A Single-Stranded Amphipathic α-Helix in Aqueous Solution: Design, Structural Characterization, and Its Application for Determining α-Helical Propensities of Amino Acids†

Nian E. Zhou, Cyril M. Kay, Brian D. Sykes, and Robert S. Hodges*

Department of Biochemistry and the Protein Engineering Network of Centres of Excellence, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

Received January 4, 1993; Revised Manuscript Received April 2, 1993

ABSTRACT: In order to investigate the positional effect of α-helical propensities of amino acids in an amphipathic α-helix, an amphipathic α-helical model peptide (Ac-Glu-Ala-Glu-Lys-Ala-Ala-Lys-Glu-Ala-Glu-Lys-Ala-Ala-Lys-Glu-Ala-Glu-Lys-amide) was designed and characterized by circular dichroism and 2D-NMR spectroscopies. This peptide contains 65% α-helical structure in solution, and its monomeric molecular weight in aqueous solution was determined by size-exclusion chromatography. The independence of α-helical structure and stability on peptide concentration demonstrates that helix formation of this peptide is a monomolecular process. To compare the effect of substitutions in the hydrophobic and hydrophilic face of the helix on structure and stability, a leucine, alanine, or glycine was individually substituted in the hydrophobic face (position 9) or hydrophilic face (position 7) of the model peptide. The change in helix content and stability upon substitution was measured by circular dichroism studies in the absence and presence of TFE or urea. The results indicate that each amino acid has a different helix propensity when it is located in the hydrophobic face versus hydrophilic face and the effect of substitution is more significant in the hydrophobic face. This single-stranded amphipathic α-helical peptide provides an appropriate model system to determine helix propensities of amino acids on both hydrophobic and hydrophilic faces.

The α-helix is the most abundant secondary structure in proteins, and it is therefore of considerable importance to determine the α-helix-forming tendencies of amino acid residues to aid in our understanding of protein folding and protein structure (Scholtz & Baldwin, 1992; Ptitsyn, 1992). Each amino acid residue has a distinct conformational preference based on the frequency of its occurrence in different secondary structures in proteins of known structures (Fasman, 1989; Chou & Fasman, 1978; Richardson & Richardson, 1988). A notable approach for experimentally determining α-helix-forming tendencies of amino acid residues is to compare the contribution of each amino acid residue to α-helical content and stability of synthetic model peptides in an α-helical conformation. Scheraga and co-workers (Scheraga, 1978; Sueki et al., 1984; Wojcik et al., 1990) have determined the thermodynamic parameters σ and s, which are related to nucleation and propagation of a helix, respectively (Zimm & Bragg, 1959), by incorporating amino acids at low concentration as "guests" into synthetic copolymers containing hydroxyalkylated glutamine "host" side chains. Since this pioneering work, numerous examples of natural and synthetic short α-helical peptides in aqueous solution have been reported (Bierzynski et al., 1982; Fairman et al., 1989; Shoemaker et al., 1985, 1987; Strehlow & Baldwin, 1989; Marqusee & Baldwin, 1987; Merutka & Stellwagen, 1990; Merutka et al., 1990). These short helices afford a convenient model system for conducting host–guest studies. Baldwin and co-workers measured the helix-forming tendencies of five natural nonpolar amino acids (Padmanabhan et al., 1990) and three nonnatural amino acids (Padmanabhan & Baldwin, 1991) in a soluble, alanine-based peptide. Lyu and co-workers derived the relative contribution to helix formation for 10 natural (Lyu et al., 1990) and 4 nonnatural amino acids (Lyu et al., 1991) in a highly polar peptide containing alternating blocks of Glu and Lys residues (succinyl-YSEEEEKKKKXXX-EEEEKKKK-NH_2) (Lyu et al., 1989). O'Neil and DeGrado (1990) determined the free energy contribution to helix formation for 20 naturally occurring amino acids by measuring the dimer ⇌ monomer equilibrium constant for a two-stranded α-helical coiled-coil upon substituting each amino acid into a solvent-exposed position of this model system (Hodges et al., 1981, 1988, 1990; Lau et al., 1984a).

However, it has been demonstrated recently that the effect of an Ala to Gly substitution (Chakrabartty et al., 1991) as well an Ala to Glu, His, Arg, Lys, or Phe (Fairman et al., 1991) on α-helical structure was strongly dependent upon the position of substitution in the α-helix. A previous study from our laboratory (Zhou et al., 1990) has also shown that peptides with the same amino acid composition, but different sequence, have different α-helical contents. The more amphipathic the peptide, the higher the helicity (Zhou et al., 1990). In addition, the amino acid distribution in α-helices of proteins is not random. The distributions of hydrophobic and charged amino acids are highly restricted; that is, the hydrophobic amino acids Leu, Ile, Val, and Phe are concentrated in one longitudinal quadrant of the helix, while the four charged amino acids Lys, Arg, Asp, and Glu are uniformly absent from this axial hydrophobic strip (Torgerson et al., 1991). These results raise an important question: Does each amino acid have a similar helical propensity when it is located on either the hydrophobic or the hydrophilic face of the α-helix? It is therefore essential to systematically investigate the positional effect on helical propensities of amino acids in an amphipathic helix. In addition, the study of amphipathic helices is very relevant to native peptides and proteins (Mant et al., 1993), since the amphipathic feature of helices is generally observed in biologically active peptides and proteins

† This research is an integral part of the Protein Engineering Network of Centres of Excellence Program supported by the Government of Canada.

* Author to whom correspondence should be addressed.

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(Segrest et al., 1990) and approximately 50% of all helices in proteins are amphipathic (Cornette et al., 1987). Unfortunately, previous measurements of helical propensities of amino acids were conducted either on a nonamphipathic single-stranded α-helical peptide or on a solvent-exposed site in a model coiled-coil. One of the reasons that amphipathic α-helical model peptides were not used previously is that an amphipathic α-helix tends to aggregate in aqueous solution (Marqusee et al., 1989; Bradley et al., 1990). A limitation of the coiled-coil model system is that the helical propensities of amino acids cannot be determined in the hydrophobic face where the side chains are involved in intermolecular interactions (Hodges et al., 1990; Zhou et al., 1992a).

The goal of our research is to determine the helical propensities of 20 commonly occurring amino acids on both the hydrophobic and hydrophilic faces of an amphipathic α-helix. The first crucial step toward this goal was the design of an α-helical model peptide with the appropriate amphipathicity to avoid self-aggregation in aqueous solution, in order to ensure that the peptide remains single-stranded. The second aspect of this investigation was to compare the effect of substitutions in the hydrophobic and hydrophilic face of the helix on structure and stability.

MATERIALS AND METHODS

All peptides were synthesized by the solid-phase technique (Erickson & Merrifield, 1976) using standard procedures as described previously by Hodges et al. (1988) on an Applied Biosystems 430A peptide synthesizer. Copoly(styrene, 1% divinylbenzene)benzhydrylamine hydrochloride resin was used. All amino acids were protected at their α-amino group with the *tert*-butyloxycarbonyl (*t*-Boc) group, and the side-chain-protecting groups used were as follows: benzyl (Glu) and 2-chlorobenzyloxycarbonyl (Lys). The peptides were cleaved from the resin by reaction with hydrogen fluoride (20 mL/g of resin) containing 10% anisole and 2% 1,2-ethanedithiol for 1 h at −5 °C. The crude peptides were purified by reversed-phase chromatography (RPC)[1] on a Synchropak RP-P semipreparative C18 column (250 × 10 mm i.d., 6.5-μm particle size, 300-Å pore size) (Synchrom, Layfayette, IN), with a linear AB gradient (ranging from 0.2% to 1.0% B/min, depending on the peptide) at a flow rate of 2 mL/min, where solvent A was 0.05% aqueous trifluoroacetic acid (TFA) and solvent B was 0.05% TFA in acetonitrile. The purified peptides were homogeneous as determined by analytical RPC and confirmed by amino acid analysis and mass spectroscopy. For amino acid analysis, purified peptides were hydrolyzed in 6 N HCl containing 0.1% phenol at 110 °C for 24 or 1 h at 160 °C in evacuated sealed tubes. Amino acid analysis was performed on a Beckman Model 6300 amino acid analyzer (Beckman Instruments Inc., Fullerton, CA). The correct primary ion molecular weights of peptides were confirmed by time of flight mass spectroscopy on a BIOION-20 Nordic (Uppsala, Sweden).

Size-exclusion chromatography (SEC) was carried out on a Spherogel TSK 2000SW column (300 × 7.5 mm I.D., particle size 10 μm, pore size 125 Å) (Beckman Instruments, Japan) at a flow rate of 0.2 mL/min at room temperature. The eluent was a 0.1 M KCl/50 mM PO_4, pH 7, buffer. 7-, 14-, 21-, 28-, and 35-residue peptides of the sequences Ac-K-C-A-E-G-E-L-(K-L-E-A-G-E-L)$_n$-amide, where n = 0, 1, 2, 3, or 4, were used as molecular weight standards (Zhou et al., 1990).

Circular dichroism (CD) spectra were recorded on a Jasco J-500C spectropolarimeter (Jasco, Easton, MD) equipped with a Jasco IF500II interface, and an IBM PS/2 running the Jasco DP-500/PS-2 system version 1.33a software, and a Lauda (Model RMS) water bath (Brinkmann Instruments, Rexdale, Ontario, Canada), used to control the temperature of the cell. Constant N_2 flushing was employed. The instrument was routinely calibrated with an aqueous solution of recrystallized *d*-10-(+)-camphorsulfonic acid at 290 nm. Molar ellipticity at 220 nm is reported as mean residue molar ellipticity ($[\theta]_{220}$, deg·cm²·dmol⁻¹) and calculated from the equation:

$$[\theta] = [\theta]_{obs}(\text{mrw})/10lc$$

where $[\theta]_{obs}$ is the ellipticity measured in degrees, mrw is the mean residue molecular weight (molecular weight of the peptide divided by the number of amino acid residues), c is the peptide concentration in grams per milliliter, and l is the optical path length of the cell in centimeters. CD spectra were the average of four scans obtained by collecting data at 0.25-nm intervals from 250 to 190 nm. Urea denaturation studies were carried out by preparing mixtures of a stock solution of peptide in buffer (0.1 M KCl/50 mM PO_4, pH 7), buffer alone, and a solution of 10 M urea in buffer where the ratios of buffer and 10 M urea solutions were varied to give the appropriate final urea concentrations. Peptide concentrations were determined by amino acid analysis.

The thermal dependence of ellipticity at 220 nm in aqueous solution was analyzed by assuming that the folding/unfolding is a two-state transition only involving the helix form and coil form of the peptide, in which case an equilibrium constant (K) for the transition of helix (H) ⇌ coil (C) can be expressed by $K = [C]/[H] = ([\theta]_H - [\theta])/([\theta] - [\theta]_C)$, where $[\theta]$ is the ellipticity and $[\theta]_H$ and $[\theta]_C$ are the ellipticities of the helical and the coil forms of peptide, respectively. The $[\theta]_H$ was obtained from TFE titration (Marqusee & Baldwin, 1987; Marqusee et al., 1989; Padmanabhan et al., 1990), and the value of $[\theta]_{220}$ in 50% TFE ($[\theta]_{220} = -29\,000$ deg·cm²·dmol⁻¹) was taken to represent the formation of 100% α-helix in this peptide. The thermal melting curves can be fitted with the van't Hoff equation: $\ln K = \ln[([\theta]_H - [\theta])/([\theta] - [\theta]_C)] = -\Delta H/R(1/T - 1/T_m)$ in which T_m is the temperature at the midpoint of the transition where $([\theta]_H - [\theta])/([\theta] - [\theta]_C) = 1$. The values of $[\theta]_C$ and T_m were obtained by fitting the observed ellipticities at different temperatures to the van't Hoff equation using the SigmaPlot program.

^{1}H-NMR spectra were obtained on a Varian VXR-500 NMR spectrometer with an operating frequency of 500 MHz for protons. Chemical shifts were referenced to the trimethylsilyl resonance of 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) at 0.00 ppm. Spectra were taken at 25 °C. NOESY spectra (Kumar et al., 1980) and DQF-COSY (Rance et al., 1983) were obtained in the phase-sensitive detection mode by using standard pulse sequences. Spectra were acquired with 2048 complex points in t_2 and 256–512 complex FIDs in t_1 with 24–64 transients for each FID. The carrier was centered on the H_2O resonance, and the spectral width was 6000 Hz in both dimensions. NOESY spectra were collected with mixing times of 400 ms. For measurement of NMR spectra, the peptide AA9 was dissolved in a 1:1 (v/v) mixture of trifluoroethanol-d_3 (TFE-d_3) (Cambridge Isotopes Laboratory) and H_2O containing 0.1 M KCl/50 mM potassium phosphate buffer. The pH of the sample was adjusted to 5.2

[1] Abbreviations: CD, circular dichroism; TFA, trifluoroacetic acid; TFE, trifluoroethanol; RPC, reversed-phase chromatography; SEC, size-exclusion chromatography; NOESY, nuclear Overhauser enhancement spectroscopy; NMR, nuclear magnetic resonance; DQF-COSY, double quantum filtered correlation spectroscopy.

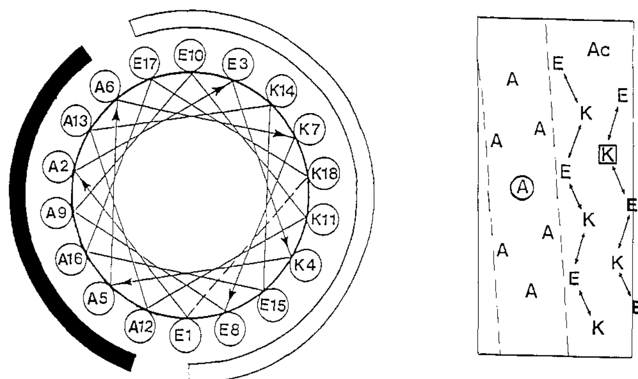

FIGURE 1: Amino acid sequence of the designed amphipathic α-helical peptide AA9 represented as a helical wheel (left panel) and a helical net (right panel). Since the α-helix has 3.6 residues per turn, adjacent side chains in the sequence are separated by 100° of arc on the wheel. The hydrophobic and hydrophilic faces are observed on opposing sides of the helix as indicated by the solid and open bars, respectively. Arrows indicate the possible intrahelical ($i,i+3$) and ($i,i+4$) ionic attractions between side chains of Glu and Lys residues. The circle in the helical net of AA9 denotes an alanine residue at position 9 which is replaced by a leucine in peptide AL9 or by a glycine in AG9. Similarly, the square denotes a lysine residue at position 7 which is replaced by an alanine in AA7, by a leucine in AL7, or by a glycine in AG7.

with dilute HCl and KOH. pH meter readings were measured at room temperature and were not corrected. The final peptide concentration is approximately 5 mM.

Secondary structure of peptides was predicted by the methods of Chou and Fasman (1978), Garnier et al. (1978), Eisenberg et al. (1984b), and homology search (Levin & Garnier, 1988), using the software package SEQSEE written by R. Boyko and D. Wishart, University of Alberta.

RESULTS AND DISCUSSION

Design of a Single-Stranded Helix Which Is Monomeric in Aqueous Solution. The amino acid sequence of the designed peptide is Ac-Glu-Ala-Glu-Lys-Ala-Ala-Lys-Glu-Ala-Glu-Lys-Ala-Ala-Lys-Glu-Ala-Glu-Lys-amide, which has a high potential to form an amphipathic helix. The distribution of the hydrophobic and hydrophilic amino acid residues is displayed in the helical wheel and helical net diagram (Figure 1). As shown in the helical net (right panel of Figure 1), the glutamate/lysine ion pairs located in the i and $i+3$ or i and $i+4$ positions along the sequence could provide additional stability to the α-helical structure by side-chain electrostatic interactions (Marqusee & Baldwin, 1987; Merutka & Stellwagen, 1991; Lyu et al., 1992). Secondary structure predictions using the methods of Chou and Fasman (1978), Garnier et al. (1978), Eisenberg et al. (1984b), and homology search (Levin & Garnier, 1988) predict that every residue in peptide AA9 should adopt an α-helical conformation. The model peptide is denoted as AA9 where the first A refers to the peptide with seven alanines in the hydrophobic face including position 9 (A9). When the alanine residue at position 9 was substituted by a leucine or glycine, the peptide analog is denoted as AL9 or AG9, respectively. Similarly, when the lysine at position 7 is substituted by an alanine, leucine, or glycine, the peptide analogs are denoted as AA7, AL7, or AG7, respectively.

In the design of this model peptide, alanine, glutamic acid, and lysine residues were selected specifically because of their high intrinsic helical propensities (Chou & Fasman, 1978; Wojcik et al., 1990; O'Neil & DeGrado, 1990), alanine as an apolar residue, and glutamic acid and lysine as potentially negatively charged and positively charged residues, respectively. In order to decrease the hydrophobicity on the nonpolar face of the helix which consequently decreases the tendency to aggregate, alanine residues were used instead of more hydrophobic residues, such as leucine, in the nonpolar face. Our recent study (Zhou et al., 1992d) showed that the peptide with seven Leu residues in the hydrophobic face self-associates in aqueous solution and required at least 25% trifluoroethanol (TFE) to keep the peptide monomeric. Eisenberg's mean helical hydrophobic moment ($\langle\mu\rangle$) (Eisenberg et al., 1982, 1984a) was used to express the helical amphipathicity of these peptides. This method consists of the vector sum of the hydrophobicity values of the amino acids, taking into account their periodic orientation in an α-helix, i.e., one residue every 100° or 3.6 residues in a turn. The value of the mean helical hydrophobic moment is 0.59 for peptide AA9 using the normalized consensus hydrophobicity scale (Eisenberg et al., 1984a), which is more amphipathic than most native amphipathic helices which have values in the range of 0.12–0.54 for the seven different classes of amphipathic helices (Segrest et al., 1990).

Characterization of the Single-Stranded α-Helical Structure by Circular Dichroism. The far-ultraviolet CD spectra of peptide AA9 in the absence and presence of the α-helix-enhancing solvent TFE (Sönnichsen et al., 1992) are illustrated in Figure 2A,B. At low temperature (5 °C), the double minima at 220 nm ($n-\pi^*$ transition) and 207 nm ($\pi-\pi^*$ transition) as well as the maximum at 192 nm ($\pi-\pi^*$ transition) in the CD spectra of peptide AA9 (Figure 2A,B) are characteristic of an α-helix (Holzwarth & Doty, 1965). The ellipticity at 220 nm was monitored in subsequent experiments as an indication of helical content. The mean residue molar ellipticities of −18 400 and −29 000 deg·cm^2·dmol^{-1} at 220 nm for peptide AA9 in 0.1 M KCl/50 mM PO_4 buffer, pH 7, in the absence and presence of TFE, respectively, indicated that peptide AA9 has significant α-helical content. As the temperature is increased, the intensity of the minimum at 220 nm is decreased, which corresponds to the transition from α-helix to random coil structure (Figure 2C). CD spectra taken through the transition showed an isodichroic point at 202 nm, which is consistent with the presence of just two conformations, either the α-helix or the random coil conformation (Padmanabhan et al., 1990). The absence of a plateau at low temperature implied that the peptide is not completely α-helical under these conditions. The dependence of the mean residue molar ellipticity of peptide AA9 at 220 nm on temperature in different solvent systems is shown in Figure 2C. The midpoint of the transition is 14.6 °C for peptide AA9 in 10 mM PO_4 buffer and 10.3 °C in 0.1 M KCl/50 mM PO_4 buffer, suggesting that the helix is more stable in lower salt concentration due to the increased ionic interactions of the Glu/Lys ion pairs. In the presence of the helix-inducing solvent TFE, the helix is much more stable to temperature denaturation (Figure 2C), and the transition is not cooperative, with the ellipticity decreasing roughly linearly with increasing temperature.

It is important to establish that the peptide is monomeric and the helical structure is not a consequence of the known tendency of amphipathic helices to associate intermolecularly. The monomeric molecular weight of peptide AA9 in aqueous solution was determined using SEC by comparison with 7-, 14-, 21-, 28-, and 35-residue peptide standards (Zhou et al., 1990) (Figure 3A). The amphipathic peptide with seven leucine residues in the hydrophobic face (Zhou et al., 1992d) showed the apparent dimeric molecular weight under similar

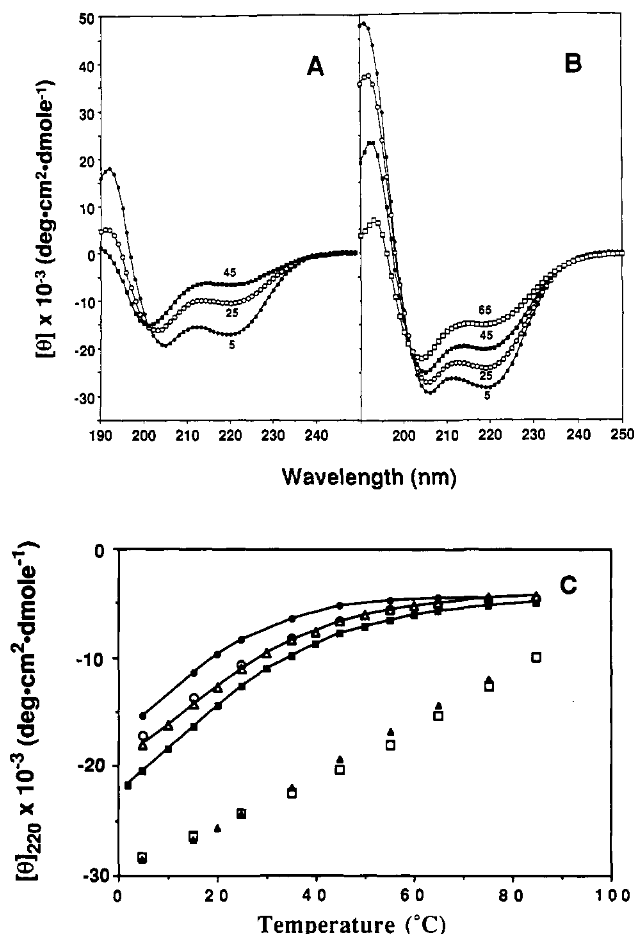

FIGURE 2: Thermal dependence of the ellipticities of peptide AA9 under various conditions. Panel A: CD spectra of peptide AA9 in 0.1 M KCl/50 mM PO_4 buffer, pH 7, at 5 (●), 25 (○), and 45 °C (■). Panel B: CD spectra of peptide AA9 in 0.1 M KCl/50 mM PO_4 buffer containing 30% TFE (v/v), pH 7, at 5 (●), 25 (○), 45 (■), and 65 °C (□). Panel C: Thermal dependence of the mean residue molar ellipticities at 220 nm for peptide AA9 in various solvents. Open triangles and open circles represent values obtained in 0.1 M KCl/50 mM PO_4 buffer, pH 7, at peptide concentrations of 59 μM (△) and 659 μM (○). Closed circles represent values obtained in 0.1 M KCl/50 mM PO_4 buffer, pH 2, at a peptide concentration of 348 μM. Closed squares represent values obtained in 10 mM PO_4 buffer, pH 7, at a peptide concentration of 59 μM. Open squares and closed triangles represent values obtained in 0.1 M KCl/50 mM PO_4 buffer containing 30% TFE (v/v), pH 7, at a peptide concentration of 593 μM (□) and in 0.1 M KCl/50 mM PO_4 buffer containing 30% TFE (v/v), pH 2, at a peptide concentration of 348 μM (▲).

conditions (data not shown). In addition, studies of helix formation as a function of peptide concentration (Figure 3B) also indicated that this alanine amphipathic α-helix is monomeric. The independence of $[\theta]_{220}$ on concentration for peptide AA9 was observed over the range from 10 to 1100 μM at 5 and 20 °C in aqueous solution, pH 7. In contrast, for two amphipathic α-helices which interact to form a dimeric coiled-coil, the helicity of the peptide increased as the peptide concentration increased (Zhou et al., 1992b,c). In addition, for peptides which associate into multimeric structures, such as dimeric α-helical coiled-coils (Zhou et al., 1992b,c) or four-helix bundles (Ho & DeGrado, 1987), the unfolding transitions from a multimeric folded structure to the random coil monomer are strongly dependent on peptide concentration. However, peptide AA9 showed essentially identical unfolding curves at two different concentrations (59 and 659 μM) (Figure 2C). These results demonstrate that helix formation of peptide AA9 is a monomolecular process and is not the result of aggregation.

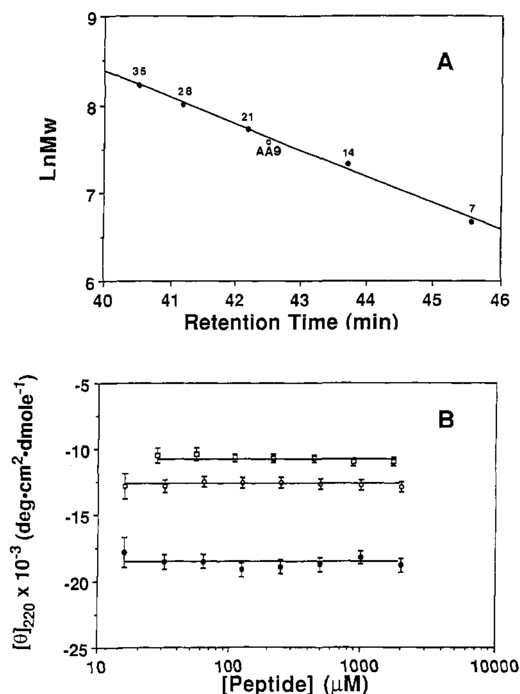

FIGURE 3: Top panel: Standard plot of ln of the molecular weight versus the retention time of the synthetic peptides in size-exclusion chromatography (SEC). The conditions for SEC are described under Materials and Methods. The samples were dissolved in 0.1 M KCl/ 50 mM PO_4, pH 7, buffer (~1 mg/mL). The numbers 7, 14, 21, 28, and 35 represent the number of residues in the size-exclusion chromatographic standard peptides (Zhou et al., 1990) of the sequences Ac-K-C-A-E-G-E-L-(K-L-E-A-G-E-L)$_n$-amide, where n = 0, 1, 2, 3, or 4. Bottom panel: Concentration dependence of the mean residue molar ellipticities at 220 nm for peptides AA9 at 5 °C (●) and 20 °C (○) and for AL9 at 20 °C (□). All measurements were performed in 0.1 M KCl/50 mM PO_4 buffer, pH 7, with a 0.5-mm path-length cell.

In order to investigate the effect of intrahelical ionic interactions on helix formation, studies were performed with peptide AA9 as a function of pH (Figure 4). In aqueous buffer, peptide AA9 shows maximum helical content at neutral pH, and the helicity decreases at both basic and acidic pH, which is the result of protonation of the side chains of glutamic residues at acidic pH and deprotonation of the side chains of lysine residues at basic pH. These results indicate that the intrahelical ionic interactions are important for α-helix stabilization. In addition, the apparent pK value for Glu residues in the peptide is ~4, which is less than the pK for noninteracting acidic side chains in proteins of 4.6 (Creighton, 1984), and the apparent pK value for Lys residues in the peptide is ~11.2, which is greater than the pK of 10.4 for the noninteracting amino group of Lys side chains (Creighton, 1984). This finding suggests that acidic and basic residues are preferentially involved in intrahelical ionic interactions which would decrease the pK of the Glu side chains and increase the pK of the Lys side chains (Merutka & Stellwagen, 1991). However, the decrease in helicity corresponding to the protonation of Glu and the deprotonation of Lys is not identical (Figure 4C). The smaller decrease in helicity caused by protonation of the Glu side chain can be interpreted as being due to the compensation effect of the higher intrinsic helical propensity of the protonated or neutral carboxyl group compared to the ionized carboxyl group (Wojcik et al., 1990). It is interesting to note that the helicity and stability of peptide AA9 are independent of pH in the presence of TFE (Figures 2C and 4C). This suggests that the stabilizing effect of TFE on the helix is not a consequence of its smaller dielectric

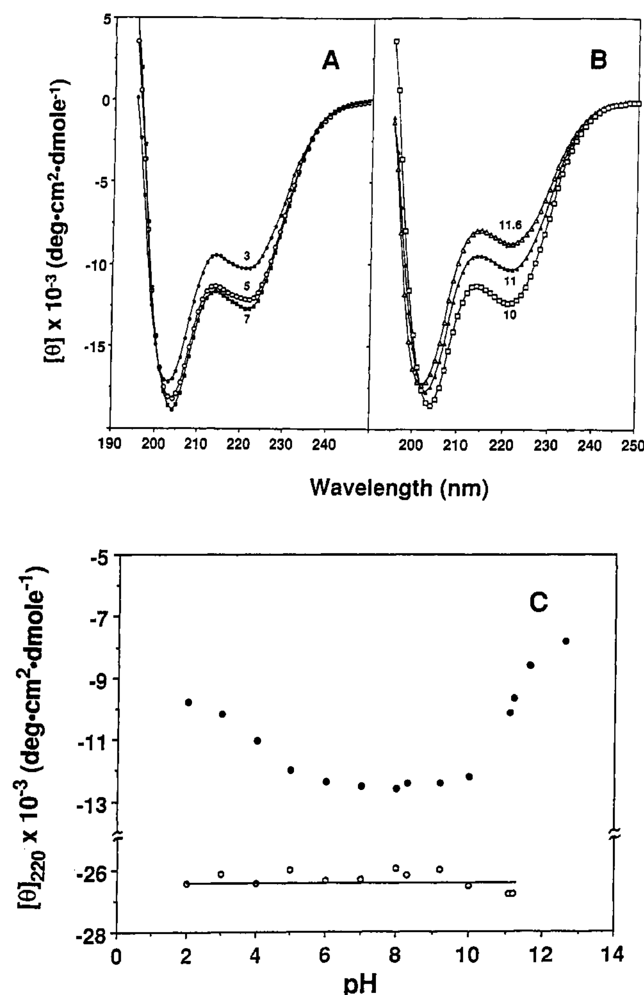

FIGURE 4: pH dependence of the ellipticities of peptide AA9. All measurements were performed in 0.1 M KCl/50 mM PO_4 buffer at 20 °C. Panel A: CD spectra of peptide AA9 at pH 3 (●), 5 (○), and 7 (■). Panel B: CD spectra of peptide AA9 at pH 10 (□), 11 (▲), and 11.6 (△). Panel C: Plot of the mean residue molar ellipticity at 220 nm vs pH for peptide AA9 in 0.1 M KCl/50 mM PO_4 buffer (closed circles) and in 0.1 M KCl/50 mM PO_4 buffer containing 30% TFE (v/v) (open circles).

constant which should enhance ionic interactions between charged groups compared to water. Previous studies (Llinas & Klein, 1975; Nelson & Kallenbach, 1986) have demonstrated that the stabilizing effect of TFE is predominantly caused by its weaker basicity which can decrease hydrogen bonding of amide protons to the solvent and consequently strengthen intramolecular hydrogen bonds. The pH independence in TFE may simply be a consequence of stabilizing hydrogen bonding between the side chains of Lys and Glu. Furthermore, TFE, due to its increased hydrophobicity relative to water, disrupts intermolecular hydrophobic interactions and can act as a denaturant of tertiary and quaternary structure (Lau et al., 1984b). Our recent results (Zhou et al., unpublished data) show that the more hydrophobic the nonpolar face of the amphipathic α-helix is, the more stable the α-helix is in TFE solutions. This implies that increased stability of the single-stranded helix upon addition of TFE is a result of enhancing hydrophobic interactions between peptide and solvent molecules. It has been estimated recently that hydrogen bonding and hydrophobic interactions are the two most important stabilizing forces in the folded globular protein (Pace, 1992; Shirley et al., 1992). These two stabilizing forces, which may also stabilize a single-stranded helix (Scholtz et al., 1991), could be enhanced to outweigh the relative smaller

Table I: Proton Chemical Shifts (ppm) of Peptide AA9[a]

residue	NH	$C_\alpha H$	$C_\beta H$	$C_\gamma H$	$C_\delta H$	$C_\epsilon H$
Glu-1	8.46	4.15	2.05, 2.13	2.42		
Ala-2	8.43	4.19	1.48			
Glu-3	8.29	4.11	2.16, 2.09	2.38		
Lys-4	7.84	4.05	1.97	1.76	1.58, 1.46	3.01
Ala-5	8.14	4.16	1.54			
Ala-6	8.09	4.19	1.57			
Lys-7	8.11	4.15	2.09, 1.96	1.76	1.58	3.01
Glu-8	8.35	4.14	2.23	2.43, 2.51		
Ala-9	8.25	4.21	1.58			
Glu-10	8.12	4.06	2.24	2.50		
Lys-11	8.00	4.01	2.02	1.63	1.47	3.01
Ala-12	8.19	4.17	1.57			
Ala-13	8.12	4.16	1.55			
Lys-14	8.11	4.15	2.05, 1.97	1.76	1.62, 1.46	3.01
Glu-15	8.22	4.15	2.18	2.41, 2.51		
Ala-16	8.00	4.23	1.56			
Glu-17	7.87	4.20	2.20	2.41, 2.53		
Lys-18	7.76	4.25	1.94	1.76	1.63, 1.54	3.01
NH_2-19	7.30					
	6.98					

[a] In 50% TFE-d_3 and 50% H_2O containing 0.1 M KCl/50 mM phosphate, pH 5.2, at 25 °C.

FIGURE 5: Summary of the sequential and medium-range NOEs observed for peptide AA9. Solid lines indicate observed NOEs, dashed lines indicate ambiguous NOEs due to spectral overlap, and widths of the lines indicate the intensities of the NOEs.

contributions of ionic interactions in the presence of TFE.

Helical Structure Determined by ^1H-NMR. The assignments of amide proton, α-CH proton, and other proton chemical shifts were achieved by using the standard sequential assignment (Wüthrich, 1986) and main-chain-directed approach (Englander & Wand, 1987) on the basis of 2D-NMR spectra (DQF-COSY and NOESY). The proton chemical shifts of peptide AA9 are given in Table I. The NOESY spectrum of peptide AA9 showed all the patterns that are characteristic of an α-helical conformation. In an ideal α-helix, $d_{NN}(i,i+1)$ and $d_{\alpha N}(i,i+1)$ distances are 2.8 and 3.5 Å, respectively. These distances are 4.3 and 2.2 Å, respectively, in an extended chain (Billeter et al., 1982). Therefore, the $d_{NN}(i,i+1)$ cross-peak should be much more intense than the $d_{\alpha N}(i,i+1)$ cross-peak in an α-helical structure (Bradley et al., 1990). Starting with Lys-2 and extending up to Lys-18, strong NOEs corresponding to distances d_{NN} between most of the adjacent amide protons and weaker sequential connectivities $d_{\alpha N}(i,i+1)$ were observed (Figure 5). In addition, the presence of both medium-range NOEs of $d_{\alpha N}(i,i+3)$ and $d_{\alpha\beta}(i,i+3)$ (Figure 5) is another indication of helix formation (Wüthrich, 1984). It is well-known that the α-proton chemical shifts of amino acid residues tend to upfield values (relative to the random coil value) in an α-helical structure (Pastore & Sandek, 1990; Wishart et al., 1991). A plot of the difference between the chemical shifts of the α-protons and those in a random coil of the same sequence versus the position along

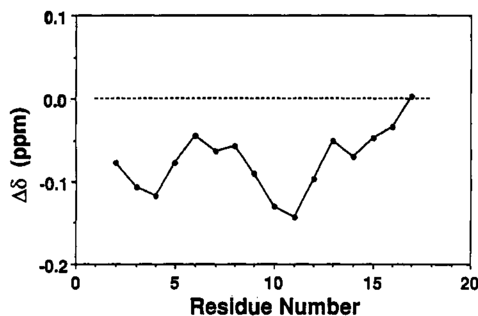

FIGURE 6: Plot of the difference between the observed chemical shifts of CαH protons of peptide AA9 and random coil values ($\Delta\delta = \delta_{obs} - \delta_{random\ coil}$ versus the position along the sequence. The random coil values were taken from Wishart et al. (1991).

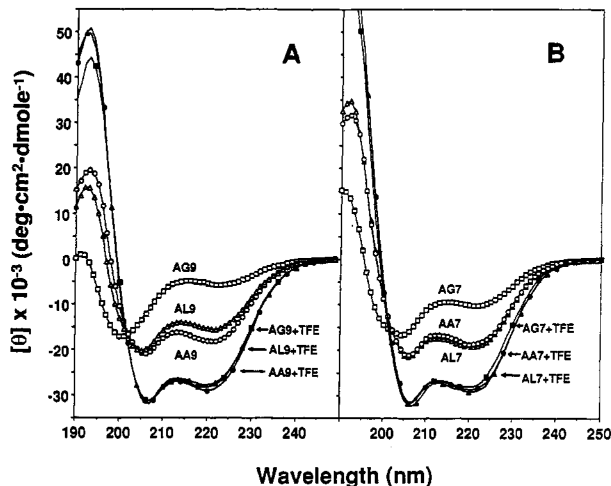

FIGURE 7: Panel A: CD spectra of peptides AA9 (circles), AL9 (triangles), and AG9 (squares) in 0.1 M KCl/50 mM PO_4 buffer, pH 7, at 5 °C in the absence (open symbols) and presence (closed symbols) of 50% TFE (v/v). Panel B: CD spectra of peptides AA7 (circles), AL7 (triangles), and AG7 (squares) in 0.1 M KCl/50 mM PO_4 buffer, pH 7, at 5 °C in the absence (open symbols) and presence (closed symbols) of 50% TFE (v/v).

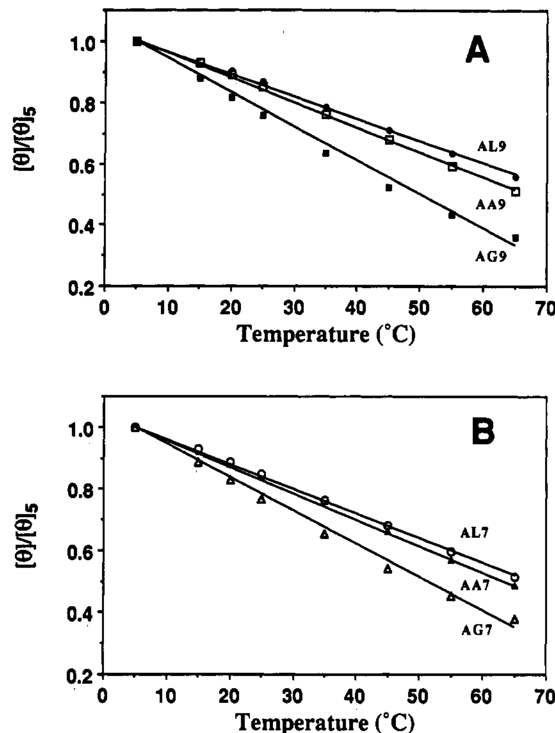

FIGURE 8: Thermal melting profiles of AA9 (□), AL9 (●), AG9 (■), AA7 (▲), AL7 (○), and AG7 (△) in 0.1 M KCl/50 mM PO_4 buffer containing 30% TFE (v/v), pH 7. $[\theta]/[\theta]_5$ represents the ratio of the ellipticity at 220 nm at the indicated temperature to the ellipticity at 5 °C.

the peptide sequence (Figure 6) is a simple and effective way to relate chemical shifts to features of secondary structure (Pastore & Sandek, 1990; Pastore et al., 1991). The curve is smoothed by averaging the value at each point with its ±1 neighbors to average out local effects (Pastore & Sandek, 1990). When the criteria for secondary structure determination based on chemical shifts (Wishart et al., 1991) were applied the negative values for adjacent α-CH protons from residue Lys-2 to Ala-16 indicate α-helix formation through the majority of the sequence. This observation is in agreement with the CD data.

Dependence of Helical Propensity on the Position of Substitution in the α-Helix. In order to investigate the positional effect of helical propensity in an amphipathic helix, a leucine, alanine, or glycine was individually placed in the hydrophobic face (position 9) and hydrophilic face (position 7) of peptide AA9 (Figure 1). The change in helix content and stability upon substitution was measured by circular dichroism studies in the absence and presence of TFE or urea. As shown in Figure 7, the same substitution (Ala → Leu or Ala → Gly) at two different positions on the α-helix has different effects on helical content. In benign medium, the decrease in ellipticity at 220 nm (in deg·cm²·dmol⁻¹) caused by the substitution of Ala → Leu or Ala → Gly in the hydrophobic face was 2700 (−15 570 + 18 270) or 12 510 (−5760 + 18 270), respectively, while the corresponding value in the hydrophilic face was only −520 (−19 330 + 18 810) or 8470 (−10 340 + 18 810) (Table II). These results suggest that substitutions in the hydrophobic face have a larger effect on helical propensity compared to substitutions in the hydrophilic face. However, in the presence of TFE, all these peptides showed essentially the same CD spectra with an average ellipticity of −28 600 deg·cm²·dmol⁻¹ (range from −27 960 to −29 140 deg·cm²·dmol⁻¹) at 220 nm (Figure 7). TFE, known to promote helix formation in peptides with helical propensity (Sönnichsen et al., 1992; Lehrmann et al., 1990), was used to obtain the value of [θ] for maximal helix formation (Marqusee et al., 1989). The TFE titration of peptide AA9 showed that helix formation is increased as TFE concentration increased and reached the maximal value ($[\theta]_{220} = -29\,140$ deg·cm²·dmol⁻¹) at 30% (v/v) TFE. Further increase in TFE concentration did not change the α-helical content (data not shown). Moreover, because all these peptides showed essentially the same value of $[\theta]_{220}$ in 50% TFE, the different values of $[\theta]_{220}$ in the absence of TFE must reflect true differences in helicity. Though all the peptides have essentially the same α-helical content in the presence of TFE at 5 °C (Figure 7), differences in helical propensity are observed in TFE as the temperature is increased (Figure 8).

The best comparison for the positional effect on helical propensity is to examine the changes in free energy corresponding to the same substitution at different positions. The free energy (ΔG) of helix formation for the peptides can be calculated by assuming the helix–coil transition is a totally cooperative two-state transition (Merutka & Stellwagen, 1990; Merutka et al., 1990) accoding to the equation: $\Delta G = -RT \ln[([\theta] - [\theta]_C)/([\theta]_H - [\theta])]$, where $[\theta]$ is the observed ellipticity and $[\theta]_H$ and $[\theta]_C$ are the ellpiticities of the completely helical and random coil forms of the peptide, respectively. $[\theta]_H$ was obtained from TFE titration (Marqusee & Baldwin, 1987; Marqusee et al., 1989; Padmanabhan et al., 1990), and the value of $[\theta]_{220}$ in 50% TFE was taken to represent the formation

Table II: Comparison of Helix Formation Parameters for the Substitution of Ala, Leu, or Gly in the Hydrophobic and Hydrophilic Face of an Amphipathic α-Helix

amino acid	hydrophobic face					hydrophilic face						
	$[\theta]_{220}$[a]	f_H[b]	ΔG_f[c]	ΔG_u[d]	$\Delta\Delta G_{(X-G)}$[e,h]	$[\Theta]_{222}$[a]	f_H[b]	ΔG_f[c]	ΔG_u[d]	$\Delta\Delta G_{(X-G)}$[e,h]	$\Delta\Delta G$[f,h]	$\Delta\Delta G$[g,h]
Ala	−18270	0.65	−0.33	−0.35	−0.96	−18810	0.67	−0.40	−0.39	−0.64	−0.77	−0.52
Leu	−15570	0.58	−0.18	−0.21	−0.81	−19330	0.68	−0.41	−0.41	−0.65	−0.62	−0.40
Gly	−5760	0.24	0.63	−0.60	0	−10340	0.40	0.22	0.25	0		

[a] Mean residue ellipticity (deg·cm²·dmol⁻¹) of the peptide at 220 nm was measured at 5 °C in 0.1 M KCl/50 mM PO_4 buffer, pH 7. [b] Fraction of helix was calculated from the equation $f_H = ([\theta] - [\theta]_C)/([\theta]_H - [\Theta]_C)$, where $[\theta]_C$ and $[\Theta]_H$ are the ellipticities corresponding to random coil and 100% helix, respectively. The value of $[\Theta]_C$ (1500 deg·cm²·dmol⁻¹) was determined by averaging the ellipticities of the peptides in the presence of 8 M urea (Figure 9A). The ellipticity in the presence of 50% TFE was used as the value of $[\theta]_H$. See the text for details. [c] The free energy (kcal/mol) of helix formation was calculated from the equation of $\Delta G_f = -RT \ln[f_H/(1 - f_H)]$ on the basis of an all-or-none two-state model (Lyu et al., 1990). The uncertainty for ΔG is ±0.05 kcal/mol. [d] The free energy (kcal/mol) of helix formation was determined from urea denaturation experiments and was estimated by extrapolating the free energy at each individual concentration of urea to zero concentration assuming a linear relationship. The uncertainty for ΔG is ±0.05 kcal/mol. [e] The free energy (kcal/mol) of helix formation relative to Gly calculated by subtracting ΔG for each peptide from ΔG for the corresponding Gly peptide. For example: $\Delta\Delta G(A-G) = \Delta G_f(A) - \Delta G_f(G) = -0.33 - 0.63 = -0.96$ kcal/mol. The $\Delta\Delta G_{(X-G)}$ values calculated from helical content and urea denaturation are essentially identical, and the average value is shown. [f] The free energy (kcal/mol) of helix formation relative to Gly determined by O'Neil and DeGrado (1990) on a synthetic α-helical coiled-coil. [g] The free energy (kcal/mol) of helix formation relative to Gly determined by Lyu et al. (1990) on a single-stranded nonamphipathic helix. [h] The $\Delta\Delta G_{(X-G)}$ values were calculated on the basis of a two-state model for this study, O'Neil and DeGrado (1990) and Lyu et al. (1990), which allows direct comparison.

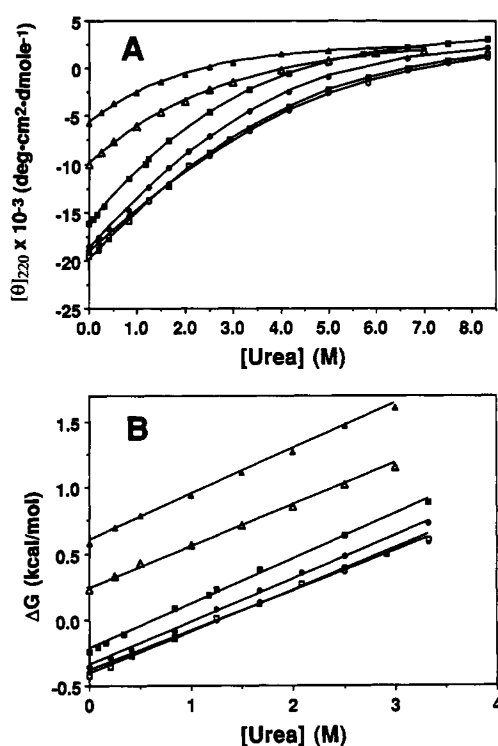

FIGURE 9: Panel A: Urea concentration dependence of the mean residue molar ellipticities at 220 nm for peptides AA9 (●), AL9 (■), AG9 (▲), AA7 (○), AL7 (□), and AG7 (△) in 0.1 M KCl/50 mM PO_4 buffer, pH 7, at 5 °C. Panel B: Corresponding plots of the free energy of helix formation (ΔG) versus concentrations of urea. Calculations were made based on a two-state model (C ⇌ H) as described in the text. The symbols are the same as in panel A.

of 100% α-helix in these peptides. The $[\theta]_C$ value was determined by urea denaturation of these peptides (Figure 9A), and the average value of 1500 deg·cm²·dmol⁻¹ in 8 M urea was used for $[\theta]_C$. In addition, the free energy for helix formation was also estimated from the urea denaturation experiments by extrapolating the free energy at each individual concentration of urea to zero concentration (Figure 9B). Similar values of the free energy were obtained for both methods (Table II). The helix propensity of each amino acid was expressed as the difference in the free energy of helix formation for each amino acid relative to Gly [$\Delta\Delta G_{(X-G)}$] for comparison with values determined by other researchers using different systems (Table II) (O'Neil & DeGrado, 1990; Lyu et al., 1990). As shown in Table II, the $\Delta\Delta G$ values of Ala and Leu determined on the hydrophilic face (position 7) are essentially identical. However, larger and different $\Delta\Delta G$ values for Ala and Leu were observed in the hydrophobic face compared to those in the hydrophilic face, implying each amino acid has a different helix propensity when it is located in the hydrophobic versus the hydrophilic face and the effect of substitution is more significant in the hydrophobic face. The values obtained on the hydrophilic face in this model system are close to those values obtained by substituting amino acids either at a solvent-exposed position in the coiled-coil (O'Neil & DeGrado, 1990) or in a highly polar single-stranded helical peptide (succinyl-YSEEEEKKKKXXXEEEEKKKK-NH_2) (Lyu et al., 1990). Differences in helical propensity of amino acid side chains have also been shown to vary with the distance from the end of the α-helix (Chakrabartty et al., 1991). These authors demonstrated that the effect of an Ala → Gly substitution in a 17-residue nonamphipathic α-helical peptide was strongly dependent upon the position of substitution, that is, the largest decrease in α-helical content was produced in the center of the helix, and substitutions close to either end had comparatively small effects. However, only a small difference was observed between two closely related positions in the α-helix (~22% helical content at position 9 and ~27% helical content at position 7 were predicted by the Lifson–Roig equation; Chakrabartty et al., 1991). This effect would not account for the differences observed in this study (24% for position 9 and 40% for position 7, Table II). In conclusion, the results from this study suggest that it is important to determine helix propensities of the 20 commonly occurring amino acids on both hydrophobic and hydrophilic faces of a single-stranded amphipathic α-helix and that this amphipathic α-helical peptide is an appropriate model system to determine these values.

ACKNOWLEDGMENT

We thank Paul D. Semchuk and Bob Luty for their skilled technical assistance in synthesis and purification of peptides and performing the circular dichroism measurements.

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