Distinguishing Helix Conformations in Alanine-Rich Peptides Using the Unnatural Amino Acid TOAC and Electron Spin Resonance

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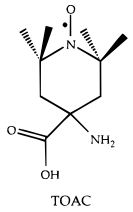
Electron spin resonance (ESR) of doubly spin labeled peptides reports on 3_{10}-helix/α-helix coexistence in Ala-rich sequences.[1−5] Nitroxide spin labels have been attached through disulfide linkages to pairs of Cys residues placed $i \rightarrow i + 2$ through $i \rightarrow i + 4$ in series of analogous peptides. Helical conformation was determined by the relative strength of the biradical interaction. To further resolve the conformation of helical peptides, we report here on a series of doubly labeled peptides containing the rigid $C^{\alpha,\alpha}$-dialkylated glycine TOAC (4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl-4-carboxylic acid). Figure 1 shows the structure of TOAC. TOAC is very similar in structure to the $C^{\alpha,\alpha}$-dimethylated amino acid α-aminoisobutyric acid (Aib).[6−8] TOAC has been used in previous studies of angiotensin,[9,10] but only recently has TOAC been exploited in double labeling experiments.[11] By studying two series of designed peptides, we show that TOAC double labeling, placed $i \rightarrow i + 2$ through $i \rightarrow i + 4$, is able to clearly distinguish 3_{10}-helix from α-helix. Further, we find that TOAC greatly increases helix stability for designed peptides.

Two series of doubly labeled peptides were prepared (Figure 1). The hexapeptides are insoluble in water, while the longer 3KT, 16-residue, peptides were designed along the lines of the 3K peptides previously studied by this and other laboratories[1−4,12] and are water soluble. The hexapeptides were examined in the helix-supporting solvent MeOH. Figure 2 shows the spectra of the hexapeptides in MeOH and the 3KT peptides in water. In the motionally narrowed regime, the line shapes arising from the biradical interaction are determined primarily by the exchange integral J. In the strong exchange region, where $J \gg a_N$ (a_N is the isotropic hyperfine coupling constant), the ESR spectrum is characterized by a five-line pattern with intensities 1:2:3:2:1. In flexible biradicals, fluctuations in the distance between the radicals result in a modulation of J that can preferentially broaden the second and fourth lines.[13] The strength of the biradical interaction is exponentially attenuated by distance[14] and thus may be used to obtain qualitative distance ($D_{i,j}$) rankings between the spin label positions.

Sequence	Abbreviation
Boc - TOAC - Ala - TOAC - Ala - Ala - Ala - OtBu	*Hex - 1,3*
Boc - TOAC - Ala - Ala - TOAC - Ala - Ala - OtBu	*Hex - 1,4*
Boc - TOAC - Ala - Ala - Ala - TOAC - Ala - OtBu	*Hex - 1,5*
Ac-Ala-Ala-Ala-TOAC-Lys-TOAC-Ala-Ala-Ala-Lys-Ala-Ala-Ala-Ala-Lys-Ala-NH_2	*3KT - 4,6*
Ac-Ala-Ala-Ala-TOAC-Lys-Ala-TOAC -Ala-Ala-Lys-Ala-Ala-Ala-Ala-Lys-Ala-NH_2	*3KT - 4,7*
Ac-Ala-Ala-Ala-TOAC-Lys-Ala-Ala-TOAC-Ala-Lys-Ala-Ala-Ala-Ala-Lys-Ala-NH_2	*3KT - 4,8*



TOAC

Figure 1. Chemical structure of TOAC and the two series of peptides examined in this study. Peptide sequences have their residues represented by their respective three-letter amino acid codes (Ala, alanine; Lys, lysine); Boc, *tert*-butyloxycarbonyl; Ac, acetyl; OtBu, *tert*-butoxy.

For the hexapeptide spectra in Figure 2, the strength of the biradical interaction, as revealed by the five-line pattern, is greatest in Hex-1,4. The biradical interaction is weaker in Hex-1,5 and barely detectable in Hex-1,3. These spectra indicate a ranking of $D_{\text{Hex-1,4}} < D_{\text{Hex-1,5}} < D_{\text{Hex-1,3}}$. Molecular models ($\phi$ and ψ values from ref 15) of labeled peptides in both the α- and 3_{10}-helical conformations give the distances between the nitroxide nitrogens as follows:

	$D_{i,i+2}$, Å	$D_{i,i+3}$, Å	$D_{i,i+4}$, Å
3_{10}-helical conformation	9.90	6.94	10.82
α-helical conformation	10.78	7.70	7.96

The observed distance ranking for the hexapeptides is consistent with the 3_{10}-helix conformation. Previously we showed that a 1,4 doubly labeled pentapeptide crystallized in the 3_{10}-helical conformation.[11,15] The pentapeptide and Hex-1,4 both give the same five-line pattern, which further supports the assignment of 3_{10}-helix.

Spectra of the water soluble 3KT-4,7 and 3KT-4,8 peptides, at 274 K, reveal a superposition of a broad signal and a sharp three-line signal. The broad signal arises from the electron−electron dipolar interaction, the strength of which is governed by a $1/D_{i,j}^3$ dependence. Extreme motional narrowing theory does not apply for these slow tumbling 16-residue peptides, and hence, the five-line spectrum is no longer observable.[16] However, distance rankings may be determined from the line width of the broad dipolar-broadened spectral component. The sharp component in the 3KT-4,7 and 3KT-4,8 spectra comprises about 5% of the integrated spectrum and may result from a small population of weakly interacting spins (see supporting information). Fourier deconvolution techniques[17] were employed to separate the two spectral components, and the separated broad component is shown by a dotted line in Figure 2. Assuming that the rotational correlation time is equivalent among the 3KT peptides, we conclude from the differing spectral widths that

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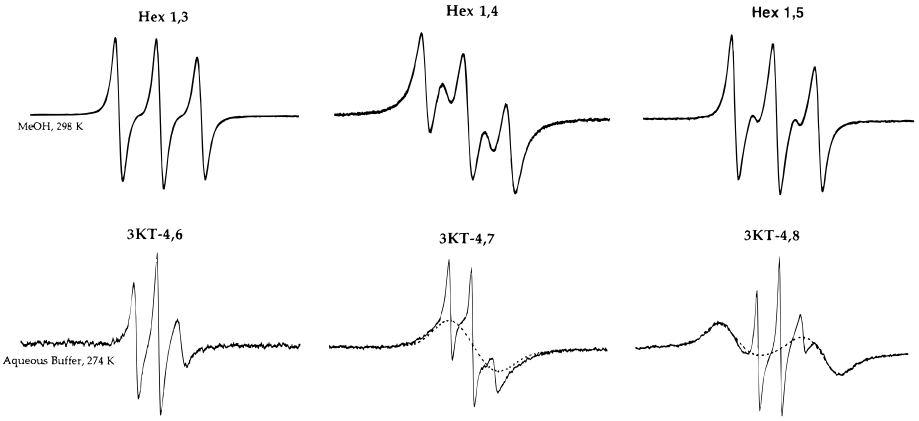

Figure 2. ESR spectra of hexapeptides and the longer 16-residue peptides. All spectra were acquired with a Bruker ESP 380 operating in continuous wave mode equipped with a dielectric resonator. A modulation frequency of 100 KHz, amplitude of 0.30 G, and scan widths of 80 G (above) and 250 G (below) were used for acquiring the spectra. Hexapeptides were dissolved in MeOH while the 3KT peptides were dissolved in aqueous buffer (Mops, pH 7.1).

$D_{4,8} < D_{4,7} < D_{4,6}$. Thus, the 3KT doubly labeled peptides are primarily in an α-helical conformation.

Previously, we examined doubly labeled analogs of the 3K peptides, in which flexible spin labels were attached to pairs of L-Cys.[1−5] Spin-labeled Cys exhibits a helix propensity similar to that of Ala.[18] Distance rankings from the 3K-4,7 and 3K-4,8 peptides indicated a *coexistence* of 3_{10}- and α-helices.[1,4] We subsequently showed that increasing helix content, by modification of sequence or by increasing the chain length, gave the ESR signals expected for an α-helix.[2,3] In addition, the denaturant guanidinium chloride eliminated biradical broadening for both the 3K-4,7 and 3K-4,8 analogs as expected for a random coil peptide.[3] Thus, Cys-attached double labeling gives the expected ESR signals for well-structured α-helix and for random coil. Because the incompletely folded 3K peptides exhibit mainly a strong 4,7 biradical interaction, we argued that 3_{10}-helix is a thermodynamic folding intermediate between random coil and α-helix.[1]

Relative to helix-neutral spin-labeled Cys, C^{α}-tetrasubstituted amino acids, such as Aib and TOAC, are strong helix promoters.[6−8] Short peptides ($n < 8$) containing two or more Aib's crystallize as 3_{10}-helices. In contrast, longer peptides ($n > 10$) containing between one and four Aib's crystallize exclusively as α-helices.[6] The results presented here suggest that parallel conformational trends exist in solution. Introduction of a TOAC is expected to stabilize the helical structure, and circular dichroism spectra of the 3KT peptides (supporting information) confirm that these peptides are substantially more helical than their 3K counterparts. On the basis of our findings as discussed above, the more helical 3KT peptides should favor α-helix over 3_{10}-helix as has been found. The results presented here are consistent with both crystallographic studies[6−8] and our previous work on Cys-labeled, Ala-rich sequences.[1−5]

It is worthwhile considering whether the position of the TOACs in the sequence influences the 3_{10}/α-helix equilibrium in the two peptide sequences studied here. For example, can the 3KT-4,6 adopt a different conformation than the 3KT-4,8? Basu et al. have recently addressed a similar problem by showing that the relative positions of two L-amino acid guest residues in Aib-rich octamers did indeed control the 3_{10}/α-helix equilibrium.[19] However, they also found that such conformational sensitivity occurs only when the peptide length is just at the 3_{10}/α-helix threshold ($n \approx 8$). The hexamers and the 3KT peptides are, respectively, below and above this critical chain length and are therefore unlikely to be influenced by the detailed positions of the TOAC residues.

In three recent molecular dynamics studies on uncharged 10- or 11-residue poly(Ala)$_n$ and poly(Aib)$_n$ peptides, our interpretation of the 3K peptide experiments has been questioned.[20−22] In all of these computational works, the peptides were confined to fully helical conformations. The poly(Ala)$_n$ α-helix was found to have a lower free energy than 3_{10}-helix by approximately 10 (ref 20) to 16 (ref 21) kcal/mol. What these three papers fail to address is the ensemble nature of a real 3K peptide. A partially helical peptide exists as an ensemble of different helix lengths, and our interpretation is that the shorter members of the ensemble readily adopt the 3_{10}-helical conformation. The energetics of short helices have been addressed by Tobias and Brooks.[23] For an Ala tripeptide they found that 3_{10}-helix is higher in energy than α-helix by only 0.6 kcal/mol. Building upon this finding, Sheinerman and Brooks[24] have developed a modified Zimm−Bragg theory in which they show that 3_{10}-helix is significantly populated for partially helical peptides. Finally, computational work by Sung[25] on the actual 3K sequence has revealed a coexistence of 3_{10}- and α-helices.

The incorporation of TOAC has provided unique spectroscopic signatures useful for distinguishing α-helix from 3_{10}-helix. Furthermore, TOAC is a strong helix stabilizer, and in water-soluble peptides it appears that a new method exists for stabilizing the α-helix conformation.

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Supporting Information Available: A further description regarding details of both CD and ESR experiments (3 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(26) Smythe et al. (*J. Am. Chem. Soc.* **1995**, *117*, 10555−10562) have recently reported similar studies on TOAC-labeled peptides. Despite reaching similar conclusions, we offer a fundamentally different interpretation of the role TOAC plays in stabilizing helix structure.