α-Helical versus 3_{10}-Helical Conformation of Alanine-Based Peptides in Aqueous Solution: An Electron Spin Resonance Investigation†

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Received May 8, 1995®

Abstract: Due to the difficulties in experimentally differentiating between the α- and 3_{10}-helical conformations in solution, isolated helical peptides have been assumed to be in the α-helical conformation. However, recent electron spin resonance (ESR) studies have suggested that such peptides, in particular short alanine-based peptides, are 3_{10}-helical (Miick, S. M.; *et al. Nature* **1992**, *359*, 653−5). This result prompted us to further investigate the helical conformations of alanine-based peptides in solution using electron spin resonance spectroscopy. Unlike previous investigations with a flexible link connecting the spin-label to the peptide backbone, we used a conformationally constrained spin-label (4-amino-4-carboxy-2,2,6,6-tetramethylpiperidine-1-oxyl, Toac) that is rigidly attached to the peptide backbone. From a combination of molecular modeling and ESR spectroscopy investigations, it was concluded that these alanine-based peptides exist primarily in the α-helical conformation, and not the 3_{10}-form as previously suggested for an analogous set of peptides in aqueous environments. This discrepancy is thought to be due to the differences in flexibility of the spin-labels employed. The conformationally constrained spin-label Toac used in this study should accurately reflect the backbone conformation. Free energy surfaces, or potentials of mean force, for the conformational transition of the spin-label used in previous studies (Miick S. M.; *et al. Nature* **1992**, *359*, 653−5) suggest that this spin-label is too flexible to accurately distinguish between the α- and 3_{10}-helical conformations.

Introduction

An understanding of the principles governing protein structure is an active goal of many investigations. Since the original *in vitro* refolding of ribonuclease A,[1] significant effort has been applied to understanding how primary sequence dictates tertiary structure. Experimental analysis of protein folding has revealed that secondary structure is stabilized very early in the folding process, while tertiary structure is acquired later. Consequently, one of the earliest steps in the folding process is thought to be the formation of secondary structure elements, which may or may not be accompanied by hydrophobic collapse.[2]

It is, therefore, important to characterize the conformations of short peptides in solution and to decipher the factors which determine the tendency of peptides to populate various conformational states in solution and initiate secondary structure elements. Consequently, the effects of peptide length,[3,4] peptide sequence,[5−8] the dielectric of the environment,[4,9−11] and other environmental influences[12−14] that stabilize helical conformations have been studied. Several examples now exist of isolated secondary structure in solutions, including the helical peptides like the C-terminal peptide of ribonuclease A,[15−17] Pα5 from BPTI,[18] and the Baldwin peptides.[19,20] Due to the limited availability of experimental data that delineate between α- and 3_{10}-helices in solution, it has been generally assumed that these isolated helices were of the α-helical conformation rather than the 3_{10}-form. However, a recent electron spin resonance (ESR) study has suggested that such peptides, in particular short alanine-based peptides, are 3_{10}-helical.[21] This surprising result

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† Abbreviations: Standard single letter codes for the amino acids are used, except B is 4-amino-4-carboxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (**2**) and U is 1-aminocyclohexanecarboxylic acid (**3**); DIC, 1,3-diisopropylcarbodiimide; DCM, dichloromethane; DIPEA, diisopropylethylamine; DMF, *N,N*-dimethylformamide; Fmoc, 9-fluorenylmethyloxycarbonyl; fs, femtosecond; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; TFA, trifluoracetic acid; TLC, thin layer chromatography.

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® Abstract published in *Advance ACS Abstracts,* October 1, 1995.

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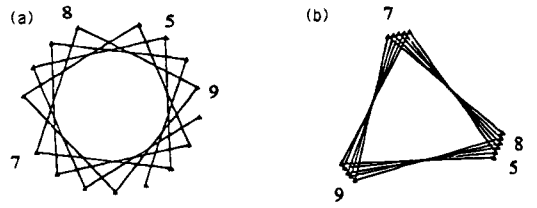

Figure 1. Helical wheels for peptides in both (a) α-helix and (b) 3_{10}-helix conformations.

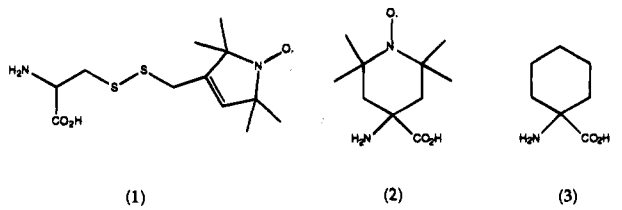

Figure 2. Structure of the spin-label 1 (C, Table 3) used by Miick *et al.*,[21] the spin-label 2 (B, Table 1) used in this study, and the control amino acid 3 (U, Table 1) used to replace the spin-label. Hydrogens are omitted from carbons for clarity.

in view of many theoretical studies on relative helical stability[9,10,22,23] prompted us to further investigate the conformation of these alanine-based helical peptides using ESR spectroscopy.

ESR spectroscopy provides information on the structure and dynamics at the site of one, or more, unpaired electrons.[24] Double-label ESR spectroscopy provides information on the relative distances between labels, and is the method of choice in characterizing helical content in peptides.[21,25-32] The experimental strategy of these ESR investigations[21] is shown in Figure 1. By synthesizing three peptides that contain spin-labels at position i and the $i + 2$, $i + 3$, or $i + 4$ position, the geometry of the α- and 3_{10}-helices gives rise to different distances of separation between the spin-labels (Figure 1). For example, using spin-labels attached to the α-carbons and letting $d(i,j)$ represent the distance between side chains at positions i and j, for $i = 5$, an α-helix requires $d(5,8) \approx d(5,9) < d(5,7)$, whereas the 3_{10}-helix would require $d(5,8) < d(5,7) \approx d(5,9)$.[21,26]

A previous investigation of the helical conformation of peptides using ESR spectroscopy[21] has used long, flexible spin-label 1 (Figure 2). In a doubly labeled peptide, the two spin-labels are removed from the backbone by 10 rotatable bonds. This conformational freedom of the spin-label may cause problems[10,22] and warrants further investigation as the measured average distance between the nitroxides may not accurately reflect the backbone conformation due to the accessible conformational space explored by the flexible spin-labels. To more accurately determine the conformation of the backbone, a spin-labeled amino acid is required that is attached to the backbone in such a way as to more closely mirror the backbone conformation. The spin-label 4-amino-4-carboxy-2,2,6,6-tetramethylpiperidine-N-oxyl (Toac, 2; Figure 2)[33] meets these criteria. The nitroxide group is relatively fixed with respect to the backbone, and its α,α-dialkyl substituents restrict the conformational space to areas surrounding the α- and 3_{10}-helices.[34,35] The ESR spectra of the doubly labeled peptides containing spin-label 2 should more accurately reflect the helical backbone conformation of peptides which is the focus of this paper.[1]

Table 1. Sequences[a] of Unlabeled, Singly Labeled, and Doubly Labeled Peptides Synthesized[b]

Unlabeled Peptides	
(5-8-U)	Ac-AAAAUKAUAAKAAAAKA-NH_2
Singly Labeled Peptides	
(5-7-M)	Ac-AAAABKUAAAKAAAAKA-NH_2
(5-8-M)	Ac-AAAABKAUAAKAAAAKA-NH_2
(5-9-M)	Ac-AAAABKAAUAKAAAAKA-NH_2
Doubly Labeled Peptides	
(5-7-D)	Ac-AAAABKBAAAKAAAAKA-NH_2
(5-8-D)	Ac-AAAABKABAAKAAAAKA-NH_2
(5-9-D)	Ac-AAAABKAABAKAAAAKA-NH_2

[a] Standard single letter codes for the amino acids are used, except the following: B is 4-amino-4-carboxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (2) and U is 1-aminocyclohexanecarboxylic acid (3). [b] These peptides contain the spin-label 2 (B in this table).

Experimental Section

Modeling. As the experimental ESR measurement reflects the average distance between the two spin-labels, one must calculate the average distances between the spin-labels of each peptide in both α- and 3_{10}-helical conformations in order to interpret the experimental observations and characterize the helical content of the peptides in solution. This is required for the three peptides substituted at position i and either the $i + 2$, $i + 3$, or $i + 4$ position. For this study, the peptides (5-7-D, 5-8-D, and 5-9-D) shown in Table 1 were investigated. The doubly labeled peptides and their unlabeled and singly labeled controls were designed to resemble each other as much as possible. Each contains two α,α-dialkylamino acids, two Toac residues for the doubly labeled peptides, one Toac and one 1-aminocyclohexanecarboxylic acid residue (3) for the singly labeled controls, and two 1-aminocyclohexanecarboxylic acid residues (3) for the unlabeled peptides. This was to ensure that the perturbation of the α,α-dialkylamino acids to the helical preferences would be consistent across the series of peptides studied.

The distances between the Toac (2) spin-labels were calculated using molecular dynamics (MD) simulations. For these calculations, the nitroxide functional group (N−O) of the spin-label 2 was replaced with a methylene group (CH_2) to negate the requirement for nitroxide parameters. The AMBER/OPLS force field,[36,37] as implemented in Macromodel,[38] was used as it has been shown to provide a good description of the conformational energetics of alanine,[22,39] and the hydration free energies of small organic compounds.[40] The GB/SA solvation model of Macromodel was used to approximate solvation

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Table 2. A Summary of the Calculated Distances between Spin-labels **2** in the 5-7-D, 5-8-D, and 5-9-D Peptides[a]

	5-7-D	5-8-D	5-9-D
α-helix	11.2 ± 0.1	8.4 ± 0.1	7.6 ± 0.7
3_{10}-helix	10.3 ± 0.1	7.9 ± 0.2	10.6 ± 0.7

[a] Uncertainty estimates were calculated by block averages.

Table 3. Sequences[a] of Doubly Labeled Peptides Synthesized by Miick *et al.*[21,b]

(4-6-D)	Ac-AAACKCAAAKAAAAKA-NH_2
(4-7-D)	Ac-AAACKACAAKAAAAKA-NH_2
(4-8-D)	Ac-AAACKAACAKAAAAKA-NH_2

[a] Standard single letter codes for the amino acids are used, except the following; C is the spin-labeled Cys (**1**). [b] These peptides contain the spin-label **1** (C in this table).

effects.[41] The three peptides were simulated in both the α- and 3_{10}-helical conformations. In order to restrict the conformational sampling to the desired helical regions, we restrained the backbone conformation to average values derived from peptide crystal structures.[42] These correspond to a ϕ and ψ of −63 and −42° for the α-helix, and −57 and −30° for the 3_{10}-helix. A force constant of 1000 (kJ/mol)/rad^2 was used as the restraining potential. Prior to the molecular dynamics simulation, the system's energy was minimized to a gradient of 0.01 (kJ/mol)/Å, with the restraints in place. MD simulations were carried out at 298 K, using a 2 fs time step, and coordinates were saved every 50 fs for further analysis. Each simulation was run for a total of 400 ps and the distances between the spin-labels were averaged after 20 ps of equilibration. The data are shown in Table 2.

Potentials of Mean Force (pmf). Potential of mean force calculations were used to investigate the range of distances between the two spin-labels **1** incorporated in the three peptides studied (Table 3) by Miick *et al.*[21] for both the α- and 3_{10}-conformations. This was achieved by "driving" the distance between the flexible spin-labels (using a series of restrained MD simulations) for the doubly labeled peptides 4-6-D, 4-7-D, and 4-8-D (Table 3) in both the α- and 3_{10}-helical conformations from 20 to 4 Å. We have previously used pmf calculations to calculate the free-energy surface for the transition between α- and 3_{10}-helices.[9,10,43]

The potential of mean force as a function of a coordinate ξ is $W(\xi) = -kT \ln(p(\xi))$, where k is Boltzmann's constant, T is the absolute temperature, and $p(\xi)$ is the probability density along that coordinate. Modified dynamical methods can be used to ensure adequate conformational sampling of transitions of interest. One such approach, the umbrella sampling technique,[44,45] is used here to determine $p(\xi)$. Umbrella sampling uses an auxiliary "window" potential $U(\xi)$ to improve the uniformity of the sampling along ξ. Systematically shifting the minimum of $U(\xi)$ along the coordinate ξ, results in sampling conformations in a series of overlapping windows (simulations) that are centered around different values of ξ. The resulting potential of mean force W^* is then given by $W^*(\xi) = W(\xi) + U(\xi) + C$ and is related to the probability density p^* (that probability calculated in the presence of the umbrella potential) by $W^*(\xi) = -kT \ln(p^*(\xi))$.[44] The constants C are different for each window and are calculated by requiring that $W(\xi)$ be a continuous function of ξ and by arbitrarily selecting the zero of the free energy $W(\xi^\circ) = 0$.[44,45] The reaction coordinate used for this study was to linearly decrease the distance between the spin-labels from $r_o^{\alpha,\alpha} = 20$ Å to $r_o^{\alpha,\alpha} = 4$ Å in 1 Å increments while the peptide backbone (ϕ and ψ) was restrained to either the α- or 3_{10}-helical conformation.

The starting 3_{10}- and α-helical conformations were model built using the INSIGHT II software from Biosym Inc.[46] Simulations were carried out at 300 K and were conducted on a Silicon Graphics R4400 workstation using the CVFF force field in the DISCOVER program.[47] The simulations used a 1 fs time step, and coordinates were saved every 100 steps (100 fs) for further analysis. These gas phase simulations used a 100 Å nonbonded cutoff and a dielectric constant of 1.0. The ϕ and ψ torsion angles of the peptide backbone were restrained to either the α-helical (-63 and −42°, respectively)[48] or the 3_{10}-helical (−57 and −30°, respectively)[48] conformation using a force constant of 1000 (kcal/mol)/rad^2 (the same value of the restraint as used in the study of the Toac-containing peptides).

For these simulations, the nitroxide functional group (N−O) of the spin-label **1** was replaced with a methylene group (CH_2) to negate the requirement for nitroxide parameters. The distance between the "spin-labels" (i.e., the distance between the CH_2−CH_2 atoms that replaced the N−O functional group) was restrained using a 2.5 (kcal/mol)/Å^2 force constant for the reaction coordinate distance being studied.

Prior to molecular dynamics simulations, the peptide was minimized (in either the α- or 3_{10}-helical conformation) using steepest descent (to a gradient of less than 10.0 (kcal/mol)/Å^2) followed by a conjugate gradient (to a gradient of less than 1 (kcal/mol)/Å^2) and finally va09A (to a gradient of less than 0.001 (kcal/mol)/Å^2) with a $r_o^{\alpha,\alpha} = 20$ Å. After 100 ps of molecular dynamics, the resulting structure was used to generate the starting point for the $r_o^{\alpha,\alpha} = 19.0$ Å. This conformation was restrained to 19.0 Å and minimized using the same protocol above. In this fashion, the reaction coordinate was "walked" from the $r_o^{\alpha,\alpha} = 20$ Å to the $r_o^{\alpha,\alpha} = 4$ Å window.

The modeling studies indicated that the relative distances between the Toac spin-labels in the 5-7-D, 5-8-D, and 5-9-D peptides (Table 1) could be used to distinguish the backbone conformations.

Materials and Methods. Infrared (IR) spectra were obtained using a Perkin-Elmer 1710 FT-IR spectrometer. Mass spectra were recorded on a VG ZAB-SE spectrometer. Electron impact spectra were performed at 8 keV. Fast atom bombardment (FAB) mass spectra were recorded on the same instrument in a thioglycerol/glycerol matrix. Ion-spray mass spectra were recorded on a Vestec VT20 instrument. Melting points were obtained on a Thomas Hoover capillary melting point apparatus and are uncorrected. For thin layer chromatography (TLC) 250-nm silica gel GF precoated uniplates (Analtech) were used with the solvent system indicated. For flash chromatography, columns packed with silica gel 60 (Merck) were used. Analytical high-performance liquid chromatography (HPLC) was performed on a Spectra-Physics instrument with an SP8800 ternary pump, using a Vydac C_{18} column (0.36 × 25 cm, particle size 5 μm) at a flow rate of 1.0 mL/min, UV detection at 220 nm, and solvents (A) 0.05% trifluoracetic acid in H_2O and (B) 0.038% trifluoracetic acid in 90:10 acetonitrile/H_2O. Preparative HPLC was performed on a Ranin instrument, using a Dynamax C_{18} (2.1 × 25 cm, particle size 5μm) at a flow rate of 15 mL/min, UV detection at 220 nm, and solvents (A) 0.05% trifluoracetic acid in H_2O and (B) 100% acetonitrile. Amino acid analysis was performed for each peptide and consisted of vapor phase hydrolysis of the peptide for 24 h at 110 °C with constant boiling 6 N HCl, dabsylation of the constituent amino acids, and RP-HPLC analysis on a Beckman C_{18} Ultrasphere ODS-DABS column (4.6 mm i.d. × 25 cm, 5 μm) using a Beckman System Gold instrument.

4-[(9-Fluorenylmethyloxycarbonyl)amino]-4-carboxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (7). The synthesis of spin-label **2** is shown in Figure 3. **2** was first reported as its active ester by Jackson and Puett[49] and then as its free carboxylic acid by Marchetto *et al.*[33] Briefly, a solution of 2,2,6,6-tetramethyl-4-oxo-piperidine (**4**) and 0.88 g of sodium tungstate in 64 mL of water was added to 14 mL of 30% hydrogen peroxide at 5 °C and stirred vigorously.[50,51] The reaction was allowed to warm to room temperature and after workup yielded the oxidation product **5** in 85% yield. The hydantoin **6** was synthesized in 90% yield by the addition of ammonium carbonate in 28 mL of

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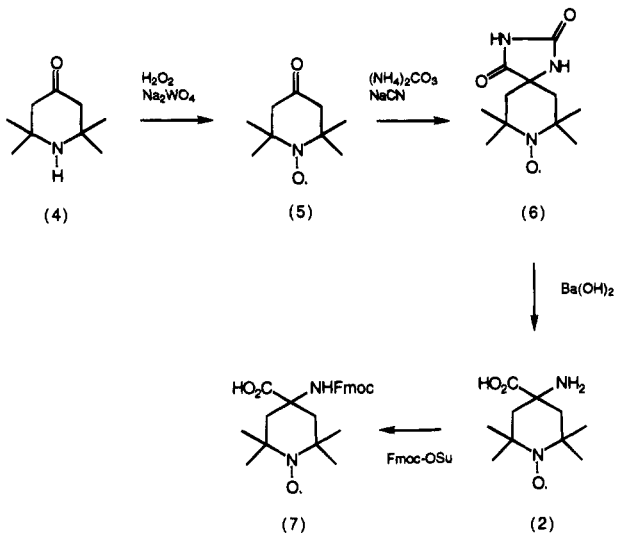

Figure 3. Synthetic scheme used to synthesize the labeled amino acid 4-[(9-fluorenylmethyloxycarbonyl)amino]-carboxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (**7**).

ethanol/water (3:2 v/v) to the nitroxide **5** and sodium cyanide (2.9 g) in 9.4 mL of water.[51] Hydrolysis of the hydantoin **6** (4 g, 16.6 mmol) was achieved with barium hydroxide (20 g) in 100 mL of water, at 140 °C, for 48 h in 92% yield.[51,52] The free amine **2** was protected as its Fmoc derivative by the addition of *N*-[(9-fluorenylmethoxycarbonyl)-oxy]succinimide (1.1 g, 3.2 mmol) in 5 mL of acetonitrile to a solution of amine (0.6 g, 3 mmol) in 5 mL of water and 1 equiv (0.4 mL) of triethylamine.[53] The resulting product was purified by chromatography (3% CH_3OH/CH_2Cl_2, 0.1% CH_3CO_2H; TLC, 10:90:3 $CH_3OH/CHCl_3/CH_3CO_2H$, R_F of product 0.7) and then recrystallized from ethyl acetate/hexane (1:1).

4-[(9-Fluorenylmethyloxycarbonyl)amino]-4-carboxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl-4-amino-4-carboxylic acid (8). (9-Fluorenylmethyloxycarbonyl)aminocyclohexanecarboxylic acid was synthesized from 1-aminocyclohexanecarboxylic acid (**3**) by the procedure reported by Milton *et al.*[53]

Synthesis of Labeled Peptides. The unlabeled, singly labeled, and doubly labeled peptides shown in Table 1 were synthesized according to the general procedure given below. *p*-Methylbenzhydrylamine (pMBHA) resin (3 g, 0.62 mmol/g substitution) was neutralized by two successive 10-min washes with 10% DIPEA/DCM. The first amino acid (Fmoc-Ala-OH) was attached to the resin as its preformed symmetrical anhydride. The remaining groups were blocked by acetylation using acetic anhydride (0.7 mL) in DCM-containing pyridine (0.15 mL) for 20 min at room temperature. On a 50-mg (0.03-mmol) scale of resin, the remaining amino acids (see Table 1 for peptides synthesized) were coupled on an Advanced Chemtech MPS 350 automatic peptide synthesizer. Each coupling comprised a 3-fold excess of amino acid, 3.3 M excess of DIC, and 4.5 M excess of HOBt. Each coupling reaction was carried out for 100 min in duplicate. The amino acids added directly after the α,α-dialkylated amino acids **2** and **3** were coupled using a 4-fold excess, followed by acetylation with acetic anhydride. After addition of the last amino acid, the resin was deprotected with 50% piperidine/DMF, washed, and acetylated with acetic anhydride. The peptides were cleaved off the resin using HF in the presence of the anisole, precipitated with ether, filtered, extracted with 5% acetic acid and water, and then lyophilized. The crude labeled peptides were then treated with base ($CH_3CO_2NH_4$, pH 9) for 3 h to regenerate the spin-label[33] and subsequently purified using reversed-phase HPLC. Characterization was achieved by fast atom bombardment, or electron spray, mass spectrometry, and amino acid analysis and agreed with expectations.

Circular Dichroism. CD spectra of 5-7-D, 5-8-D, and 5-9-D were recorded at 1 °C in 5 mM MOPS (pH 7.1), on a JASCO J600 spectrometer in a 0.1-cm path length cuvette. Peptide concentrations

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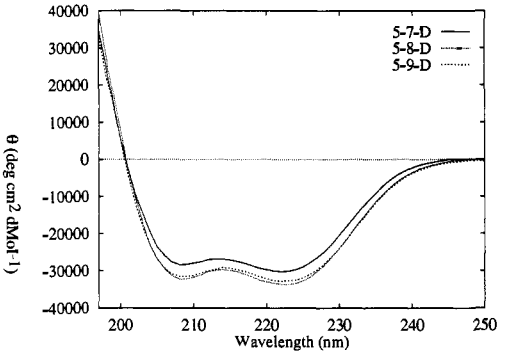

Figure 4. Circular dichroism spectra of peptides 5-7-D, 5-8-D, and 5-9-D (Table 1) recorded at 1 °C in 5 mM MOPS, pH 7.1, buffer.

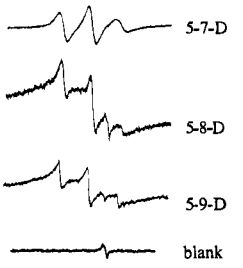

Figure 5. Continuous wave ESR spectra of doubly labeled peptides 5-7-D, 5-8-D, and 5-9-D (Table 1).

were determined from double integration of the ESR spectra and comparison to a 1.0 mM 2,2,6,6-tetramethyl-4-hydroxypiperidine-1-oxyl standard. The spectra are shown in Figure 4.

Electron Spin Resonance. Since the results of the CD spectroscopy illustrated that the peptides are highly helical, the helical character of the peptides (α vs 3_{10}) could be determined by ESR spectroscopy. Continuous wave ESR spectra of doubly labeled peptides (5-7-D, 5-8-D, and 5-9-D) were measured at 1 °C in 5 mM MOPS (pH 7.1) buffer (Figure 5). Measurements were made on a Bruker ESP 200 equipped with a TE_{102} rectangular cavity operating in continuous wave mode with modulation amplitude 1.25 G, frequency 100 kHz, and a 100-G scan width. Temperature control was achieved with a variable temperature unit from Bruker. The ESR sample tube contains a paramagnetic impurity, as illustrated by the blank spectrum (Figure 5). Spectra were recorded under a wide range of concentrations (0.13−1.6 mM) and showed no evidence of concentration dependence. Spectra were also recorded at room temperature, in the presence of a denaturant (guanidium hydrochloride), and the spectrum of 5-8-D was also recorded after titration with the unlabeled peptide 5-8-U.

Results

Modeling. The process of determining α- vs 3_{10}-helical conformation of peptides in solution[21] required a modeling investigation to estimate the distance between the spin-labels coupled with an experimental ESR investigation of the doubly labeled peptides. The distances between Toac spin-labels were calculated from a molecular dynamics study using an implicit solvation model of water, and the results are given in Table 2. These data show that the relative distances between the Toac spin-labels follow the order of 5-9 ≈ 5-8 < 5-7 for an ideal α-helix, while for an ideal 3_{10}-helix, an order of 5-8 < 5-7 ≈ 5-9 was calculated. The relatively large difference (2−3 Å) in distance between one of the distance pairs for either helical type and the small variation (±0.1−0.7 Å) indicate that interpretation of the spectra will be unambiguous regarding helical type.

Potential of Mean Force. In this study, we employ the conformationally constrained spin-label **2**. Previous studies have used a more flexible spin-label (**1**). A consequence of using flexible spin-labels on determination of helical conformation is illustrated in the pmfs shown in Figures 7 and 8. These figures

Table 4. A Summary of the Distances between Spin-Labels of Each Peptide in the α- and 3_{10}-Helical Conformations[a]

peptide	distance (Å)	peptide	distance (Å)
4-6-D α-helix	5.8−8.0, 10.7−21.1	4-6-D 3_{10}-helix	11−17.6
4-7-D α−helix	7.2−18.5	4-7-D 3_{10}-helix	7.1−18.5
4-8-D α−helix	5.7−15.0, 18.0−19.1	4-8-D 3_{10}-helix	5.2−19.9

[a] These data represent the distance range between the spin-labels in conformations that are within 2 kcal/mol of the free-energy minimum.

show the free-energy surface of the flexible spin-labels in the peptides 4-6-D, 4-7-D, and 4-8-D (Table 3) in the α- and 3_{10}-helical conformations. The surfaces demonstrate that the distances between spin-label **1** in doubly labeled peptides can fluctuate widely. This is shown by the data in Table 4 which summarize the distance ranges between the spin-labels in conformations that are within 2 kcal/mol of the free-energy minima. Because of the overlap of possible distances available to any of the three spin-label pairs regardless of helical type, the interpretation of ESR spectra based on distances between such flexible spin-labels in the doubly labeled peptides studied by Miick *et al.*[21] is problematic. Experimental conditions could easily change the distribution of side chain conformers and modify the average distance between spin-labels independent of helical conformation. This is dramatically different from the Toac-containing peptides as indicated by the data in Table 2.

Synthesis. It was necessary to synthesize the spin-label **7** (Figure 3), the control amino acid **8**, and the doubly labeled, singly labeled, and unlabeled peptides (Table 1) for characterization of the helical conformation in aqueous solution. The spin-labeled amino acid **7**[27,33] and the control amino acid **8** were synthesized and incorporated into the peptides shown in Table 1 by conventional solid phase techniques. The amino acids which were coupled to the α,α-dialkyl residues **2** and **3** were used in 4-fold excess, followed by acetylation to cap any unreacted peptide polymer in accord with known difficulties in coupling to α,α-dialkylamino acids.

Circular Dichroism. CD indicates the total helix content within a peptide, and is used here as a gauge of peptide helicity. The CD spectra of the doubly labeled peptides (Figure 4) exhibit minima at 208 and 222 nm, which is the characteristic signature for helical secondary structure. Experimental estimates of helical content were determined from the $[\theta]_{222}$ measurements. The helix percentage was calculated using an equation developed by Baldwin *et al.*[25] ($-40000(1-2.5/n)$; n = number of residues), where −40000 and 0 (deg cm^2)/dmol are the values for 100% and 0% helix, respectively. Using this formula, the 5-7-D, 5-8-D, and 5-9-D peptides were found to be 88%, 98%, and 95% helical, respectively, at 1 °C in 5 mM MOPS buffer, pH 7.1. While such equations are commonly used to estimate the percentage of helical conformations in proteins, there are several problems involved with this procedure.[54−56] It is used here only as a qualitative estimate of peptide helicity.

Electron Spin Resonance. Electron spin resonance spectra of the doubly labeled peptides in combination with the modelling studies allows the characterization of helical conformation in solution. The peak-to-peak hyperfine line width, δ, of a biradical spectrum broadens as the distance between spins decreases.[57,58] The reciprocals of the center ($W_i = 0$) spectral line widths (δ^{-1}) are proportional to the relative distances between nitroxides in the doubly labeled peptides. These line widths were used to rank the distances between the spin-labels in each peptide.

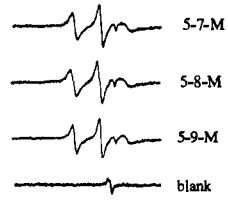

Figure 6. Continuous wave ESR spectra of singly labeled peptides 5-7-M, 5-8-M, and 5-9-M (Table 1).

The ESR spectra of the doubly labeled peptides at 1 °C are shown in Figure 5. The spectra of the doubly labeled peptides 5-8-D and 5-9-D appear to be a superimposition of two species, a very broad component (~95% as determined by spectral integration) and a characteristic three-line nitroxyl spectrum. The spectra were recorded in a range of concentrations (0.13−1.6 mM) and were very similar (data not shown). In addition, ESR spectra of 5-8-D in the presence of unlabeled peptide 5-8-U showed no differences (data not shown). Such experiments suggest that the peptides are not aggregating and that the perturbation to the ESR spectra seen in the doubly labeled peptides arises from intramolecular interactions of the spin-labels rather than from intermolecular interactions. The spectra of the singly labeled peptides at 1 °C show the characteristic three-line nitroxyl spectrum (Figure 6).

The spectra of the doubly labeled peptides 5-8-D and 5-9-D are very similar and are substantially broadened in comparison to the 5-7-D spectra. These data suggest that the relative distances between spin-labels follow the order 5-9-D ≈ 5-8-D < 5-7-D. Taken in conjunction with the modeling, these data indicate that the major component of these alanine-based peptides is the α-helical conformation.

In order to determine the conformation of the minor component, spectra of the doubly labeled peptides 5-7-D, 5-8-D, and 5-9-D have been recorded at room temperature and under denaturing conditions (addition of guanidinium hydrochloride). The line widths of these spectra are reported in Table 5. Importantly, under denaturing conditions, the line widths of the spectra are similar to those of the minor component at room temperature, suggesting that this conformation is extended in nature.

Discussion

Those factors which stabilize isolated secondary structures and the characterization of isolated secondary structural units in solution have implications for the protein folding problem. Several groups have been interested in the factors that stabilize the α- and the 3_{10}-helices, largely because the 3_{10}-helix is thought to be an intermediate between the extended and the α-helical conformations.[59−61] In fact, theoretical studies have suggested that for particular peptides the two helical forms may belong to the same conformational state,[9,10] and that at certain helical lengths the two helical conformations are equally favored.[51] All of these studies would suggest that peptides of length greater than 10−12 residues would dramatically favor the α-helical conformation in water. The recent electron spin resonance study of Miick *et al.*,[22] however, concluded that a

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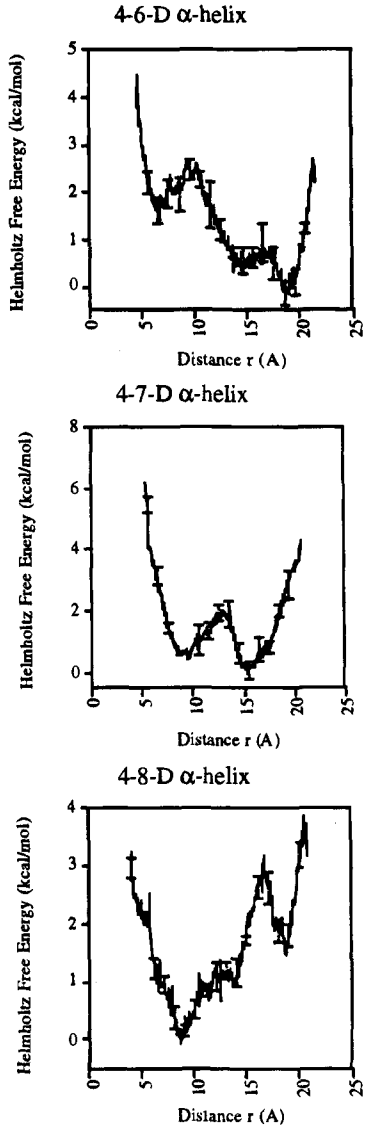

Figure 7. Computed potentials of mean force for the distance between the spin-labels in the peptides 4-7-D, 4-8-D, and 4-9-D (Table 3) in the α-helical conformation. Statistical uncertainties were calculated according to the method of block averages.

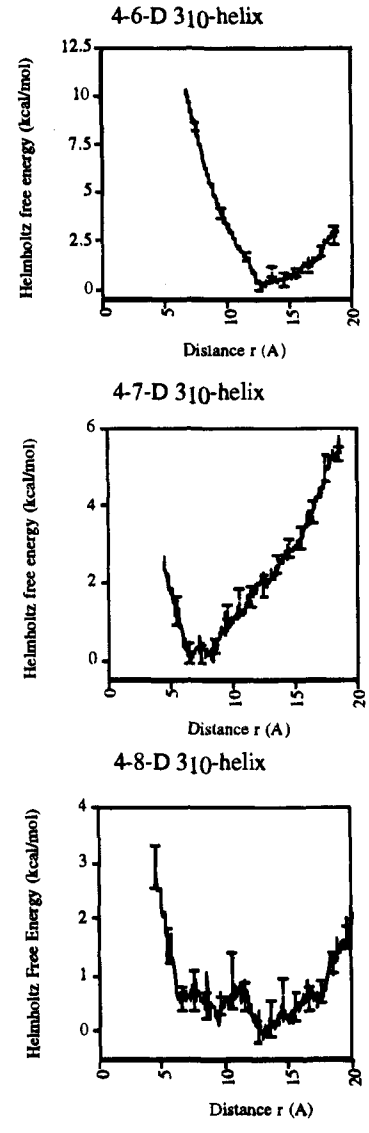

Figure 8. Computed potentials of mean force for the distance between the spin-labels in the peptides 4-7-D, 4-8-D, and 4-9-D (Table 3) in the 3_{10}-helical conformation. Statistical uncertainties were calculated according to the method of block averages.

16-residue alanine-based peptide was 3_{10}-helical in aqueous environment. Why does this discrepancy exist between theoretical results and experimental results? It has been suggested[9,10,22] that a possible explanation is due to the conformational flexibility of the spin-label employed in the ESR investigation of Miick *et al.*[22] which used a long, flexible tether to connect spin-label **1** (Figure 2) to the peptide backbone. Consequently, due to the conformational averaging by the flexible spin-labels, data using this approach do not accurately reflect the backbone conformation.

This prompted us to repeat the experiments of Miick *et al.*[21] using the more conformationally constrained spin-label **2** incorporated into the peptides shown in Table 1. These peptides are analogous to those used by Miick *et al.*[21] (which are variations of the Baldwin peptides[20]), but contain an additional alanine at the N-terminus. This moves the spin-label one amino acid further from the C-terminus, thus diminishing artifacts in the interpretation of the data due to the fraying of the termini of the helices.[25] Furthermore, the α,α-dialkylated spin-label **2** would be expected to increase 3_{10}-helix propensity.[35,62] It has been reported that longer peptides favor the α-helical conformation;[3,4,43] therefore, the additional alanine residue helps to compensate for the expected increase in 3_{10}-helical propensity due to the introduction of two α,α-dialkylamino acids into the sequence (as discussed below). As is evident (Table 1), a series of peptides that contain one, two, or no spin-label have been prepared. For the singly labeled and unlabeled peptides, we replaced either one or both spin-labels with 1-aminocyclohexanecarboxylic acid (**3**; Figure 2), thus preserving the conformational restriction of the backbone in the control peptides.

Table 5. A Summary of the Experimental Reciprocal Line Widths[a] of the Minor Conformation of the Doubly Labeled Peptides 5-7-D, 5-8-D, and 5-9-D

peptide	5-7-D (minor species)	5-8-D (minor species)	5-9-D (minor species)
At Room Temperature ($W_i = 0$)			
	0.4	0.6	0.6
At Room Temperature, after Addition of Guanidine Hydrochloride			
	0.4	0.6	0.7

[a] Experimental reciprocal line widths (δ^{-1} (G^{-1})) are the reciprocal of the peak-to-peak width of the center ($W_i = 0$) nitroxide hyperfine line.

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The CD spectra of the doubly labeled peptides at 1 °C are shown in Figure 4 and indicate that the peptides are strongly helical. The ESR spectra of the doubly labeled peptides at 1 °C are summarized in Figure 5. The spectra of the singly labeled peptides at 1 °C show the characteristic three-line nitroxide spectrum (Figure 6). The spectra of the doubly labeled peptides 5-8-D and 5-9-D appear to be a superimposition of two species, a very broad component (~95% in each case) and a typical nitroxide three-line spectrum. Since the spin-label is rigidly tethered to the backbone, one would expect that these two species would accurately reflect different backbone conformations. It is concluded that the broad component of the spectra is not due to peptide aggregation, on the basis of the following experiments. The ESR spectra are very similar within a concentration range of 0.13−1.6 mM. In addition, ESR spectra of 5-8-D in the presence of excess unlabeled peptide 5-8-U were not altered. If the peptides were aggregating, then the spin-labels would be expected to interact by Heisenberg exchange and/or electron dipole−dipole interactions, which shorten the effective relaxation rates and lead to increased line width and decreased signal intensity.[63] Therefore, the addition of unlabeled peptide would "dilute" the spin-labels in the aggregate, resulting in a sharpening of the spectral lines and an increase in signal intensity. The fact that the spectra of 5-8-D show no difference upon titration with unlabeled peptide (5-8-U) suggests that the peptides are not aggregating.

The very broad components of the spectra of 5-8-D and 5-9-D are indicative of spin-labels that are in close proximity to each other. From examination of the calculated distances between spin-labels in the α- and 3_{10}-helical conformations (Table 2) and the ranking of the distances from the ESR spectra ($d(5,8) \approx< d(5,9) < d(5,7)$), we must conclude that the major conformer in water is the α-helical conformation, in contrast to results reported by Miick *et al.*[8] for a similar series of peptides.

What is the conformation of the minor component? The line widths of this component at room temperature are summarized in Table 5. Addition of guanidinium hydrochloride gave spectra more indicative of a typical three-line spectrum. Under these denaturing conditions, the line widths (Table 5) are very similar to those of the minor component in water, suggesting that the conformation of the minor component is extended in nature.

While ESR spectroscopy is one of the core techniques used in biochemistry and biophysics, it requires the incorporation of spin-labels into a "native" peptide sequence. These spin-labels may have some form of structural perturbation on the peptide conformation (which is certainly probable for Toac), or the inherent conformational flexibility of the spin-labels may mislead the interpretation of the experimental results. Anytime a reporter group is introduced into a peptide sequence, one has perturbed the native structure and runs the risk of altering the original conformational distribution. With knowledge of the conformational effects of α,α-dialkylamino acids and these limitations in mind, we argue that the differences in helical states between our results and those of Miick *et al.*[21] are probably due to the differences in conformational flexibilities of the spin-labels employed. The spin-label **2** used in our investigation closely reflects the backbone conformation of the peptide and, hence, is better suited for determination of helical conformations.

This conclusion is partially based on pmf calculations that help quantify the potential problems of using flexible spin-labels (such as **1**) when determining helical conformations using ESR spectroscopy. We calculated the pmf for the transition of the spin-labels from being 4−20 Å apart in the peptides studied by Miick *et al.*[21] A relatively restricted reaction coordinate was used which involved restraining the ϕ and ψ torsion angles of the peptide to the desired α- or 3_{10}-helical conformation while the distance between the spin-labels was driven from 20 to 4 Å in 1 Å increments. Consequently, the effect of the conformation of the side chain spin-labels on the backbone (ϕ, ψ) conformations was not considered. However, the side chains and the ω backbone torsion angles of individual residues are unrestricted. Consequently, the free-energy surfaces calculated can be considered as upper limits due to the possibility of more facile transitions due to concerted changes in backbone (ϕ, ψ) conformations. The data are shown in Figures 7 and 8 and Table 4. The data in Table 4 show the distance ranges between the spin-labels of conformations that are within 2 kcal/mol of the free-energy minimum. These data show that the spin-labels in the 4-7-D, 4-8-D, and 4-9-D peptides have common distance ranges of 10.7−15.0 and 11−17.6 Å in the α-helical and 3_{10}-helical conformations, respectively. Thus, any peptide in the α- or 3_{10}-helical conformation is capable of having spin-labels separated by between 11 and 15.0 Å. Such flexibility is problematic when the distance between the spin-labels is used to assign the class of helical conformation. As the side chain−spin-label combination is quite hydrophobic, one might expect some association of the spin-label with the hydrophobic surface of the adjacent helix in aqueous solution which could alter any rotameric averaging of position.

These conclusions differ significantly from the calculations of Miick *et al.*[21] in which the distances between the side chains in the i and $i + 3$ residues were found to be 7.8 ± 0.7 Å for the 3_{10}-helix and 8.6 ± 1.0 Å for the α-helix and the distances between the side chains in the i and $i + 4$ residues were found to be 12.4 ± 0.9 Å for the 3_{10}-helix and 8.4 ± 0.7 Å for the α-helix.[30] Primarily, this is due to the different computational techniques employed. In this study, we have used umbrella sampling techniques[36,37] to ensure adequate sampling of the conformational transitions between minima available to the spin-labels. This overcomes the difficulties in sampling conformational space using ordinary molecular dynamics methods as used by Miick *et al.*[21]

The key to the characterization of peptide helicity using ESR spectroscopy is that the different geometries of the α- and 3_{10}-helices give rise to spin-labels with different distances of separation (Figure 1). Essential to preserving these differences in helical geometry is limited flexibility of the spin-labels employed. If the spin-labels used are too flexible, then this conformational freedom will eliminate the required differences in spin-label positions necessary to distinguish between the two helical conformations. The pmf calculations described above suggest that the spin-label **1** used by Miick *et al.*[21] is too flexible and would not accurately reflect the backbone conformation. In addition, the computational results reported here allude to the potential problems of interpreting experimental data in terms of theoretical calculations. We are currently experimentally validating the expected influences of environment on spin-label **1** conformations using ESR spectroscopy.

Alternatively, the differences between the results of Miick *et al.*[21] and those found here may not be due to the flexibility of the spin-labels (as argued above), but may arise because the incorporation of the α,α-dialkylated residues force the peptide from the 3_{10}-helical conformation observed by Miick *et al.*[21] to the α-helical conformation found here. We think that this is unlikely. The addition of α,α-dialkylated residues should, if anything, increase the population of 3_{10}-helical conformations[35]

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and not α-helical conformations. When an alanine is switched to an α-methylalanine, the enhanced stability of the 3_{10}-helix over the α-helix is 0.75 (kcal/mol)/residue according to the *in vacuo* calculations of Clark *et al.*[64] More recently, Zhang and Hermans[23] have estimated the enhanced stabilization in water of the 3_{10}-helix over the α-helix by the introduction of a single α-methylalanine residue to be 2.0 kcal/mol. Thus, the difference of two α,α-dialkylamino acids (**2** and **3**) in the peptides studied here would be expected to increase the stability of the 3_{10}-helical form by between 1.5 and 4.0 kcal/mol. The extra alanine residue was added to help compensate for the length dependence of helix preference which was estimated to be approximately 2.5 kcal/residue favoring the α-helix by Huston and Marshall.[35] However, the impact of the differences in peptide sequence used in these studies and those used by Miick *et al.*[21] cannot be ignored. The increased helicity of the peptides used in this study compared to those used by Miick *et al.*[21] (as determined by comparison of CD spectra) is due to the incorporation of α,α-dialkylated residues which are sterically restricted to helical regions of ϕ, ψ space.

Conclusion

The experiments of Miick *et al.*[21] which suggested that isolated peptide helices of 16 residues in aqueous solution favored significantly the 3_{10}-helical conformation was contrary to theoretical studies of the relative stabilities of the α- and 3_{10}-helical states. A conformationally constrained spin-label (Toac) that is fused to the backbone when incorporated into peptides was incorporated into analogous sequences. Toac is an ideal "marker" of the backbone conformation when trying to decipher between the different backbone conformations of α- and 3_{10}-helices in solution.[65] The CD spectra indicate that these peptides are, as expected, highly helical. The ESR spectra of the doubly labeled peptides indicate unambiguously an α-helical conformation. These results question the conclusion of Miick *et al.*'s previous experiments[21] that used a significantly more flexible spin-label (**1**) in a study on similar peptides.

Acknowledgment. We would like to thank Drs. Tom Lin and Vladimir Kouskov for assistance with ESR spectroscopy, Drs. Carl Frieden and Mary Holtzer for access to CD equipment and helpful discussions, Drs. David Cafiso (University of Virginia), Stephen Kolodziej, David Chalmers, and Shawn Huston for many stimulating discussions, and Dr. Reinaldo Marchetto for his contribution to the original synthesis of 4-[(9-fluorenylmethyloxycarbonyl)amino]-carboxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl. This work was supported by the Center for Molecular Design and, in part, by NIH Grant GM24483.

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(65) While this paper was under review, work by Toniolo et al.[32] was published in which Toac residues were incorporated into a pentapeptide and used for doubly labeled peptide ESR measurements.