The Secondary Structure of a Membrane-Modifying Peptide in a Supramolecular Assembly Studied by PELDOR and CW-ESR Spectroscopies

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Abstract: The new technique of pulsed electron−electron double resonance in electron spin−echo (PELDOR) in combination with the CW-ESR method has been used to investigate the secondary structure of a double spin-labeled peptide (the [TOAC-1,8]-analogue of the peptaibol antibiotic trichogin GA IV) that is hidden into a tetrameric supramolecular assembly of unlabeled peptide molecules. The magnetic dipole−dipole relaxation of spin labels has been experimentally studied in glassy solutions of the double-labeled peptide frozen to 77 K in a mixture of chloroform−toluene with an excess of unlabeled peptide. The PELDOR signal oscillations have been observed at high degrees of dilution with unlabeled peptide. The intramolecular distance between the spin labels of the peptide molecule in the aggregate has been determined from the oscillation frequency to be 15.7 Å which is close to the value of ≅14 Å calculated for a 3_{10}-helical structure. Estimation of the fraction of this ordered secondary structure shows that about 19% of the peptide molecules in aggregates are folded in the 3_{10}-helical conformation. The present experimental results are consistent with our molecular model presented in *J. Am. Chem. Soc.* **2000**, *122*, 3843−3848, wherein four amphiphilic 3_{10}-helical peptide molecules form a vesicular system with the polar amino acid side chains pointing to the interior, and the apolar side chains, to the exterior of the cluster. The experimental data were compared with the results obtained with other techniques.

Introduction

The methods of pulsed ESR spectroscopy have been recently exploited to analyze the 3D-structure of the supramolecular cluster of spin-labeled peptides in frozen glassy solutions by extracting information about the *intermolecular* distances between spin labels from dipole−dipole couplings.[1−4] In references[3,4] the method of pulsed electron−electron double resonance in electron spin−echo (PELDOR) has been used in combination with the CW-ESR technique to clarify details of aggregation of the spin-labeled peptaibol antibiotic trichogin GA IV molecules in apolar glassy solvents at 77 K. On the basis of the obtained experimental data we showed that the number of peptide molecules in the aggregate is close to four. A molecular model has been proposed for such aggregates.[3] The CW-ESR results testified the possible existence of aggregates in the same solutions, but in the liquid phase at room temperature. It has been demonstrated that increasing the solvent polarity by adding ethanol leads to dissociation of the aggregates.[3]

The data on magnetic dipole−dipole relaxation obtained by PELDOR in frozen glassy solutions of *single*-labeled peptides indicate that the aggregates include peptide chains with a fixed structure where the intermolecular distances between spin labels show minor spreading.[3,4] This observation allows one to assume a fairly ordered spatial structure for the peptide building blocks of the tetrameric peptide cluster. Therefore, the problem is of interest to experimentally determine the nature of the secondary structure adopted by the peptide constituents that form aggregates.

One of the possible methods for solving this problem is the study of the *intramolecular* magnetic dipole coupling of two spin labels incorporated at well-defined positions in the peptide chain. In this way one can get information about the distance between labels of an individual peptide molecule. This distance should be different for different peptide conformational states. For instance, when the backbone torsion angles of peptide I (see Table 1) were adjusted to theoretical values corresponding to α-, 3_{10}-, 2_7-, and 2_5-helices, the following distances between the NO radicals were calculated: 11, 14, 22, and 28 Å, respectively. A comparison of the experimental distance with the calculated one can give the evidence of a particular peptide conformational state.[1,2]

We have studied the *intramolecular* dipole−dipole couplings of spin labels in *double*-labeled peptide I in a frozen glassy solution, containing an excess of unlabeled peptide II under

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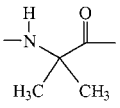
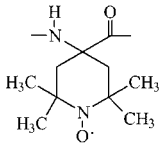
Table 1. Primary Structure of Peptides I and II; in Addition, the Chemical Structures of Amino Acids TOAC and Aib Are Shown

Peptide I	*n*Oct-TOAC[1]-Gly-Leu-Aib-Gly-Gly-Leu-TOAC[8]-Gly-Ile-Leu[11]-OMe
Peptide II	*n*Oct-Aib[1]-Gly-Leu-Aib-Gly-Gly-Leu-Aib[8]-Gly-Ile-Leu[11]-OMe



Aib



TOAC

conditions that aggregates are formed. The primary structures of these peptides are shown in Table 1. In peptide I two α-aminoisobutyric acid (Aib) residues have been substituted by paramagnetic labels, that is, 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid (TOAC) residues. It has been shown before that the replacement of an Aib by a TOAC residue does not influence the peptide conformation.[5,6]

As has been published previously,[1−4] to study the magnetic dipole−dipole coupling of spin labels of the trichogin analogues in frozen solutions we have used the PELDOR technique. This technique makes it possible to determine the weak magnetic dipole couplings of spins in solids, hidden by the inhomogeneous broadening of ESR spectra lines, and to calculate accurate distances between paramagnetic particles.

The PELDOR technique, which has been reviewed in detail in references,[7,8] is a modification of the electron spin−echo method (ESE). The PELDOR signal is the usual two-pulse Hahn ESE signal observed at frequency ω_A but measured by switching on additional pumping pulse at frequency ω_B that modulates the dipole−dipole coupling of spins. The pumping pulse is applied between two ω_A pulses at time T after the first pulse. The spins are marked as spins A (at ω_A) and spins B excited by the pumping pulse at ω_B. The pumping pulse induces transitions between the Zeeman levels of spins B, thus changing local magnetic fields at spins A. This effect results in the additional dephasing of spins A and hence in a decrease of the ESE amplitude. The main decay of the PELDOR signal occurs within a characteristic time $T \approx 1/D$, where D is a typical value for the dipole−dipole spin coupling. Usually, in the experiments time τ between ω_A pulses is fixed, and an analysis is made of the dependence of the PELDOR signal amplitude V on time T between the first ω_A pulse and the pumping ω_B pulse. As the pumping pulse changes the spin−spin interaction between spins only, PELDOR allows one to distinguish the spin−spin interaction among other independent mechanisms of magnetic phase relaxation, such as the electron spin−echo modulation due to electron−nuclear interaction (ESEEM) or other sources of inhomogeneous spectral broadening. As compared with the usual two-pulse ESE technique, the PELDOR method also makes it possible to partially exclude the dead time of the spectrometer due to "ringing" of the cavity (leaving limitations connected with the pulse width) and to substantially extend the range of studied dipole couplings.

To unambiguously determine the intramolecular dipole−dipole coupling of spin labels in the molecule of double-labeled peptide I, it is necessary to set this contribution apart from the total dipole−dipole coupling in the system, also including the spin coupling between different molecules of peptide I in aggregates and the spin coupling between aggregates. To this end, the required amount of double-labeled peptide I was added to a given amount of unlabeled peptide II. Then, this mixture was dissolved in the apolar chloroform−toluene solvent combination in a 7:3 ratio by volume. According to reference 3, under these conditions, forming a transparent glass at 77 K, aggregates are generated from single spin-labeled peptide molecules. Assuming formation of supramolecular structures similar to those of single-labeled trichogin by double-labeled peptide I and unlabeled peptide II, it is expected that in this latter case aggregates will form that contain both unlabeled and double-labeled peptides. With a sufficient excess of unlabeled peptides, the aggregates will mainly contain one double-labeled peptide per aggregate, as the fraction of aggregates with two and more double-labeled peptides can be neglected. In this way the PELDOR technique can be exploited to unravel and study in detail the intramolecular coupling of spin labels inside one of the building blocks of the tetrameric peptide cluster, thereby excluding the interaction with other peptides of the aggregate. By analyzing the dependence of magnetic relaxation on the total concentration of spins for these samples, one can readily exclude the contribution of intermolecular (inter-aggregate) dipole coupling.

For comparison, in this paper the data on magnetic phase relaxation are also given for the double spin-labeled peptide I in a polar solution of 2,2,2-trifluoroethanol (TFE). In this case, according to reference 2, no aggregation of peptides is observed.

Experimental Section

In the experiments synthetic, double TOAC-labeled[5] and unlabeled[9] peptides (Table 1) were used. In both *n*-octanoylated (*n*-Oct) peptides I and II the C-terminal 1,2-amino alcohol, leucinol, of trichogin GA IV was replaced by leucine methyl ester (Leu-OMe). It has been shown before that the antibiotic activity as well as the membrane-modifying properties of trichogin are not changed by these substitutions.[5,6,9]

PELDOR experiments have been carried out on a homemade X-band ESE spectrometer supplied with a bimodal resonator and a device for creating pumping pulses at frequency ω_B.[7] The registration frequency of the spectrometer was 9476 MHz. The difference between registration and pumping frequency was about 100 MHz. The durations of the first and second ω_A pulses forming the spin−echo were 40 and 70 ns, respectively. The pumping pulse duration was about 40 ns.

The samples (glass ampules, 5.0 mm in diameter) contained about 0.1 mL ($\cong 10^{-2}$ M) of the solution studied. A chloroform−toluene mixture in a 7:3 ratio by volume and TFE were used as the solvents. Spectrograde solvents were used without additional purification. The samples were frozen by inserting ampules into liquid nitrogen. When freezing, the solutions formed transparent glasses.

CW-ESR spectra of the spin-labeled peptide were recorded on an X-band ESP-380 Bruker spectrometer at a modulation frequency of 100 kHz in the absence of spectrum saturation.

In the CW-ESR and PELDOR experiments at 77 K the samples were placed inside the nitrogen-cooled finger of a Dewar flask, which was located in the cavity of the spectrometer. The number of spin labels in samples was controlled by comparing the double integrals of the CW-ESR spectra of the sample with similar values for $CuCl_2 \cdot H_2O$ crystals

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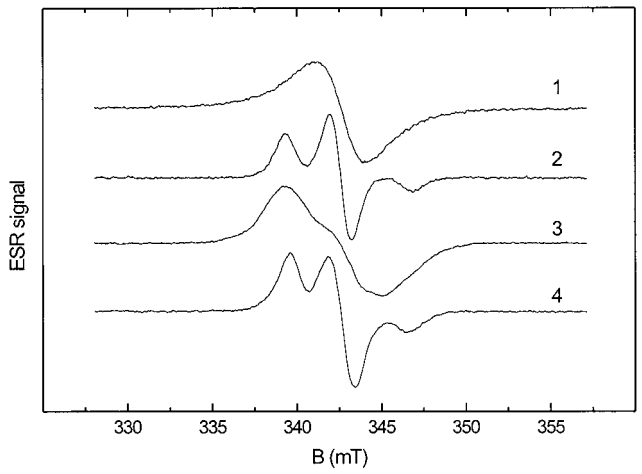

Figure 1. CW-ESR spectra of peptide I at 77 K. 1: Peptide I in the dried powder form; 2: solution of peptide I in TFE; 3: solution of peptide I in a chloroform−toluene mixture (7:3 ratio by volume); 4: mixture of peptides I and II (ratio 1:10) in a frozen chloroform−toluene mixture.

containing a given number of paramagnetic centers. The concentration of spin labels in the samples studied ranged from 4×10^{18} cm^{-3} to 8×10^{18} cm^{-3}.

Results and Discussion

Figure 1 shows the CW-ESR spectra of peptide I in the dry powder form at 77 K. The spectrum (curve 1) is a single line whose width between the extrema is 3.2 mT. By contrast, the ESR spectrum in frozen glassy TFE ($\cong 10^{-2}$ M) (curve 2) is an anisotropic triplet typical for a diluted glassy solution of a peptide labeled with the nitroxide-containing TOAC residue.[1,2] The difference between spectra 1 and 2 is probably due to the strong exchange coupling of spin labels due to their high concentration in the powder.

Of interest is the considerable difference between the spectra of peptide I in polar TFE (curve 2) and in apolar chloroform−toluene solution (curve 3). In the case of the chloroform−toluene mixture a substantial broadening of the components and a general change in the spectral shape are seen. Since the difference in concentration of peptide I in the two solutions is negligible, this change might be explained by formation of aggregates, as has been found previously for single spin-labeled trichogin peptides in nonpolar solvents.[3,4] Note that in this case the effect of aggregation leads to a more considerable change in the ESR spectral shape than has been revealed for the aggregates consisting of single spin-labeled peptides.[3,4] Thus, the CW-ESR data for the double spin-labeled peptide I confirm the previous observed phenomenon of aggregation of single spin-labeled peptides in liquid apolar solvents.

Curve 4 refers to a mixture of double-labeled peptide I and unlabeled peptide II in a frozen chloroform−toluene mixture. The ratio between the amount of double-labeled and unlabeled peptides is 1:10. It turns out that a decrease in the fraction of double-labeled peptide molecules in aggregates, owing to the addition of unlabeled peptide, causes a substantial narrowing and higher resolution of the ESR spectrum. These changes are related to a decrease in the intermolecular dipole−dipole coupling of spin labels with a decreasing fraction of spin-labeled peptide molecules inside the aggregates. Note that the ESR spectral shape becomes close to the ESR spectrum of peptide I in TFE. This is an obvious result from the point of view of magnetic dipole interactions in the tetramer, as the dilution from eight to two spins per cluster is the same as that taking place in the dissociation of the aggregate to the monomeric peptide in a polar solvent.

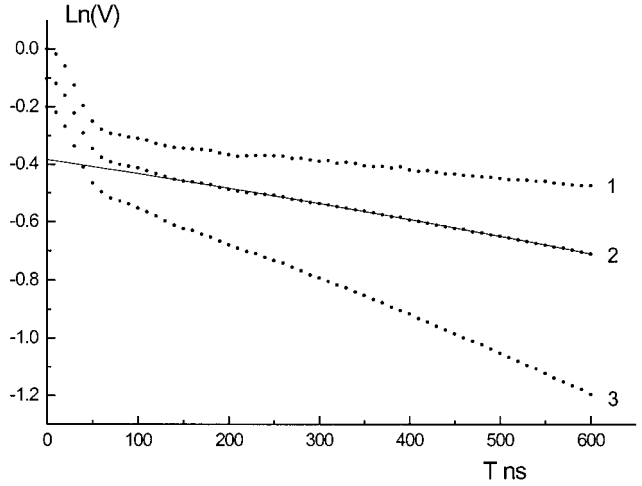

Figure 2. Dependence of the logarithm of the PELDOR signal V on time T for glassy solutions of mixtures of peptides I and II (ratio 1:3) in chloroform−toluene at 77 K. The concentrations of peptide I for curves 1, 2 and 3 are 1×10^{-3} M, 2.5×10^{-3} M and 5×10^{-3} M, respectively.

Our conclusions regarding the aggregation of peptide I in chloroform−toluene are confirmed by the PELDOR data. Figure 2 shows the logarithm of PELDOR signal amplitude V versus T for a mixture of peptides I and II in a 1:3 ratio dissolved in chloroform−toluene at 77 K. Curves 1−3 were obtained for different total concentrations of the peptide and by keeping the ratio of peptide I and II constant. The concentration-independent fast decrease of V at $T < 100$ ns, followed by weak signal oscillations, refers to the coupling of spin labels inside the peptide cluster. At $T > 100$ ns, the decrease of the amplitude is dependent on peptide I concentration and can be referred to the interaggregate spin coupling. Since this latter coupling is almost independent of the spin-label coupling inside the cluster, the total decay of the PELDOR signal is considered as the product of two time dependencies: the signal decay due to the label coupling *inside* the tetramers, V_{intra}, and the signal decay due to the label coupling *between* the peptide clusters, V_{inter}.[8]

$$V(T) = V(T)_{intra} V(T)_{inter} \quad (1)$$

As time T increases, the PELDOR signal oscillations attenuate (curves 1 and 2), owing to the averaging of the dipole−dipole coupling of spin labels inside the aggregates over the angles and distances between the spin labels. Because of the oscillation attenuation, the value of V_{intra} becomes T-independent and tends to its limiting value, V_p.[8] This behavior of V_{intra} makes it possible to split the contributions of label coupling inside and between aggregates using eq 1. To this end, after the initial fast signal decay, the experimental dependence ln(V) was considered to decay with time only due to the spin coupling between aggregates and represented as the second-order polynomial with regard to T in the form of a smooth nonoscillating curve ln(V_{inter}).[1,4] As an example, this dependence is shown in the form of a smooth curve for experimental curve 2. By subtracting the ln(V_{inter}) dependence from the experimental curve 2 we found the V/V_{inter} ratio close to V_{intra} and sufficient to estimate the oscillation frequencies as well as the V_p values.

Curves 1−5 in Figure 3 are the dependencies of V/V_{inter} on T at 77 K, obtained by the method described above, for different ratios of double-labeled and unlabeled peptides in chloroform−toluene (curves 1−4) as well as for peptide I in TFE (curve 5).

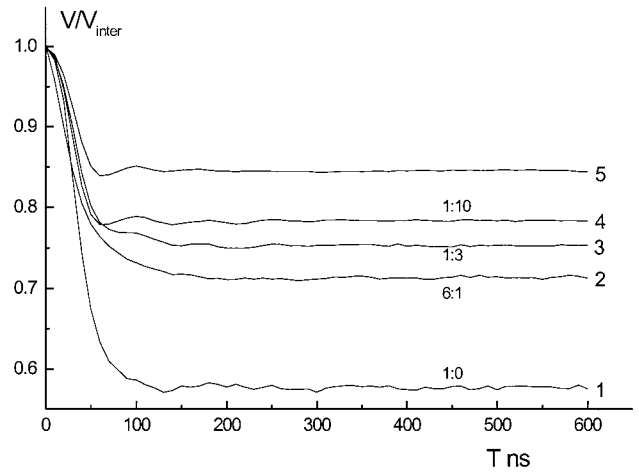

Figure 3. Dependence of V/V_{inter} on T for the frozen glassy solutions of the mixtures of peptides I and II at different ratios of peptides. 1: Peptide I in chloroform−toluene; 2: peptides I and II in a 6:1 ratio in chloroform−toluene; 3: peptides I and II in a 1:3 ratio in chloroform−toluene; 4: peptides I and II in a 1:10 ratio chloroform−toluene; 5: peptide I in TFE.

In general, Figure 3 shows a fast decrease in the PELDOR signal at $T < 100$ ns with subsequent passage beyond the limiting V_p value, accompanied in some cases by rapidly attenuating oscillations.

A fast decrease in the PELDOR signal at short times with subsequent oscillations of the signal indicates the existence of compact groups of spin labels in the system studied.[8] A strong dipole−dipole coupling of spin labels inside the groups leads to a fast dephasing of spins at $T < 100$ ns. These dependencies were repeatedly observed for frozen glassy solutions of different kind of biradicals,[8] double-labeled peptides in other solvents[1,2] and aggregates of single-labeled peptides.[3,4] According to references,[3,8] the limiting value of the PELDOR signal $V_p = V_{intra}(T \rightarrow \infty)$ is related to the number of spins in groups via the equation

$$V_p = (1 - p_b)^{N-1} \qquad (2)$$

where N is the number of spins in the group and p_b is the probability of spin B flip induced by the pumping pulse at ω_B.

By comparing curves 1 and 5 (Figure 3), it is seen that the V_p value for peptide I in the aggregated form (curve 1) is smaller than that for the same peptide in the monomeric form (curve 5). The V_p value observed for curve 5 corresponds to two spin labels per monomeric peptide. According to our previous publication, the number of molecules in the aggregate is about four.[3,4] Therefore, the number of spins per aggregate for curve 1 should be about eight. According to relation (2), V_p will decrease with increasing number of spin labels, N, in the aggregate. This corresponds to the change of the limiting value of $V(T)$ observed in each experiment (Figure 3, curves 1−4).

Figure 3 shows that dilution of double-labeled peptide I by unlabeled peptide II leads to both an increase in the V_p value and the appearance of oscillations in the dependence of V/V_{inter} on time T. The oscillation period determined for curve 4 recorded for the peptides I/II mixture (at a 1:10 ratio) in chloroform−toluene is 75 ± 5 ns. This value is, within experimental error, equal to the oscillation period of curve 5 for peptide I in TFE. This independency of solvent effect is also reflected in the CW-ESR spectra shown in Figure 1, where spectrum 2 recorded for peptide I in TFE is almost identical to spectrum 4 for a mixture of peptides I and II (at a 1:10 ratio) in chloroform−toluene.

An increase in V_p and changes in the ESR spectra by dilution of double-labeled peptide I by unlabeled peptide II correspond to a decrease in the mean number of spin labels in the aggregates. Thus, at a fairly high degree of dilution by unlabeled molecules, the fraction of aggregates containing more than one double spin-labeled peptide molecule, becomes small. Then, the main contribution to the dependence of V/V_{inter} on time T will be made by the intramolecular coupling of labels in the isolated double spin-labeled molecules which are hidden in the aggregates. In this case the PELDOR signal oscillations are due to the dipole−dipole couplings of spin labels in the aggregate that is formed from one double-labeled and three unlabeled molecules. This makes it possible to estimate the intramolecular distance between two spin labels. According to ref 8, the oscillation frequency is related to the distance between spin labels via the equation

$$\omega = 2\pi/\Delta = \gamma^2\hbar/r^3 \qquad (3)$$

where ω and Δ are the frequency and the oscillation period, respectively, γ is the gyromagnetic relation for an electron, $\hbar$ is the Plank constant, and r is the distance between spin labels.

The oscillations of V/V_{inter} on time T in Figure 3 indicate that some fraction of peptide I molecules, in aggregates as well as in TFE, has a fixed structure in which the intramolecular distances between spin labels are determined with a minor spread. In this case the mean distances r between spin labels in this fraction can be estimated from the experimental oscillation frequencies using the frequency-distance relation 3. In particular, using eq 3 and the Δ value 75 ± 5 ns, we get an intramolecular distance between spin labels of peptide I in the aggregate of 15.7 Å. According to eq 3 the error for r depends on the error for the oscillation period by the relation $\delta r/r = \delta\Delta/3\Delta$. Using this relation and an experimental error for the period of 7% we get an error for r of about 2.3%.

A similar estimation of the distance between spin labels for double-labeled peptide I in TFE (Figure 3, curve 5) gives the value 15.4 Å. Within experimental error, this value is equal to 15.3 Å, that has been reported earlier for the distance between labels in the double-labeled peptide in TFE.[2] The analysis performed in references[1,2] shows that this distance most closely corresponds to a 3_{10}-helical structure.[10] Two remarks have to be made at this stage of discussion. First, the peptide conformation is, considering the flexible nature of peptides, most likely the average of different segmental conformations. Second, the suggested conformation is in agreement with the secondary structure that has been proposed earlier on the basis of a set of intermolecular distances between spins of single-labeled peptides in the aggregate and measured with the PELDOR technique.[3] Thus, the data obtained confirm the possibility of the 3_{10}-helical conformation for at least a part of the peptide molecules in the aggregate. The coincidence of oscillation frequencies and the close values of oscillation amplitudes for peptide I in the aggregated state and glassy TFE indicate a similar spatial structure of peptide I under these two different conditions.

As mentioned in ref 2, when studying the frozen TFE solution of peptide I, the observed amplitude of oscillations is much smaller than that expected from theoretical calculations for a pair of spins at a fixed distance.[8,11] A similar behavior is shown by peptide molecules included in aggregates (Figure 3, curve

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4). This is indicative for the fact that the aggregated labeled peptides do not all have the 3_{10}-helical structure. Indeed, some fraction of them has a significant spread of intramolecular distances between spin labels and therefore gives only a fast decay in $V(T)$ without any oscillation. The fast decay of V_{intra} at short T should be attributed to all types of spin-labeled peptides.

The relationship between the depth of the fast decay and the oscillation amplitude makes it possible to estimate the fraction of aggregated peptides with a 3_{10}-helical conformation. To determine this fraction, we use the equation for the oscillation amplitude in the case of a fixed pair of spins randomly oriented in the magnetic field.[8,11] In the absence of any exchange interaction between these spins, the two equations (3 and 4) in ref 11 are reduced, and for $T \gg 1/\omega$ the V_{intra} behavior will have the form

$$V_{intra} \cong 1 - p_b[1 - ((\pi/12)\omega T)^{1/2} \cos(\omega T - \pi/4)] \quad (4)$$

where ω is given by relation 3 and $p_b = 1 - V_p$. According to eq 4, the attenuation of the V_{intra} oscillations should correspond to low $1/T^{1/2}$. The analysis of the experimental oscillation attenuation (Figure 3, curve 4) shows that it is faster than $1/T^{1/2}$. This finding indicates that we have to consider some spread in oscillation frequencies and a corresponding spread of distances d_r around the mean value of $r = 15.7$ Å obtained from the experimental oscillation period.

To estimate the frequency spread, let us assume for simplicity that this spread is small in comparison with the oscillation frequency, and the frequency distribution function is a Lorentzian line centered at the frequency ω_0 with the half-width $\delta\omega_{1/2}$ at the half-height ($\delta\omega_{1/2} \ll \omega_0$). In this case, the mean value of the decay function $V_{intra}(T)$ could be obtained by integration as

$$\langle V_{intra} \rangle = \int_\omega [(V_{intra}/\pi\delta\omega_{1/2})/(1 + ((\omega - \omega_0)/\delta\omega_{1/2})^2)]d\omega = 1 - p_b[1 - A_{(T)} \cos(\omega_0 T - \pi/4)] \quad (5)$$

$$A_{(T)} \cong ((\pi/12)\omega_0 T)^{1/2} \exp(-\delta\omega_{1/2} T) \quad (6)$$

where $\langle ... \rangle$ means averaging over the Lorentzian distribution of ω, ω_o is the mean value of oscillations of $V(T)$ and $A_{(T)}$ is the oscillation amplitude. Relation 5 makes it possible to estimate $\delta\omega_{1/2}$ from the experimental $A_{(T)}$ decay and therefore to find the corresponding distance spread d_r.

The ratio of the oscillation amplitude $A_{(T)}$ at the peak points of the $\cos(\omega_o T - \pi/4)$ function which are distant at the oscillation period $\Delta = 2\pi/\omega_o$ will be

$$A_{(T+\Delta)}/A_{(T)} = (T/(T + \Delta))^{1/2} \exp(-\delta\omega_{1/2}\Delta) \quad (7)$$

This gives for the distance spread, as $d_r/r = \delta\omega_{1/2}/3\omega_o$, the following relations

$$d_r/r = \pm(1/6\pi) \ln[D] \quad (8)$$

$$D = A_{(T+\Delta)}(T + \Delta)^{1/2}/A_{(T)}T^{1/2} \quad (9)$$

where D is the attenuation of oscillations amplitude of $\langle V_{intra} \rangle$ only due to the distance spread between spin labels.

From the experimental V_{intra} measurements (Figure 3, curve 4) it is evident that the D value is practically the same at any oscillation period chosen. In our particular case $D = 0.83 \pm 0.02$. According to refs 8 and 9 this value gives a corresponding distance spread between spin labels of $d_r/r = \pm 0.01$. This value qualitatively reflects the geometric rigidity of the corresponding peptide conformations that are responsible for the observed oscillations in V_{intra}. It is worthwhile to mention that the distance spread measured by this method is less than the experimental error of the mean distance measurements from the experimental oscillation frequency.

The relation 5 now could be used to determine the fraction of aggregated peptides with a 3_{10}-helical conformation. Using eq 5, the general dependence of V_{intra} on T for $T > 1/\omega_0$ may be written in the form

$$V_{intra} \cong X(1 - p_b[1 - A_{(T)} \cos(\omega_0 T - \pi/4)]) + (1 - X)(1 - p_b) \quad (10)$$

where X is the fraction of peptides with a 3_{10}-helical structure. The first term in eq 10 is related to 3_{10}-helical peptides, while the second is related to peptides having different conformations. After the fast decay of V_{intra}, when $T > 1/\omega_0$, the peak values of eq 10 are reached by $\cos(\omega_0 T - \pi/4) \cong 1$. In this case eq 10 makes it possible to estimate the value of X:

$$X = (V_{intra} - V_p)/p_b A_{(T)} = (12\omega_0 T/\pi)^{1/2} \exp(\delta\omega_{1/2} T)(V_{intra} - V_p)/p_b \quad (11)$$

By substitution of the experimental values of $\omega_o = 8.37 \times 10^7$ rad/s, V_{intra} and T (at the first peak of curve 4 in Figure 3), $\exp(\delta\omega_{1/2}T) = 1.28$ and $p_b = (1 - V_p) = 0.22$ into eq 11, we obtain the fraction of aggregated peptides with 3_{10}-helical conformation, that is, $X = 0.19$ ($\pm$ 0.03). This value is close to the estimated fraction of peptides with 3_{10}-helical structures for peptide I in TFE solution, as has been reported previously.[2]

From our data there is no direct evidence for the conformation of the remaining fraction (~80%) of aggregated peptides. In comparison with the ESR spectra of single-labeled peptides,[2] the ESR spectrum of the double-labeled peptide in the aggregate with a high degree of unlabeled peptide (Figure 1, curve 4) indicates an additional broadening of the lines due to dipole coupling between labels inside the peptide (about 1 mT). This means that the molecules of the main fraction of our aggregated spin system have a comparatively large spread of distances between the spin labels. This spread could even include some fraction of distances which may correspond to α-helical or mixed α/3_{10}-helical structures. However, more experiments are needed to get conclusive information on the secondary structure of these aggregated peptides.

In previous works[1,2] it was found that peptide I in other types of frozen solutions may adopt different conformations, depending upon the nature of solvent. Indeed, the intramolecular distance between spin labels may vary from 15.3 Å (for TFE) to 21.8 Å (for ethanol). A partly different situation was observed in MeOH/EtOH glass at 77 K. By analyzing the CW-ESR spectra, at $g \cong 4.0$ and at half-field, it was reported that trichogin GA IV can exist in a mixed α/3_{10}-helical conformation in equilibrium with unfolded conformers.[12] The same type of mixed helical conformation was found in the crystal state by X-ray diffraction analysis for trichogin GA IV[13] and the [TOAC-4,8] analog.[14] It may be concluded that short peptides as trichogin exhibit conformational flexibility, depending upon temperature

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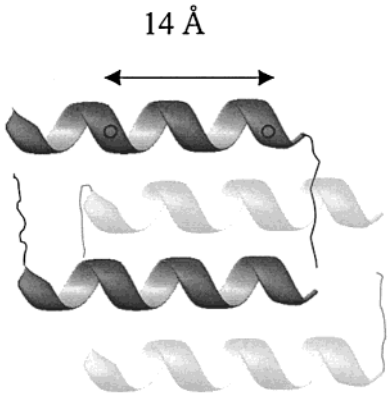

Figure 4. A ribbon drawing of the supramolecular assembly of one double-labeled and three unlabeled *n*-octanoylated trichogin peptide chains.

and the nature and organization of surrounding molecules into the matrix (glass, crystalline, etc.).

Conclusions

The PELDOR method combined with the CW-ESR technique was used to study the frozen glassy solutions of the double TOAC spin-labeled trichogin GA IV diluted by the unlabeled peptide. The difference in the CW-ESR spectra for frozen glassy polar and nonpolar solutions testifies the aggregation phenomenon of double spin-labeled peptides in the latter environment. The magnetic dipole−dipole relaxation of spin labels was experimentally investigated in the glassy solutions of the double-labeled peptide frozen at 77 K in chloroform−toluene as a function of the content of unlabeled peptide. An increase in the fraction of unlabeled peptide in frozen solution causes a decrease in the fraction of double-labeled peptides in the aggregates and weakens the *inter*molecular coupling of spin labels in the aggregates. At high degree of dilution with unlabeled peptide, we observed oscillations of the PELDOR signal as a function of time due to *intra*molecular coupling of the spin labels. The intramolecular distance between spin labels (inside the peptide molecule) was determined from the oscillation frequency to be 15.7 Å which is close to that calculated for the peptide in the 3_{10} -helical conformation ($\cong$14 Å). The fraction of 3_{10}-helices was estimated from the oscillation amplitude to be about 19% of the number of peptides in the aggregate. This result is in excellent agreement with the molecular model that was proposed previously.[3] Indeed, based on the measured set of intermolecular distances between single labeled peptides, a model was constructed from four 3_{10}-helices by adjusting the helical axes in pairs with the polar sides pointing to the center of the tetrameric peptide cluster (Figure 4).

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