; dipolar coupling

INTRODUCTION

EPR in conjunction with spin labelling provides the opportunity to determine molecular structures.[1–3] In particular, distance measurements between two-spin labels[4] have been widely used for investigations of protein structure.[5–7] Most commonly used spin labels for such studies are those of the MTS family, which can be covalently attached to cysteine amino acid residues.[8] The link between the piperidine-oxyl moiety and the protein backbone renders the label flexible. This usually allows native folding of the protein because the label can evade the steric restrictions of the protein. On the other hand, the labels have structural variability over several Ångstroms. Therefore, a distance distribution, rather than defined distances, is commonly observed. Further, the distance is not a unique parameter to describe a protein structure. Studies on spin-correlated radical pairs have shown that it is possible to obtain the orientation of radicals from EPR spectra. Measurements on short-lived spin-correlated two-spin systems (radical pairs), e.g. on $P_{700}^{\bullet+}$ $A_1^{\bullet-}$ yielded the orientation of the dipolar axis within the **g**-tensor system of the electron acceptor $A_1^{\bullet-}$ and thereby, the orientation of $A_1^{\bullet-}$ with respect to the axis connecting A_1 and the primary donor P_{700}.[9] This was later extended to a complete model for the relative arrangement of the two species $P_{700}^{\bullet+}$ and $A_1^{\bullet-}$ (for a review see Ref. 10). The potential to extract this structural information has been shown for pairs of spin labels also.[11] Most structural information can be extracted from nitroxides that are rigidly bound to the protein, adopting unique distance and orientation with respect to each other if all molecular motions have correlation times that are large compared to the timescale of EPR. This requires a rigid spin label with defined orientation to the protein backbone and solid-state conditions.

Chemical synthesis of peptides[12] provides the opportunity to incorporate rigid (non-natural) spin-labelled amino acids into peptides.[13] The spin-label TOAC[14] is an artificial amino acid and has only one degree of freedom, the conformation of the six-membered ring (Scheme 1). This label has repeatedly been used for the investigations of secondary structures of small peptides[13,15–17] taking advantage of the fact that TOAC narrows the distance distribution significantly. However, these experiments were primarily performed in liquid solutions with the proteins rotating freely and therefore, only the isotropic parts of the exchange coupling could be measured, while anisotropic parts as well as the purely anisotropic dipolar coupling are averaged out. Consequently, the orientation of the labels could not be determined.

In this work, we investigate the potential to extract not only the distance but also the complete orientational information from EPR spectra of such doubly spin-labelled peptides. By using the rigid spin-label TOAC and performing measurements under solid-state conditions, we tried to experimentally realise the concepts successfully applied to spin-polarised species for stationary two-spin systems. We investigated chemically synthesised peptides into which two TOACs are built in at different positions. These peptides were *de novo* designed to form an α-helix. Charged amino acids are arranged such that in α-helical conformation salt bridges can be formed. The goal is to find the relative orientation of the two nitroxides and to correlate this with the geometry expected for ideal helical peptides.

Although the peptides were designed for an aqueous environment, we have investigated them in different solvent

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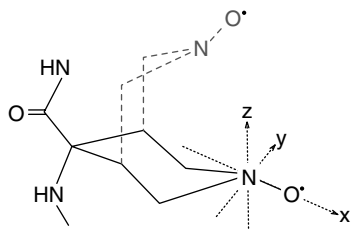

Scheme 1. The rigid spin-label TOAC built into a peptides backbone has only one degree of freedom left, which is the flip of the six-membered ring. TOAC's methyl groups are omitted for clarity.

systems to explore the influence of the environment on the protein structure. A mixture of water and glycerol, forming a glass upon cooling and therefore is a more homogeneous environment, which is also used often for biological systems. Fluorinated solvents such as TFE (2,2,2-trifluorethanol) and HFIP (1,1,1,3,3,3-hexafluorisopropanol) and their mixtures with water are considered to be structure stabilising in most cases.[18–21] We have therefore chosen to investigate, as an example, the peptide `mr18` in a mixture of TFE/H_2O.

The effect of organic solvents on protein structures has gained interest since model environments for membrane proteins are needed. $CHCl_3$ constitutes a non-polar and aprotic environment; DMSO also provides an aprotic but polar environment. Aprotic solvents that form no or only very weak hydrogen bonds do not compete with the peptides' internal hydrogen-bonding networks. The polar character of a solvent still allows it to stabilise partially charged structures. By mixing $CHCl_3$ and DMSO it is possible to adjust the polarity of the solvent to a certain degree by adjusting the ratio of the solvents.

EXPERIMENTAL

Peptides and solvents

Design and synthesis of the *de novo* peptides are already described elsewhere.[22] The primary sequences of the six peptides are listed in Scheme 2. As spin label for EPR, the artificial amino acid TOAC (4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl-4-carboxylic acid)[14] was used.

Fmoc-TOAC was synthesised following the procedure of Marchetto *et al.*[23] using TOAC (from Acros Organics) and *N*-(9-fluorenylmethoxycarbonyl)succinimide (from Novabiochem) except that Fmoc-TOAC was crystallised from a 1 : 1 mixture of ethyl ether and *N*-hexane (v/v). Peptide synthesis on 0.3 mmol PAL-PEG-PS-Fmoc (Applied Biosystems) resin began with standard solid phase peptide synthesis (SPPS) using the Fmoc strategy as described.[24] The resin was removed from the column and, before Fmoc-TOAC, was coupled manually using HATU (*N*-[(Dimethylamino)-1*H*-1,2,3-triazolo[4,5b]-pyridin-1-ylmethylene]-*N*-methylmethanaminium Hexafluorophosphate *N*-oxide) (Perseptive) and HOBt (*N*-hydroxybenzotriazole) in a 4.5 times (mol/mol) excess over the resin. The amino acid following TOAC was coupled with extended cycle times before standard SPPS was continued.

The spin-label 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) (Aldrich) was used for comparison, as it has been shown to have similar *g*-values as TOAC.[25]

The following solvents were used: TFE (99.5%, NMR grade), Aldrich; $CHCl_3$ (uvasol), Merck; DMSO (99.9%, acs), Sigma-Aldrich. For the MOPS buffer, a 0.1 molar solution of 3-morpholinopropansulfonic acid in *aq. bidest* was set to pH 7.0 with NaOH and sterilised. Solutions of the peptides were filled into EPR tubes and frozen in a liquid-nitrogen-cooled ethanol bath.

	1 5 10
mr57	NH_2 KKKLAEEA**X**K**X**LQEA $CONH_2$
mr58	NH_2 KKKLAEEA**X**KL**X**QEA $CONH_2$
mr59	NH_2 KKKLAEEA**X**KLQ**X**EA $CONH_2$
mr510	NH_2 KKKLAEEA**X**KLQE**X**A $CONH_2$
mr17	NH_2 KKKL**X**EEAAK**X**LQEA $CONH_2$
mr18	NH_2 KKKL**X**EEAAKL**X**QEA $CONH_2$

Scheme 2. Primary sequences of investigated *de novo* peptides, where **X** denotes the spin-label TOAC and the scheme of spin-label positions.

EPR

EPR spectra at 9 GHz were recorded partly with an FT-EPR spectrometer ESP 380E (Bruker) equipped with a dielectric ring resonator, and partly with a laboratory-built spectrometer. All spectra were recorded at 80 K. EPR spectra at 94 GHz were recorded with an Elexsys E680 spectrometer (Bruker).[26] For field calibration Li : LiF was used.[27]

Description of a helix

The shape of an EPR spectrum of two coupled spin labels is sensitive to the distance between the labels and their relative orientation. The relative geometry is described by six independent parameters. We have chosen the following set of parameters:

- three Euler angles, α, β, γ, transforming the **g**-tensor system of one spin label into the **g**-tensor axis system of the second spin label by the rotation operation $R_z(\beta)R_x(\alpha)R_z(\gamma)$;
- two angles ϑ, φ describing the orientation of the dipolar axis, where ϑ is the angle between the spin–spin connecting vector and the *z*-axis of the first spin labels **g**-matrix and φ is the angle between the projection of the connecting vector into the *xy* plane in this **g**-matrix and its *x*-axis (Fig. 1);
- the distance *R* between the two centres of gravity of either spin density.

The spin Hamiltonian

The EPR spectra of two coupled nitroxides are determined by the **g**-tensor of the nitroxides (electron Zeeman interaction eZ), the hyperfine coupling (hf) of the nitrogen to the electron spin and the electron–electron coupling (D) between the spins. The spin Hamiltonian of the system can be written as

$$\hat{\mathbf{H}} = \hat{\mathbf{H}}_{eZ} + \hat{\mathbf{H}}_{hf} + \hat{\mathbf{H}}_{D} \quad (1)$$

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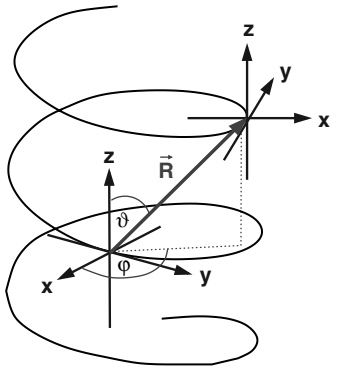

Figure 1. The relative orientation of two-spin labels is determined by their distance $|\vec{\mathbf{R}}|$, the orientation of the dipolar axis is defined by an angle ϑ between g_z and the connecting vector $\vec{\mathbf{R}}$ and the angle φ between the projection of connecting vector into the *xy*-plane and the *x*-axis of the first spin system.

$$= \mu_{\mathbf{B}} \sum_i \mathbf{B}\mathbf{g}_i\mathbf{S}_i + \sum_{ij} \mathbf{I}_j\mathbf{A}_{ij}\mathbf{S}_i + \mathbf{S}_1\mathbf{D}\mathbf{S}_2 \qquad (2)$$

In the simulations of the spectra, we omitted an exchange contribution to the coupling term $\hat{\mathbf{H}}_{\mathrm{D}}$ since for most of the peptides a separation of the spin labels of more than 8 Å is expected in any helical conformation. Only the dipolar coupling is taken into account for $\hat{\mathbf{H}}_{\mathrm{D}}$, which can be expressed as

$$\hat{\mathbf{H}}_{\mathrm{dip}} = \frac{1}{r_{ij}^3}\frac{\mu_0}{2h} g_i g_j \mu_B^2 (3\cos^2\vartheta - 1) \qquad (3)$$

The dipolar coupling shows a r^{-3} distance dependence and therefore can be used to determine the distance between the spins.

The electron Zeeman interaction and the hyperfine coupling are anisotropic and are therefore represented by tensors. The electron Zeeman anisotropy is a field-dependent effect which is expressed via the **g**-tensor, and measurements at different fields/frequencies are used to separate lines of different *g*-value. The spectral separation of two lines g_1 and g_2 is given by:[28]

$$\Delta B = \frac{h\nu}{\mu}\left(\frac{1}{g_1} - \frac{1}{g_2}\right). \qquad (4)$$

The hyperfine and the dipolar interaction, on the other hand, are field-/frequency-independent. Experiments at different frequency bands can therefore be used to separate contributions of different origin.

Two-spin order

For single spins, lowering the temperature raises the signal intensity because of the increased population difference. Furthermore, dynamic effects like protein movement and rotation of side chains are reduced.

In coupled systems, the depopulation of higher energy levels leads to a shift in population differences between different m_s levels. This effect is referred to as 'two-spin order'. Intensity shifts due to this effect can already be observed at temperatures which are an order of magnitude above the Zeeman temperature $h\nu = kT_z$.[29] At temperatures below the Zeeman temperature, mainly the lowest level is populated so that half of the transitions disappear.

At X-band, the Zeeman temperature is at $T_z = 0.4$ K, which cannot be reached with standard EPR instrumentation. At 94 GHz, temperature $T = 5$ K, close to the Zeeman temperature ($T_z = 4.5$ K), can well be obtained. In Fig. 2, the simulated spectra of two dipolar-coupled nitroxides at different temperatures are shown. The **g**-tensors of the nitroxides are collinear and the dipolar axis has an angle of 30° with the *z*-axis towards the *x*-axis. Therefore, the g_x- and g_z-components show a distinct splitting, whereas the g_y-component that lies close to the magic angle of the dipolar tensor is merely broadened. The simulations of spectra at different temperatures show distinct shifts of intensity in the dipolar-split lines. These intensity variations upon cooling can be used as additional spectroscopic information in multi-frequency studies. Along g_x the signal at higher fields loses intensity, whereas along g_z the lines at lower fields lose intensity. This can be accounted for by the sign of the dipolar coupling, which changes between *x* and *z*. Therefore, the relative orientations of the dipolar axis with respect to the **g**-tensor system can be determined.

Simulation routines

The spectra with two dipolar-coupled spin labels were simulated using the program barley[30] which diagonalises the full spin Hamiltonian. The X-band data were fitted with a routine written by E. Kirilina (Novosibirsk), based on second order perturbation theory.

The *g*-principal values and hyperfine values were taken from measurements of TEMPO in the respective solvent.

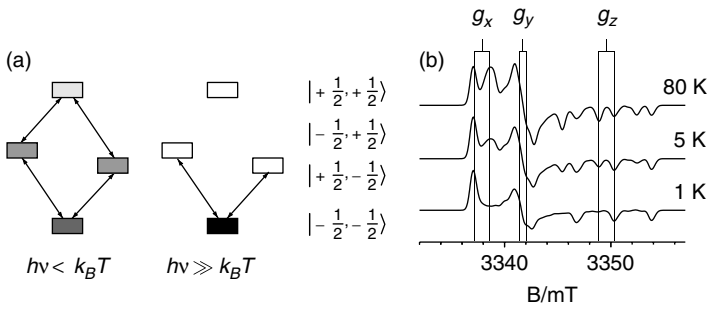

Figure 2. Two-spin order. (a) Population of the four electron spin levels in the high- and low-temperature regime. (b) Simulation of W-band EPR spectra of two coupled nitroxides at different temperatures.

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RESULTS

The primary sequences of the investigated doubly spin-labelled *de novo* peptides are shown in Scheme 2. First, we concentrated on two peptides, one with a single amino acid between the labels, called mr57, and the other with seven amino acids between the labels, called mr18. As solvent we have chosen aqueous buffer (pH 7), resembling the natural environment of soluble proteins. The X-band spectra of both peptides are quite similar (Fig. 3(A)). Both spectra display the shape of a single nitroxide that is broadened, presumably due to dipolar coupling. However, in the two peptides the labels should have completely different distances and orientations. This difference can, in this case, hardly be deduced from the X-band EPR spectra since both show line broadening. In W-band, where field-dependent components are better separated, the spectra of the two peptides are at 80 K clearly different (Fig 3(B)). At 94 GHz, the spectrum of mr57 shows substantial broadening of the g_x component, which cannot be explained by convolution with only one gaussian line. The g_y component is broadened to approximately 3.0 mT. In a well-defined peptide, a dipolar coupling of such size should lead to a line splitting since the linewidth of the spin label itself is much smaller (about 0.4–0.6 mT). Furthermore, cooling close to the Zeeman temperature (Fig. 3(C)) only leads to a general intensity shift towards the low-field region but not to the disappearance of individual lines, as demonstrated in Fig. 2.

Simulation of the experimental spectra is possible only under the assumption of a high inhomogeneous linewidth. Therefore, a large set of parameters lead to similar spectra. Also, the simulated spectra for an α- and a π-helix are very similar and both fit the experimental spectra. In a 3_{10}-helical conformation the distance between the spin labels can be estimated to be about 5 Å. In this case, the dipolar and exchange coupling between the spin labels would be so large that despite the high linewidth, a splitting should be observed. Since this is not observed, the 3_{10}-helical conformation can be excluded. Although the spectrum of mr18 at X-Band is quite similar to the spectrum of mr57, the spectrum of mr18 at W-band shows sharper features in the g_z-region (Fig. 3(B)). Again, upon cooling to 5 K rather global intensity changes are observed. Similar to mr57, the broad line features cannot be explained by a specific dipolar coupling in any of the helix types. A possible origin for the significant line broadening is an aggregation of the peptides upon cooling. However, the samples stayed homogeneous even after repeated freeze–thaw cycles. Therefore, we consider aggregation as unlikely. Another source for line broadening is the conformational variability of the spin labels. Because of the flip of the six-membered ring, four conformers giving rise to different EPR spectra are possible. But even the superpositions of calculated spectra for these four conformers result in clearly resolved spectra (data not shown) so that further mechanisms of line broadening have to be assumed.

Frozen aqueous solutions form a micro-crystalline environment, causing strain effects and, therefore, line broadening. By this, mainly g_x would be affected.[28] Another effect, which leads to a broadening of all lines corresponding to the respective g-components, would be a conformational heterogeneity of the peptide that leads to a wide distribution of dipolar couplings.

To investigate the influence of the micro-crystalline environment, we used a buffer mixture with glycerol and measured, as an example, the peptide mr18. In Fig. 4(A) the spectrum measured at W-band is shown. Compared to the spectrum of mr18 in water, the linewidth is significantly reduced, but no resolved components are visible. Therefore, no unique information about distance and orientation between the labels can be deduced. Since the absence of line splitting at the observed small linewidth indicates very small dipolar couplings, we assume that the peptide is mostly unfolded in the water/glycerol environment. As the next solvent mixture, we consider TFE/H_2O.

Spectra of mr18 in TFE/H_2O (1 : 1) were recorded again at 9 GHz (80 K) and at 94 GHz (80 K, 5 K) (Fig. 5). The spectra of mr18 in TFE/H_2O are better resolved than the

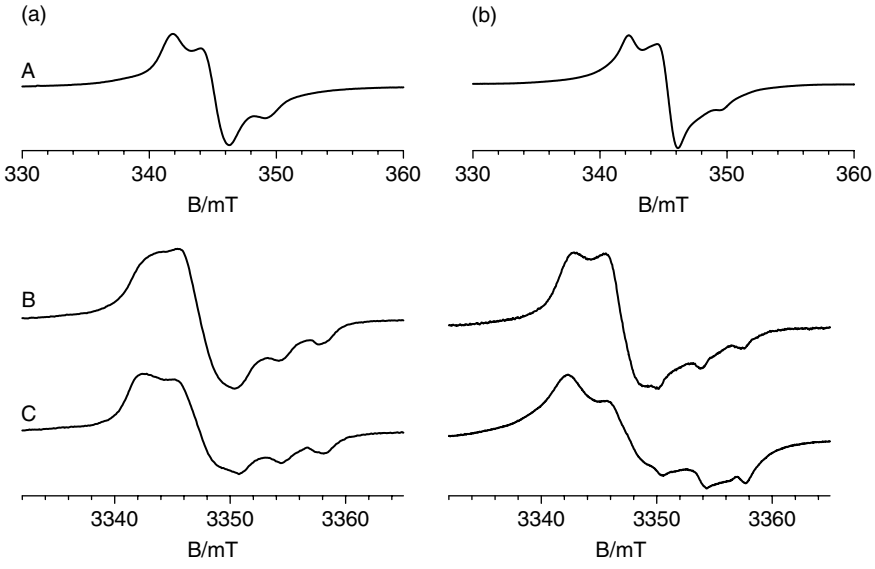

Figure 3. EPR spectra of mr57 (a) and mr18 (b). A: 9 GHz, 80 K, B: 94 GHz, 80 K, C: 94 GHz, 5 K.

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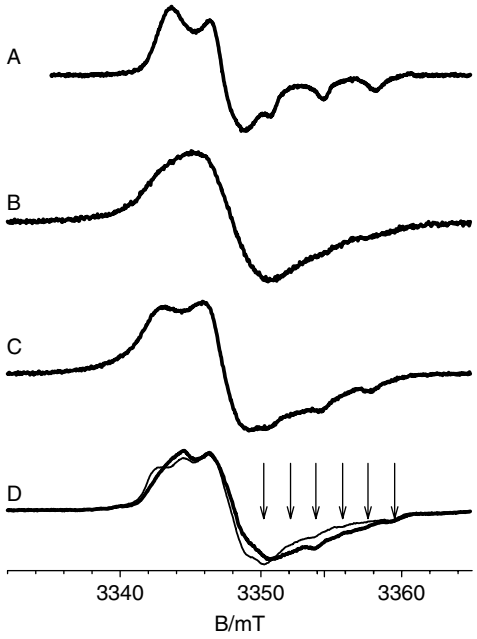

Figure 4. W-band spectra of mr18 in different solvents at 80 K. A: H_2O/glycerol (4 : 6), B: $CHCl_3$, C: DMSO, D: $CHCl_3$/DMSO (7 : 3) (thin line spectrum at 5 K). Marked is the hyperfine and dipolar splitting of the g_z component.

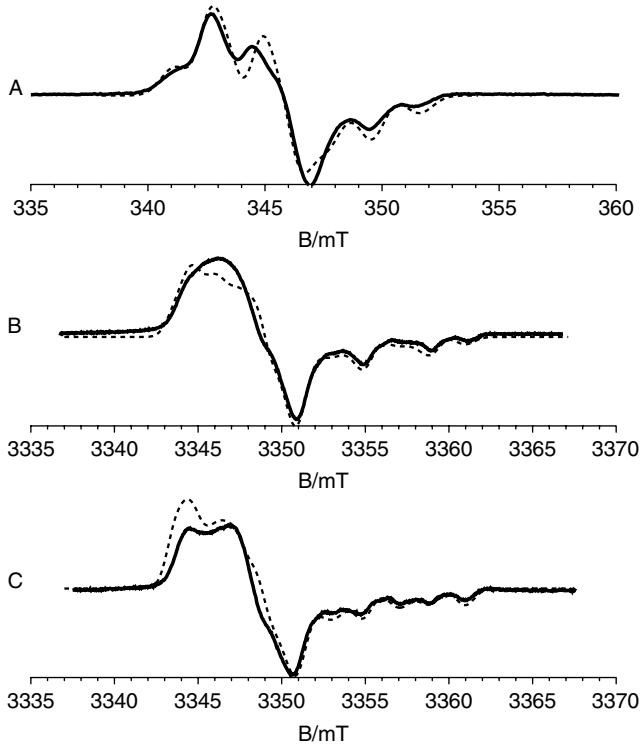

Figure 5. mr18 in TFE/H_2O. EPR spectra and simulations at 9.7 GHz (A), 80 K, 94 GHz, 5 K (B), 94 GHz, 80 K (C). Simulation parameters: $g_x = 2.0086$, $g_y = 2.0060$, $g_z = 2.0020$, $A_{xx} = 17.0$ MHz, $A_{yy} = 21.0$ MHz, $A_{zz} = 110.5$ MHz, $\alpha = 15°$, $\beta = 352°$, $\gamma = 329°$, $\vartheta = 20°$, $\varphi = 85°$, D = 1.2 mT.

spectra of mr18 in H_2O. Already at X-band, several lines are clearly recognisable. Clear splittings of the hyperfine components in the g_z region are visible in the spectra taken at W-band. Distinct changes in intensity are observable in the W-band spectra at the different temperatures. These intensity shifts mainly affect the split hyperfine lines on g_z, but prominent intensity changes also occur in the still unresolved g_x-component.

Despite the well-resolved structure of the X-band spectrum, satisfactory simulations are possible using different parameter sets. Only a simultaneous simulation of all three spectra leads to a unique simulation, which is shown in Fig. 5 (the simulation parameters are given in the figure caption).

In addition, spectra of doubly labelled peptides have been recorded in organic solvents. The spectra of mr18 in $CHCl_3$ and in DMSO at 94 GHz are shown in Fig. 4(B) and (C). The spectral features are extremely broadened and an interpretation beyond the assumption of significant structural heterogeneity is impossible.

In a mixture of $CHCl_3$/DMSO (7 : 3) the spectrum of mr18 (Fig. 4D) differs distinctly from that in water and from that in TFE/H_2O. However, in contrast to the case of pure solvents, the linewidth is strongly reduced compared with the spectrum in water and similar to the spectrum taken in TFE/H_2O. Comparing the spectra in the solvent mixtures TFE/H_2O and $CHCl_3$/DMSO, the most significant differences are visible along g_x. In TFE/H_2O the g_x-component shows no clear dipolar coupling, whereas in $CHCl_3$/DMSO g_x is made up of two lines separated by 1.6 mT. This shows that the peptide adopts a well-defined but different conformation in $CHCl_3$/DMSO than in TFE/H_2O. It is important to note here, that the centre of the split lines around g_x is shifted to lower g-values (upfield) compared to the g_x-value of TEMPO in the same solvent. To obtain additional structural information, we have recorded spectra of a series of peptides with increasing distance between the labels in the primary structure in the two solvents H_2O and TFE/H_2O.

X-band EPR spectra of the peptide series in water show repeated increase and decrease of the dipolar coupling (Fig. 6(a)). The deviation from the spectral shape of a single spin label increases from mr57 to mr59. In mr59 the dipolar coupling clearly dominates the spectrum. Analysis of the spectrum shows that it consists of two species. The central part of the spectrum (341–349 mT) presents the lineshape of a weakly coupled species while the outer features around 338 and 351 mT belong to a strongly coupled spin system. In the peptide mr510, the dipolar coupling is so weak that it only leads to a broadening of lines. The spectrum of mr17 has a peculiar shape since the line at the lowest field starts negative. A positive signal at a lower field should be present, but probably because of the strong broadening it has such little intensity that it was not observed. The spectral shape can be explained, assuming that mr17 adopts two different conformations. The well-structured signal between 340 and 350 mT belongs to a very weakly coupled spin label, whereas the signals at 337 and 357 mT belong to a strongly coupled conformation. In mr18 the spectral shape is again uniformly broadened.

The spectra of the peptide series in TFE/H_2O (Fig. 6(b)) at X-band have a linewidth of approximately 0.6 mT, which is significantly reduced, compared with the spectra in water.

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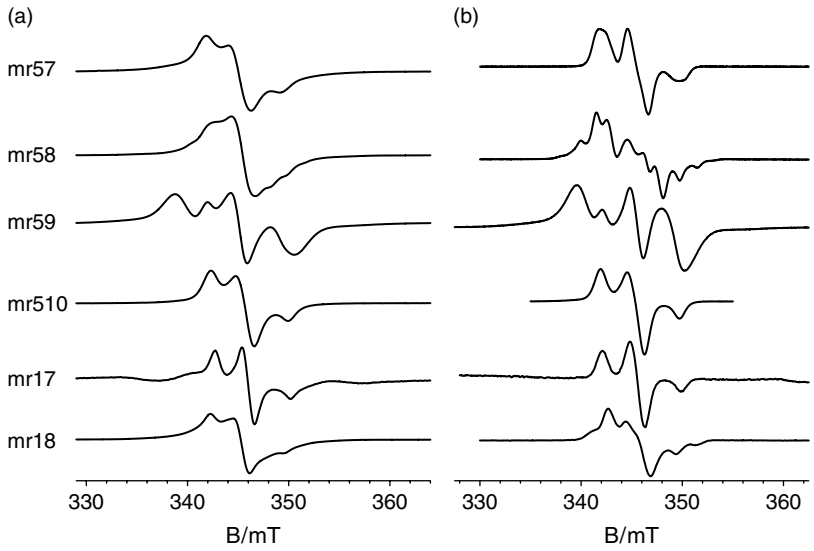

Figure 6. EPR spectra (9 GHz) of the series of peptides. (a) in water, pH 7; (b) in TFE/H_2O (1 : 1).

The spectra of the peptides in TFE/H_2O have not only a reduced linewidth compared to those dissolved in water, but some of the spectra even have completely different shapes. Especially, for mr57, mr58, mr17 and mr18 the structure of the spectra changes significantly between the solvents. As in water, the series of spectra at 9 GHz shows a periodic increase and decrease of the spectral width. However, in H_2O/TFE, the spectral width is the largest in mr58, mr59 and mr18. Closer inspection of the individual spectra shows that mr57 reveals a partially resolved splitting of the g_z component. The spectrum of mr58 is an exception in the series since it shows very narrow lines with a linewidth reduced to 0.4 mT. The spectrum of mr59 is similar to the one in H_2O but shows a smaller linewidth. The spectrum again is composed of two different species with a similar ratio between the species. In the spectrum of mr17 the strongly broadened species have disappeared. Therefore, this peptide has adopted a uniform conformation under the addition of TFE. The spectrum of mr18 has been discussed above.

DISCUSSION

In the following, we combine the results from the dual frequency analysis of individual peptides in different solvents with results derived from the measurements of the peptide series in water and H_2O/TFE. As an example, we first analyse the spectra of mr18 in H_2O and H_2O/TFE. Similar to the attempts to fit the significantly broadened spectra of mr57, no unique parameter set describing the orientation between the two-spin labels could be obtained for mr18 in H_2O. This is remarkable because the spectra of mr18 at W-band show a smaller linewidth of the hyperfine components along g_z. For mr18 in H_2O/TFE the situation is different. The simultaneous simulation of the X-band spectrum and the W-band spectra recorded at 80 and 5 K resulted in a satisfactory simulation with the parameters given in the caption of Fig. 5. In this simulation, only one specific relative geometry between the spin labels was included, even though each spin label could adopt two different conformers (Scheme 1). Therefore, we conclude that the spin labels adopt a unique conformation in mr18. The simulations fit the experimental spectra and their temperature dependence, particularly well in the g_z region. The observed intensity changes of the split hyperfine components along g_z indicate that both g_z-axes form an angle with the dipolar axis that is much smaller than the magic angle. For none of the considered ideal helix types (taking all four possible TOAC conformations into account) this situation is realised. Consequently, we assume that the orientation of the TOACs describe a very specific conformation of the peptide.

Apparently, the bulky and charged amino acid residues can lead to a modification of the helical structure and the relative geometry between the two-spin labels. Such a deviation from an ideal helical structure deduced so far from the analysis of the X- and W-band spectra of the peptide mr18 is consistent with the findings from the X-band spectra of the peptide series. Using a series of peptides, it should be possible to deduce the helix type, based on the periodicity of the dipolar broadening of the spectra. In a 3_{10}-helix, one turn is built from exactly three amino acids, resulting in an expected periodicity of three for the dipolar broadening. For a typical α-helix the dipolar broadening should have a periodicity of 3.6. In a π-helix the periodicity should be 4.4. In the peptide series in H_2O, the spectral width is high for mr59 and mr17, at least for one of the sub-species, and medium for mr58 and mr18. This pattern for the peptides in water does not correlate to any of the expected periodicities for the conventional helix types. It is possible that the incorporation of the non-natural amino acid TOAC disturbs the folding of the peptide to its preferential geometry in the absence of TOAC. Amino acids that are tetra-substituted at C^α have been found to destabilise β-sheets[31] and to support helical structures. Increased ratios of α-helical structures[19,21] but also of 3_{10} helical structures[32] have been found by CD spectroscopy. Furthermore, the observation of different spectral sub-species, at least for some of the peptides, leads to the conclusion of a structural heterogeneity of the peptides.

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In TFE/water, the spectral width of the peptide series resembles in its periodicity an α-helical shape. However, the peptide mr59 still adopts two different conformations. Analysis of the peptide mr58 shows that despite the highly resolved pattern, the spectrum could not be simulated with the given g-values and under the assumption of pure dipolar coupling. The W-band spectra of mr58 (Fig. 7) at 80 K and at 5 K show no difference. Inspection of these spectra shows that the g_z-component consists of five lines with a splitting that is half of the usual hyperfine splitting on g_z. This is possible only if the spin labels are so close that the two $S = 1/2$ systems couple to a spin $S = 1$. In that case, the spin would couple to two ^{14}N with equal hyperfine coupling, resulting in the observed pattern of five lines with an intensity pattern of 1 : 2 : 3 : 2 : 1.

However, if the spin labels in mr58 are much closer than in mr59, again a strong deviation from the α-helical shape occurs.

Finally, we analyse the spectra of the peptide mr18 in the organic solvents. A comparison of the W-band spectrum of mr18 with the spectrum of the single spin label (Fig. 8) in $CHCl_3$/DMSO shows that the g_x-component of the single spin label (3337.2 mT) is at a lower field than the lowest lying resonance in the coupled spectrum (3338.0 mT). Such a shift cannot be caused by spin–spin interactions but can only be explained by a lowering of g_x itself. The value of g_x strongly depends on the polarity of the environment and the hydrogen-bonding pattern to the nitroxide.[33–35] The environment-dependent parameters g_i and A_{ii} were determined independently by measuring the similar spin-label TEMPO in the same solvent mixture. Since TEMPO and TOAC have similar g-values,[25] the decrease of g_x has to originate from a change in the local environment of the label. In a peptide, one possibility is internal hydrogen bonding to side chains of other amino acids. Here, NH_3^+ groups of lysines are close, and a hydrogen bond to an ammonium group could lower g_x. A shift of g- and hyperfine values due to the peptide environment increases the number of free parameters from 6 to 18, since the values can again differ for the individual labels. While environment-induced g-factor changes are well established in the interior of proteins and are used to map polarity profiles,[36] we did not expect such an influence for the solvent-exposed spin labels attached to a small peptide. This finding, however, shows that for further structural investigations, singly- as well as doubly labelled peptides are required to obtain the g-values for the labels at specific sites.

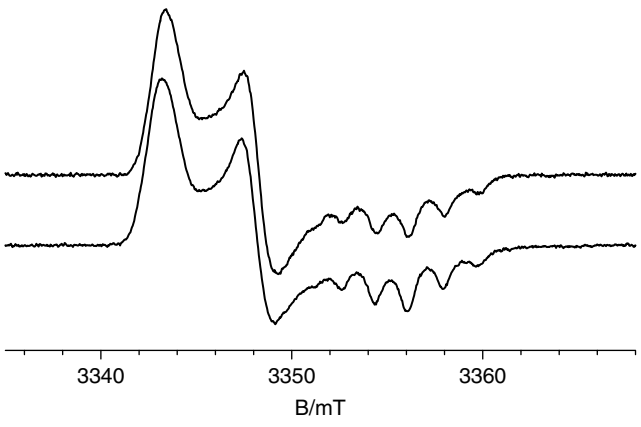

Figure 7. Spectrum of mr58 at 94 GHz and 80 K (top) and 5 K (bottom).

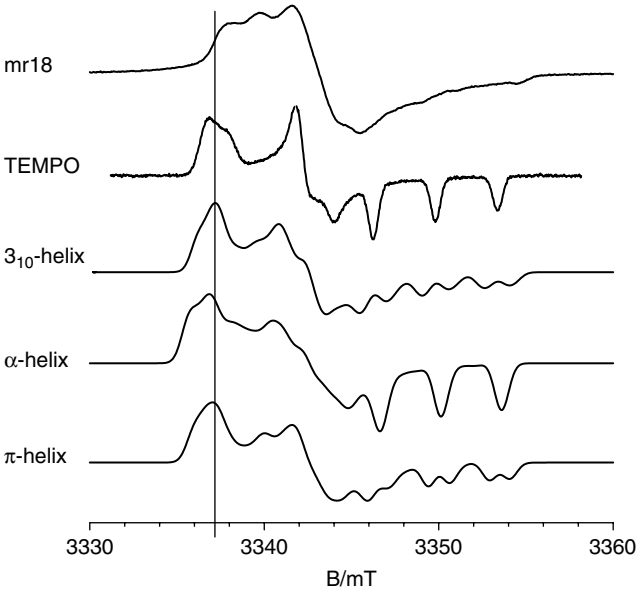

Figure 8. Experimental spectrum of mr18 at 94 GHz and 80 K, in comparison with the experimental spectrum of TEMPO in $CHCl_3$/DMSO and calculated spectra of two-spin labels orientated as in the three helix types given. g_x of TEMPO is marked by the vertical line.

In Fig. 8 the spectrum of mr18 in $CHCl_3$/DMSO is compared with simulations based on spin-label orientations in typical helix conformations. Neglecting the exact position of the spectral lines and comparing only the overall shape of the spectra, the spectrum of mr18 resembles most the simulation of an 3_{10} helix. The structure-stabilising abilities of aprotic solvents arise from the fact that they do not effectively solvate polar groups and therefore do not compete with intra-peptide hydrogen bonds. However, polar groups at the ends of a peptide cannot form intra-peptide hydrogen bonds and destabilise the structure. In tighter conformations (as a 3_{10}-helix), the number of end groups is smaller than in wider conformations (as a π-helix), rendering the latter less stable. However, theoretical[37] and experimental[38] evidence has been given for the occurrence of π-helical conformations under similar conditions. Pulsed ELDOR experiments on a similar peptide in $CHCl_3$/DMSO gave indications for a 2_7 conformation of the peptide.[6] Such a structure, however, can be excluded for `mr18`, since the labels would be 22 Å apart and no splitting would be observed in that case.

CONCLUSION

This work shows how determination of distance and orientation of two-spin labels can contribute to the structural investigation of peptides. A series of peptides doubly labelled at different positions with the rigid spin label TOAC has been investigated. By the choice of solvents, different secondary structures could be induced. It was shown that the peptide assumes a well-defined conformation in a mixture of

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TFE/water as well as in $CHCl_3$/DMSO. The structures in the different solvents differ significantly. The peptide mr18 was investigated in TFE/H_2O at different frequency bands and in different temperature regimes. From the simultaneous simulation of three spectra, a set of parameters could be obtained, which uniquely describes the relative orientation of the two TOACs. However, the conformation does not correlate with any of the typical helix types adopted by peptides. The analysis of the peptide series showed that the peptides do not adopt a uniform structure either in aqueous buffer or in TFE/water.

The difficulties in analysing the relative geometry of two rigid spin labels under solid-state conditions, which became obvious during this study, provides an explanation for the rather conflicting results that have been given for similar systems at different times by other groups.[6,13,16,17,39,40]

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