# Reports

## Fluorescence Properties of Calmodulin-Binding Peptides Reflect Alpha-Helical Periodicity

KARYN T. O'NEIL, HENRY R. WOLFE, JR., SUSAN ERICKSON-VIITANEN, WILLIAM F. DEGRADO*

**A basic amphiphilic α-helix is a structural feature common to many calmodulin-binding peptides and proteins. A set of fluorescent analogues of a very tight binding inhibitor (dissociation constant of 200 picomolar) of calmodulin has been synthesized. The fluorescent amino acid tryptophan has been systematically moved throughout the sequence of this peptide. The fluorescence properties for the peptides repeat every three to four residues and are consistent with the periodicity observed for an α-helix.**

THE PROTEIN CALMODULIN PLAYS A key role in cellular regulation by modulating the activities of a number of regulatory enzymes such as protein kinases, phosphodiesterases, nicotine amide adenine dinucleotide kinase, and adenylate cyclase (*1*). Calmodulin is also known to bind a variety of peptides competitively with respect to its target enzymes (*2–4*). It has been proposed that calmodulin recognizes a basic, amphiphilic α-helix in many of the peptides that bind to it with high affinity (dissociation constant less than 100 n*M*) (*3, 4*). To show this we synthesized peptide 1 (Fig. 1) (*4*), which embodies the quintessential elements of this structural feature but has minimal sequence homology to any of the peptides it is meant to mimic. Indeed, it binds calmodulin with a dissociation constant $K_d$ of 0.2 n*M*. While these studies were in progress, the calmodulin-binding sites from skeletal muscle (*5–7*) and smooth muscle myosin light chain kinases (MLCK) (*8*) were elucidated and also appear to conform to this structural paradigm (Fig. 1). However, direct evidence to show that peptides form helices when they bind to calmodulin has been primarily limited to difference circular dichroism (CD) measurements (*3, 6, 8*), which are complicated by the fact that calmodulin changes its conformation when it binds to peptides (*6*). One elegant nuclear magnetic resonance (NMR) study used bacterially derived perdeuterated calmodulin to show that melittin forms helices when it binds to calmodulin (*9*).

We have adopted an approach to obtain additional evidence for helix formation that should be generally applicable for studying the interactions of peptides and proteins with receptors or surfaces. A set of analogues of the peptide of interest is prepared; in each of these analogues one of the amino acids in the parent peptide is replaced with an amino acid that bears a spectroscopic probe (for example, a spin label, an amino acid enriched in a given isotope for NMR studies, or a fluorescent label). For the calmodulin-binding peptides the fluorescent amino acid tryptophan (Trp) was chosen as the spectroscopic probe (Fig. 2) because its fluorescence properties depend strongly on its environment, and because we wished to determine the positions at which an aromatic amino acid could be substituted without interrupting binding. Figure 3 illustrates the results that might be expected when the fluorescence properties of this set of peptides are examined as a function of the position of the tryptophan in their sequences. If the peptides bind to calmodulin in an α-helical conformation with their apolar residues in contact with the apolar binding site or sites of calmodulin and if the charged residues of the peptide are largely accessible to solvent, then to a first approximation many of the fluorescence properties of the peptides should be periodic with the same period as that of an α-helix. In particular, the emission maximum would be sensitive to the rigidity and solvent accessibility of the immediate environment of the tryptophan (*10*), the anisotropy $r_0$ would measure the rotational mobility of the tryptophan (*10*), and the Stern-Volmer quenching constant for fluorescence quenching by acrylamide (*10*) would provide information on the degree of accessibility of the tryptophan to a small neutral probe.

The 17 peptides illustrated in Fig. 2 were simultaneously synthesized manually with the use of the solid-phase method and a series of parallel reaction vessels. Both the fluorenylmethyloxycarbonyl- (Fmoc) (*11, 12*) and butoxy carbonyl- (Boc) based (*13*) methodologies were evaluated; the Fmoc procedure was more convenient and gave purer products. The structures of the peptides were confirmed by amino acid analysis and fast atom bombardment (FAB) mass spectroscopy.

The affinities of the peptides for calmodulin were assessed by their ability to compete with immobilized melittin for binding to isotopically labeled calmodulin (*3, 14*) (Fig. 2). Compared with the reference peptide that lacks tryptophan in its sequence (des Trp, $K_d$ = 2 n*M*), each of the analogues bound to calmodulin with high affinity ($K_d \leq 3$ n*M*) and each peptide also inhibited the calmodulin-dependent activity of smooth muscle MLCK. For ten of the analogues the dissociation constants were within a factor of 2 of that measured for the des Trp analogue; the remaining analogues had dissociation constants that were below 1.0 n*M*, which indicated higher affinity. Interestingly, the affinity was highest when the tryptophan was placed in position 3, the same position as in our alignment of the sequences of peptide 1 and the MLCK-derived peptides (Fig. 1). When a lysine at position 4, 5, 11, or 12 was replaced by

Central Research and Development Department, Experimental Station, E. I. du Pont de Nemours & Company, Wilmington, DE 19898.

*To whom correspondence should be addressed.

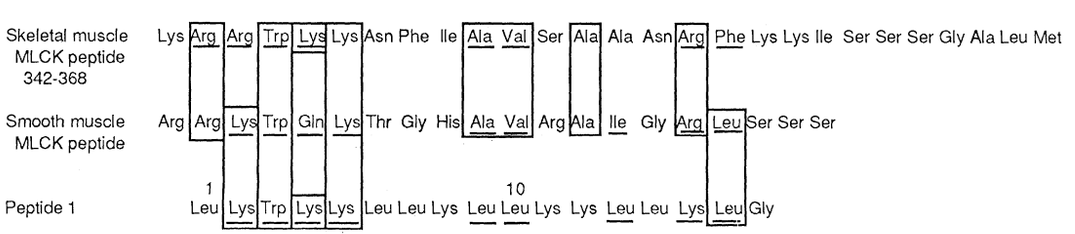


**Fig. 1.** Amino acid sequences for MLCK peptides (*5–8*) and peptide 1. Boxed residues are identical; underlined residues indicate replacements that are conservative between all three sequences.

tryptophan, the affinity of the peptide was significantly enhanced, which suggested that the removal of a single lysine resulted in the relief of unfavorable electrostatic interactions between the positively charged residues in the peptide helix. The fact that a tryptophan could be placed at any position in the chain without greatly decreasing the affinity demonstrated that its perturbational effect was small compared to the overall binding energy (approximately −12 kcal $mol^{-1}$ for the peptides that bind with the poorest affinity versus −13.2 kcal $mol^{-1}$ for $Trp^3$).

**Fig. 2.** Amino acid sequences and calmodulin affinities for 17 peptides studied. Affinities are relative to that found for des Trp ($K_d$ = 2.0 n$M$) and are calculated by dividing the value of $K_d$ for the des Trp analogues by the $K_d$ for the tryptophan-containing analogues.

| | | Affinity relative to des Trp |
|---|---|---|
| Des Trp | Leu-Lys-Leu-Lys-Lys-Leu-Leu-Lys-Leu-Leu-Lys-Lys-Leu-Leu-Lys-Leu-Gly | 1.0 |
| $Trp^1$ | Trp-Lys-Leu-Lys-Lys-Leu-Leu-Lys-Leu-Leu-Lys-Lys-Leu-Leu-Lys-Leu-Gly | 1.4 |
| $Trp^2$ | Leu-Trp-Leu-Lys-Lys-Leu-Leu-Lys-Leu-Leu-Lys-Lys-Leu-Leu-Lys-Leu-Gly | 1.0 |
| $Trp^3$ | Leu-Lys-Trp-Lys-Lys-Leu-Leu-Lys-Leu-Leu-Lys-Lys-Leu-Leu-Lys-Leu-Gly | 10.0 |
| $Trp^4$ | Leu-Lys-Leu-Trp-Lys-Leu-Leu-Lys-Leu-Leu-Lys-Lys-Leu-Leu-Lys-Leu-Gly | 5.0 |
| $Trp^5$ | Leu-Lys-Leu-Lys-Trp-Leu-Leu-Lys-Leu-Leu-Lys-Lys-Leu-Leu-Lys-Leu-Gly | 5.0 |
| $Trp^6$ | Leu-Lys-Leu-Lys-Lys-Trp-Leu-Lys-Leu-Leu-Lys-Lys-Leu-Leu-Lys-Leu-Gly | 1.8 |
| $Trp^7$ | Leu-Lys-Leu-Lys-Lys-Leu-Trp-Lys-Leu-Leu-Lys-Lys-Leu-Leu-Lys-Leu-Gly | 2.0 |
| $Trp^8$ | Leu-Lys-Leu-Lys-Lys-Leu-Leu-Trp-Leu-Leu-Lys-Lys-Leu-Leu-Lys-Leu-Gly | 1.2 |
| $Trp^9$ | Leu-Lys-Leu-Lys-Lys-Leu-Leu-Lys-Trp-Leu-Lys-Lys-Leu-Leu-Lys-Leu-Gly | 1.2 |
| $Trp^{10}$ | Leu-Lys-Leu-Lys-Lys-Leu-Leu-Lys-Leu-Trp-Lys-Lys-Leu-Leu-Lys-Leu-Gly | 0.7 |
| $Trp^{11}$ | Leu-Lys-Leu-Lys-Lys-Leu-Leu-Lys-Leu-Leu-Trp-Lys-Leu-Leu-Lys-Leu-Gly | 2.5 |
| $Trp^{12}$ | Leu-Lys-Leu-Lys-Lys-Leu-Leu-Lys-Leu-Leu-Lys-Trp-Leu-Leu-Lys-Leu-Gly | 5.0 |
| $Trp^{13}$ | Leu-Lys-Leu-Lys-Lys-Leu-Leu-Lys-Leu-Leu-Lys-Lys-Trp-Leu-Lys-Leu-Gly | 0.9 |
| $Trp^{14}$ | Leu-Lys-Leu-Lys-Lys-Leu-Leu-Lys-Leu-Leu-Lys-Lys-Leu-Trp-Lys-Leu-Gly | 0.7 |
| $Trp^{15}$ | Leu-Lys-Leu-Lys-Lys-Leu-Leu-Lys-Leu-Leu-Lys-Lys-Leu-Leu-Trp-Leu-Gly | 0.7 |
| $Trp^{16}$ | Leu-Lys-Leu-Lys-Lys-Leu-Leu-Lys-Leu-Leu-Lys-Lys-Leu-Leu-Lys-Trp-Gly | 0.8 |

In the absence of calmodulin, the tryptophan-containing peptides had CD and fluorescence spectral parameters consistent with those found for peptides in unordered conformations in dilute aqueous solution at neutral *p*H. The emission maxima of the fluorescence spectra occurred at 345 ± 2 nm (total range), which was consistent with the tryptophan being almost entirely exposed to the aqueous solvent (*10*) and with the anisotropies of the peptides in free solution being uniformly low (0.051 ± 0.014).

Under conditions in which the peptides were entirely bound in a 1:1 complex with calmodulin, the fluorescence properties were greatly modified and the degree of modification depended on the position of the tryptophan in the chain. The emission maxima were blue shifted by values that ranged from 4 nm for $Trp^{15}$ to 25 nm for $Trp^3$. The value of the wavelength for maximum emission $\lambda_{max}$ as a function of the position at which the tryptophanyl residue occurred in the sequence was periodic and repeated every three to four residues. Fourier analysis demonstrated that the data could be optimally described by a sine wave with a 3.4-residue period. This value is near that for the repeat of an α-helix (3.6) (*15*) and is consistent with the α-helical model described in Fig. 3, in which the residues on the hydrophilic side of the helix are most exposed to solvent. The bars in Fig. 4A are spaced at 3.6-residue intervals and show a good qualitative fit with the data. The values of the Stern-Volmer constants for collisional quenching (*16, 17*) of the tryptophan fluorescence were also consistent with the α-helical model (Fig. 4B). The residues that occupied the polar face of the helix had the highest accessibility to acrylamide, whereas the residues on the opposite face of the helix tended to be less accessible. Fourier analysis of the data gave a period of 3.2 residues for $K$. Finally, the anisotropies (*18*) were also periodic (Fig. 4C) and showed that the highest degree of immobilization occurred when the tryptophan was on the apolar side of the helix, which was presumably in contact with calmodulin. The repeat period for this parameter was 3.2 residues. To aid in the interpretation of the physical significance of the above three parameters, the right *y*-axes in Fig. 4 have units of the fractional change $f$ that might be expected to occur if the peptide were transferred from water to a highly apolar, rigid, solvent-inaccessible environment (Fig. 4).

As expected, there appears to be a strong linear relation between the observed values of $\lambda_{max}$, $K$, and $r_0$. The positive correlation between $\lambda_{max}$ and $K$ indicates that as the emission maximum of the tryptophan is shifted toward the blue it tends to be more inaccessible to acrylamide. Similarly, the negative correlation between either of these two parameters and $r_0$ indicates that the peptides with the lowest $\lambda_{max}$ and quenching constants have the highest anisotropies. This suggests that each parameter measures related aspects of the degree to which a given position in the peptide is involved in interaction with calmodulin. Since there are many errors and ambiguities associated with the measurement and interpretation of each of these parameters, they were combined into a single quantity that represents the degree of spectral perturbation that occurred for each peptide upon complexation with calmodulin. The mean value of $f$ for each parameter was calculated and is plotted for the set of peptides in Fig. 5. The data were optimally fit by a sine wave with a period of 3.3 residues; the smooth curve shows the theoretical curve that corresponds to this value.

The values of 3.4, 3.2, and 3.2 for the periods of $\lambda_{max}$, $K$, and $r_0$, respectively, are consistent with an α-helical conformation and eliminate the other major class of repeating secondary structure, parallel and antiparallel β-sheets, which repeat with periods of 2.0 to 2.3 residues (*15*). The correlation is not strong enough, however, to differentiate between α-helices (3.6-residue period) and $3_{10}$ helices (3.0-residue period). However, since there are no documented examples of long $3_{10}$ helices in the structures of proteins, a predominantly α-helical conformation is the simplest structural model

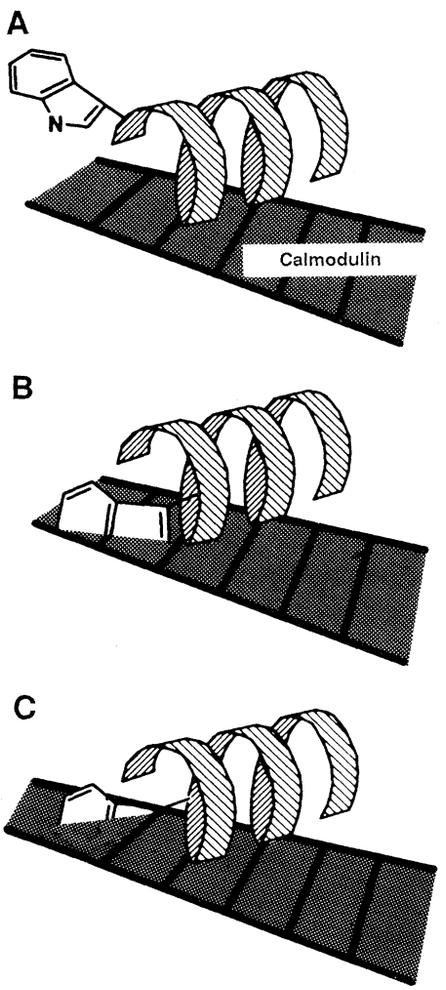


**Fig. 3.** Schematic representation of the orientation of several tryptophan-containing peptides with respect to calmodulin. In position 1 (**A**) the tryptophan is located at the N-terminus on the hydrophilic side of the helix. Peptides with the tryptophan on this face of the helix exhibit small blue shifts and low anisotropies and are accessible to quenching by acrylamide. In position 2 (**B**) the tryptophan is located at the interface between hydrophobic and hydrophilic sides of the helix and should exhibit an intermediate value for these spectral parameters. In position 3 (**C**) the tryptophan is located on the hydrophobic face of the helix and the fluorescence properties of the peptide should be maximally affected. This representation is purely schematic and not meant to imply any specific mechanism for interaction of the peptide with calmodulin.

for the peptide. In addition, the periodicity of the fluorescence properties extends throughout the peptide chain and suggests that the helix extends throughout the length of the peptide. The helical nature of the calmodulin-bound peptides is further supported by difference CD measurements. If the change in ellipticity observed at 222 nm upon binding of the peptide to calmodulin is attributed exclusively to a change in the conformation of the peptide, then the mean residue ellipticity is $-18{,}600 \pm 5{,}700$ degree cm$^2$ dmol$^{-1}$ (standard deviation) for the 16 peptides that contain tryptophan. The deviations of the data points from the sinusoidal curve in Fig. 5 are greater than can be attributed to experimental error. This deviation presumably occurs because the peptides do not bind to a planar interface that has either a dielectric, a solvent exposure, or a rigidity that varies smoothly. The structural irregularity of calmodulin's surface probably also causes the deviation of the periodicity of the fluorescence parameters from 3.6 residues. Interestingly, some of the deviations of the data from the sinusoidal curve can be explained in terms of the relative affinities of the derivatives. For example, we can consider the maxima of the curve that occur at positions on the hydrophobic face of the helix, which presumably directly interact with calmodulin. The greatest of these occurs at position 3, which is also the tryptophan derivative with the highest affinity for calmodulin ($K_d$ = 0.2 n$M$). Presumably there is a site on calmodulin that can well accommodate a tryptophanyl side chain; the tightness of the interaction is reflected in the fluorescence properties of this derivative and in its affinity. The lowest of the local maxima occurs at position 10, and this derivative is among the peptides with the lowest affinities for calmodulin ($K_d$ = 3.0 n$M$). We have yet to explain the deviations at other positions (for example, Trp[11]).

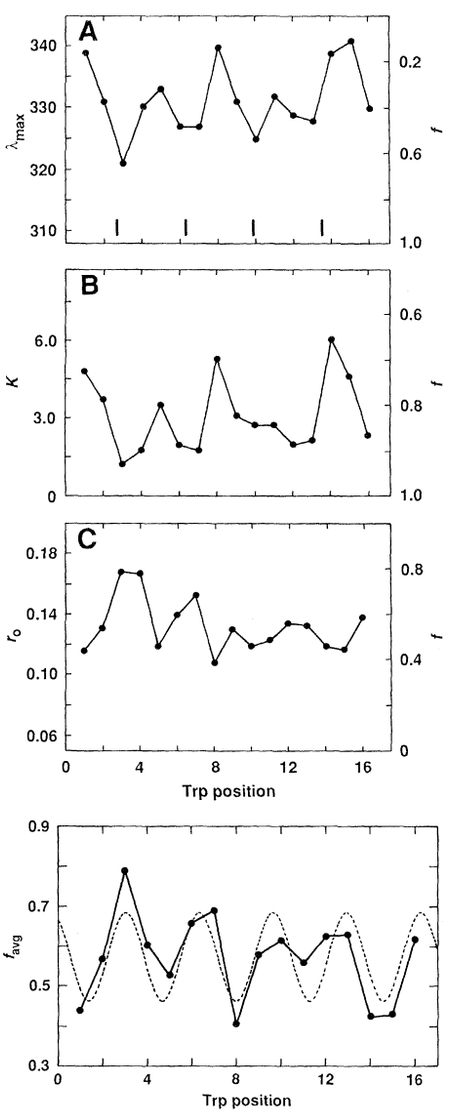


**Fig. 4.** (**A**) Wavelength maxima of peptide:calmodulin complexes as a function of tryptophan position. Fluorescence spectra were measured with a 5.0 μ$M$ peptide:calmodulin complex, 10 m$M$ tris-HCl, 0.5 m$M$ $CaCl_2$, $p$H 7.30, as described previously (*14*); excitation wavelength $\lambda_{ex}$ = 290 nm; slits, 5 nm. The spectra were corrected for the nonlinearity of the photomultiplier response and for background fluorescence. Maxima are accurate to ±2 nm. (**B**) Acrylamide quenching of peptide:calmodulin complexes as a function of tryptophan position. Acrylamide quenching data (±20%) were obtained by progressive additions of 5-μl aliquots of 5$M$ buffered acrylamide solution to 1.0 ml of 5.0 μ$M$ peptide:calmodulin complex in 10 m$M$ tris-HCl, 0.15 $M$ NaCl, and 0.5 m$M$ $CaCl_2$ at a $p$H of 7.5. Plots of $F_0/F$ versus acrylamide concentration were linear up to concentrations of at least 0.25$M$; the slope $K$ was obtained by linear regression. (**C**) Anisotropy of peptide:calmodulin complexes as a function of the tryptophan position. Anisotropies (±0.01) were measured in the L format (single channel) ($\lambda_{ex}$ = 300 nm, emission wavelength $\lambda_{em}$ = 345 nm, slits = 4 nm) with the equation $r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$ and a $G$ correction factor to account for differences in monochromator sensitivity to vertically and horizontally polarized light as described in (*10*). The parameter $f$ (right $y$-axis) was calculated using the mean values of $\lambda_{max}$, $K$, and $r_0$ for the free peptides in the absence of calmodulin. The maximal possible changes in these parameters were defined by $\lambda_{max}$ and $K$ for azurin, which contains a single, solvent-inaccessible tryptophan, and by $r_0$ for tryptophan in ethylene glycol at −60°C (*10*).

**Fig. 5.** Mean value of the three $f$ values for each peptide as a function of tryptophan position in the peptide. The data were fit to the equation $F(x) = D \sin[(\langle f \rangle + a)/n] + C$, where $\langle f \rangle$ is the mean value of $f$ for $K$, $\lambda_{max}$, and $r_0$, and $a$, $n$, $D$, and $C$ are constants obtained from the curve-fitting option of RS/1(BBN Software Products Corporation, Cambridge, MA).

These results suggest that it may be possible to apply this approach to a variety of other systems, although a tryptophan is probably too perturbing a probe to be of general use. A better choice may be one of several nitroxide spin labels (*19*) that are not only much smaller but are also highly sensitive to environment and can be detected in very small quantities. Alternatively, the use of artificial probes could be eliminated by using amino acids enriched at a specific amino acid with an isotopic label (for example, $^{13}C$ at the Cα position) and performing the appropriate NMR investigations.

## REFERENCES AND NOTES


1. C. B. Klee and T. C. Vanaman, *Adv. Protein Chem.* **35**, 213 (1982).
2. D. A. Malencik and S. R. Anderson, *Biochemistry* **23**, 2420 (1985).
3. J. A. Cox, M. Comte, J. E. Fitton, W. F. DeGrado, *J. Biol. Chem.* **260**, 2527 (1985).
4. W. F. DeGrado, F. G. Prendergast, H. R. Wolfe, J. A. Cox, *J. Cell. Biochem.* **29**, 83 (1985); L. McDowell, G. Sanyal, F. Prendergast, *Biochemistry* **24**, 2979 (1985).
5. D. K. Blumenthal *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3186 (1985).
6. R. E. Klevit, D. K. Blumenthal, D. E. Wemmer, E. G. Krebs, *Biochemistry* **24**, 8152 (1985).
7. A. M. Edelman *et al.*, *J. Biol. Chem.* **260**, 11275 (1985).
8. T. J. Lucas, W. H. Burgess, F. G. Prendergast, W. Lau, D. M. Watterson, *Biochemistry* **25**, 1458 (1986).
9. S. H. Seeholzer, M. Cohn, J. A. Putkey, A. R. Means, H. L. Cresi, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 3634 (1986).
10. J. R. Lakowicz, *Principles of Fluorescence Spectroscopy* (Plenum, New York, 1983).
11. J. Meienhofer *et al.*, *Int. J. Pept. Protein Res.* **13**, 35 (1979).
12. E. Atherton and R. C. Sheppard, *Chem. Commun.* **1985**, 165 (1985).
13. G. Barany and R. B. Merrifield, in *Peptides, Analysis, Synthesis and Biology*, J. Meienhofer and E. Gross, Eds. (Academic Press, New York, 1979), vol. 2, chap. 1.
14. S. Erickson-Viitanen and W. F. DeGrado, *Methods Enzymol.* **139**, 455 (1987).
15. D. B. Calhoun, J. M. Vanderkooi, G. R. Holton, W. S. Englander, *Proteins* **1**, 109 (1986).
16. G. E. Shultz and R. H. Shirmer, *Principles of Protein Structure* (Springer-Verlag, New York, 1979).
17. F. Prendergast and G. Sanyal have shown by examining the dependence of the fluorescence lifetime on acrylamide concentration that the peptides are quenched by the collisional mechanism (personal communication). Also, the interpretation of $K$ and $r_0$ for this set of peptides requires that the lifetimes be similar. Prendergast has found that the average lifetimes vary from 3 to 4 nsec for this set of peptides.
18. The anisotropy for the $Trp^3$ analogue reported herein is significantly greater than that we reported earlier (*3*) in our paper that described the design and synthesis of peptide 1 (Fig. 1). We recently remeasured the fluorescence anisotropy of $Trp^3$ and found a value near that reported here (0.17 ± 0.01).
19. L. J. Berliner, Ed., *Spin Labeling* (Academic Press, New York, 1976); *Spin Labeling II* (Academic Press, New York, 1978).


20. We thank F. Prendergast and G. Sanyal for helpful conversations and for sharing unpublished data, K. Smithyman for technical assistance, and B. Larsen and J. Lazar for collecting the FAB mass spectra. We also thank C. Levinthal and W. Hubbel for communicating unpublished results; they have independently adopted an approach similar to that described herein for determining the secondary structure of colicin.

2 January 1987; accepted 10 April 1987