Distribution of Helicity within the Model Peptide Acetyl(AAQAA)$_3$amide

William Shalongo, Laxmichand Dugad, and Earle Stellwagen*

Contribution from the Department of Biochemistry, University of Iowa, Iowa City, Iowa 52242

Received March 21, 1994®

Abstract: A series of peptides having the sequence acetyl(AAQAA)$_3$amide was synthesized each containing a different residue having its carbonyl carbon enriched in ^{13}C. The resonance of each enriched carbonyl carbon was examined in aqueous solvents containing different concentrations of D_2O, methanol, salt, and acid at a variety of temperatures. The thermal dependence of the carbonyl carbon chemical shift of each residue was analyzed in terms of a two-state helix/coil transition. The line shape observed for the carbonyl carbon of each residue in a D_2O/H_2O mixture was analyzed in terms of the backbone hydrogen bonding content of the amide group of the adjacent residue at equilibrium. The collective results indicate that the N-terminal region of the peptide is more helical than predicted by the traditional Lifson–Roig statistical mechanical model. The observed residue distribution of helical content can be simulated at 0 °C in water using a modified form of the Lifson–Roig model, an acetyl interaction of −1.22 kcal/mol, and propagation, s, values for the alanine and glutamine residues of 1.47 and 0.50, respectively. These values are very comparable with those obtained (Chakrabartty, A.; Kortemme, T.; Baldwin, R. L. *Protein Sci.* **1994**, *3*, 843–852) by analysis of isothermal mean residue ellipticity measurements of a series of host/guest peptides using an alternative modified Lifson–Roig model.

Introduction

The Lifson–Roig statistical mechanical model[1] has been used to obtain helix propagation values for the component residues in a variety of relatively short soluble peptides of defined sequence.[2] Utilization of this model assumes the absence of intrapeptide interactions other than backbone hydrogen bonds which would preferentially stabilize the helical conformations of the peptides considered. Recent efforts have been made to evaluate this model by measuring the helical content of individual residues in peptides of defined sequence using NMR, ESR, and circular dichroic methodology.[3] One such method[3e] involves measurement of the chemical shifts of backbone carbonyl carbon resonances of the component residues in a peptide. This methodology was developed using a peptide having the potential for significant complementary electrostatic interactions, side-chain $i,i+4$ ion-pairs and side-chain/helix macrodipole interactions, which preferentially stabilize the helical conformation. In this report, the residue helical content of a peptide sequence devoid of such interactions, acetyl-(AAQAA)$_3$amide, is considered. The carbonyl carbon resonance measurements indicate that the N-terminal sequence of this peptide is more helical than predicted by the Lifson–Roig model.

Experimental Section

Peptide Samples. All peptides were prepared by a multiple peptide synthesis procedure described previously.[4] Enriched peptides contained a single residue having its carbonyl carbon selectively enriched 20% in ^{13}C using either L-alanine-*N*-*t*-BOC (1-^{13}C,99%) or L-glutamine-*N*-*t*-BOC (1,2-^{13}C,99%) purchased from Cambridge Isotope Laboratories, Inc. Each peptide preparation was purified by reversed-phase high-performance liquid chromatography using a 10 × 250 mm preparative Vydac C_{18} column, an Isco chromatograph, and a 10%–25% acetonitrile gradient in 0.1% trifluoroacetic acid generated in 30 min at a flow rate of 5 mL/min. Each purified enriched peptide contained a single component when examined using a 4.1 × 250 mm analytical Vydac C_{18} column and yielded only two amino acids by acid hydrolysis, alanine and glutamate, the latter being the acid hydrolysis product of glutamine. All the purified enriched peptides exhibited a common chromatographic elution time, a common far ultraviolet circular dichroic spectrum, when examined using standard conditions, a mean alanine/glutamate molar ratio of 4.03(0.02),[5] and a mass/charge ratio for the main molecular ion observed by FAB-MS which is within 1 mass unit of that expected for the singly charged monomeric peptide. No material was detected at the mass/charge ratio anticipated for the dimeric form of the peptide.

Circular Dichroic Measurements. Circular dichroic spectra were obtained between 200 and 260 nm using an Aviv Model 62DS spectrometer, which was standardized as described previously.[2d] The observed ellipticity, Θ, was converted to mean residue ellipticity, [Θ], assuming that peptides acetyl-(AAQAA)$_3$amide and acetyl(AAQAA)$_3$Yamide[6] have a common apparent isodichroic value at 202 nm, −15 900 deg cm^2 (dmol res)$^{-1}$. Previous measurements have demonstrated that the aromaticity of a terminal residue does not contribute significantly to the mean residue ellipticity observed at this wavelength.[7]

NMR Measurements. Proton-decoupled carbon spectra were recorded on a Bruker AMX-600 spectrometer operated at 150.9 MHz located in the University of Iowa High Field NMR Facility. The temperature of the sample in the broad band NMR probe was maintained by the Bruker variable temperature unit, with cooling supplied by a FTS systems XRII-851 cooler. The

* Abstract published in *Advance ACS Abstracts,* August 1, 1994.

(1) Lifson, S.; Roig, A. *J. Chem. Phys.* **1961**, *34*, 1963–1974.

(2) (a) Chakrabartty, A.; Schellman, J. A.; Baldwin, R. L. *Nature* **1990**, *351*, 586–588. (b) Scholtz, J. M.; Qian, H.; York, E. J.; Stewart, J. M.; Baldwin, R. L. *Biopolymers* **1991**, *31*, 1463–1470. (c) Rohl, C. A.; Scholtz, J. M.; York, E. J.; Stewart, J. M.; Baldwin, R. L. *Biochemistry* **1992**, *31*, 1263–1269. (d) Park, S.-H.; Shalongo, W.; Stellwagen, E. *Biochemistry* **1993**, *32*, 7048–7053; 12901–12905. (e) Sholtz, J. M.; Qian, H.; Robbins, V. H.; Baldwin, R. L. *Biochemistry* **1993**, *32*, 9668–9676.

(3) (a) Lyu, P. C.; Liff, M. I.; Marky, L. A.; Kallenbach, N. R. *Science* **1990**, *250*, 669–673. (b) Mlick, S. M.; Casteel, K. M.; Milhauser, G. L. *Biochemistry* **1993**, *32*, 8014–8021. (c) Chakrabartty, A.; Doig, A. J.; Baldwin, R. L. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 11332–11336. (d) Zhou, H. X.; Lyu, P. C.; Wemmer, D. E.; Kallenbach, N. R. *J. Am. Chem. Soc.* **1994**, *116*, 1139–1140. (e) Shalongo, W.; Dugad, L.; Stellwagen, E. *J. Am. Chem. Soc.* **1994**, *116*, 2500–2507.

(4) Houghten, R. A.; DeGraw, S. T.; Bray, M. K.; Hoffman, S. R.; Frizzell, N. D. *BioTechniques* **1986**, *4*, 522–528.

(5) A number enclosed in parentheses represents one standard deviation about the mean value immediately preceding it.

(6) Scholtz, J. M.; York, E. J.; Stewart, J. M.; Baldwin, R. L. *J. Am. Chem. Soc.* **1991**, *113*, 5102–5104.

(7) Chakrabartty, A.; Kortemme, T.; Padmanabhan, S.; Baldwin, R. L. *Biochemistry* **1993**, *32*, 5560–5565.

0002-7863/94/1516-8288$04.50/0

temperature of samples within the probe was calibrated using a Physitemp BAT-12 thermocouple and using a methanol NMR thermometer.[8] NMR measurements of peptide solutions in D_2O could be obtained down to at least –4 °C, owing to the slow kinetics of freezing of this solvent.

Between 400 and 6000 scans of each sample were obtained using 16K data points, a spectral width of 38 100 Hz, a relaxation delay time of 1 s, and a 30–35° observation pulse having a 5–6-μs duration. Each FID was zero filled to 32K and subjected to an exponential line-broadening term of 2–4 Hz prior to conversion into an absorption spectrum. All chemical shifts are expressed in parts per million relative to the methyl carbon resonance of 3-(trimethylsilyl)propionate sodium salt in D_2O as the reference. Neat tetramethylsilane, neat methanol, and dioxane in D_2O exhibited resonances at 2.27, 51.34, and 68.92 ppm, respectively, relative to the reference.

Samples for carbonyl carbon chemical shift measurements contained about 3 mM enriched peptide in 99% D_2O adjusted to a pH meter reading[9] of 7.0, unless noted otherwise. Samples for amide protection measurements contained an approximate equivolume mixture of D_2O and H_2O. The exact ratio of these components was established by area analysis of the observed spectra. The pH of samples for amide exchange measurements was adjusted to a value within the range 8.68–8.86 immediately preceding NMR data collection.

Data Analysis. The thermal dependence of the carbonyl carbon chemical shift of each residue in peptide acetyl(AAQAA)$_3$amide was fit to a two-state helix/coil equilibrium in fast exchange, eq 1, using the Marquardt–Levenberg algorithm[10] for nonlinear regression, and the results were expressed as percent helicity using eq 2. In this equation, δ, δ_α, and δ_c are the observed chemical

$$\log K_{eq} = \log([\text{coil}]/[\text{helix}]) = \log[(\delta_\alpha - \delta)/(\delta - \delta_c)]_T = 2(T - T_m)/\Delta T \quad (1)$$

$$\%\ \text{helicity} = 100[(\delta - \delta_c)/(\delta_\alpha - \delta_c)]_T \quad (2)$$

shift, the chemical shift of the helix conformation, and the chemical shift of the coil conformation, respectively, at temperature T; T_m is the midpoint of the thermal transition when $\log K_{eq} = 0$; and ΔT is the width of the thermal transition, defined here as the temperature range spanning the central 80% of the transition, $\log K_{eq} = +1$ to $\log K_{eq} = -1$. The last equality in eq 1 is an empirical relationship based on an assumed linear dependenc of $\log K_{eq}$ on temperature for which the slope is $2/\Delta T$ and the intercept is $-2T_m/\Delta T$.

The chemical shift of all alanine and glutamine residues in the helix conformation was assumed to have a thermal dependence of 0.0106 ppm deg^{-1}, the value measured for the central residues in the helix conformation of peptide acetylW(EAAAR)$_3$Aamide.[3e] The chemical shift of all alanine residues in the coil conformation was assumed to have a thermal dependence of 0.0033 ppm deg^{-1}, the value measured for alanine 15 in peptide acetyl(AAQAA)$_3$-amide. The chemical shift of all glutamine residues in the coil conformation was assumed to have a thermal dependence of 0.0099 ppm deg^{-1}, the value measured for the glutamate residues in the coil conformation of peptide acetylW(EAAAR)$_3$Aamide.[3e] The presence of methanol was assumed to change the chemical shift of the carbonyl carbon resonances of all residues in the helix conformation by –0.0054 ppm (% methanol)$^{-1}$, the mean value measured at 0 °C and neutral pH for the carbonyl carbon resonances of all the central alanine residues in the helix conformation of peptide acetylW(EAAAR)$_3$Aamide. The presence of methanol was assumed to change the chemical shift of the carbonyl carbon resonances of all residues in the coil conformation by –0.0151 ppm (% methanol)$^{-1}$, the mean value measured at 0 °C and neutral pH for the carbonyl carbon resonance of all the residues in the unstructured peptide acetylARAamide.

The thermodynamic parameters associated with the two-state helix/coil transition of each residue were obtained by fitting the thermal dependence observed for each carbonyl carbon resonance using eqs 3–5. In these equations, ΔH_o and ΔS_o are the change

$$\Delta G = -RT \ln K_{eq} = \Delta H_o - T\Delta S_o + \Delta C_p[T - T_o - T\ln(T/T_o)] \quad (3)$$

$$\Delta H = \Delta H_o + \Delta C_p(T - T_o) \quad (4)$$

$$\Delta S = \Delta S_o + \Delta C_p \ln(T/T_o) \quad (5)$$

in enthalpy and the change in entropy at temperature T_o.

The effect of an amide hydrogen/deuterium exchange rate on the line shape of the ^{13}C resonance of its adjacent carbonyl is described[11] by eq 6. In this equation, $I(\delta)$ is the intensity at each

$$I(\delta) = -A_o[P + \pi P\tau(\rho_H W_D + \rho_D W_H) + QR][P^2 + R^2]^{-1} \quad (6)$$

$$P = \pi^2\tau(W_D W_H - 4S^2 + \Delta\delta^2) + \pi(\rho_D W_D + \rho_H W_H) \quad (7)$$

$$Q = \pi\tau[2S - \Delta\delta(\rho_D - \rho_H)] \quad (8)$$

$$R = 2\pi S[1 + \pi\tau(W_D + W_H)] + \pi^2\Delta\delta\tau(W_H - W_D) + \pi\Delta\delta(\rho_D - \rho_H) \quad (9)$$

$$S = 0.5(\delta_H + \delta_D) - \delta \quad (10)$$

chemical shift; A_o is an arbitrary factor which normalizes the areas of the simulated and observed spectra; δ, k, ρ, and W are the chemical shift, exchange rate, fractional area, and line width at half-height, respectively; H and D denote the solvents H_2O and D_2O, respectively; $\Delta\delta = \delta_D - \delta_H$; $\tau = \rho_D/k_H = \rho_H/k_D$; and P, Q, R, and S are defined by eqs 7–10, respectively. The retardation of an amide exchange rate of a given amide in the model peptide compared with the amide exchange rate of the same amide in an unstructured dipeptide of the same sequence measured in the same solvent[12] can be expressed as a protection factor, PF, as defined in eq 11. If a peptide amide is only protected from exchange by participation in a backbone hydrogen bond, the protection factor can be related to the fraction of the amide which is hydrogen bonded at equilibrium using eq 12 assuming exchange is rapid. These two equations are valid for a two-state

$$\text{PF} = k_{\text{dipeptide}}/k_{\text{model peptide}} \quad (11)$$

$$\%\ \text{hydrogen bonded} = 100(\text{PF} - 1)/\text{PF} \quad (12)$$

equilibrium at a constant temperature.

The traditional Lifson–Roig statistical mechanical model[1] for prediction and analysis of peptide helical content has been modified in two ways. The first modification extends the partition function of helical states by counting as helical the derivative of the (ln v) states as well as the derivative of the (ln w) states. This modification produces a nonzero helix probability at each terminus and has also been recently incorporated into an alternative[13] modification of the traditional Lifson–Roig model. The second

(8) Van Geet, A. L. *Anal. Chem.* **1970**, *42*, 679–680.
(9) The pH meter was standardized using buffers from VWR Scientific with pH values of 4.01, 7.12, and/or 10.25 at 0 °C. All pH values reflect uncorrected meter readings.
(10) Marquardt, D. W. *J. Soc. Ind. Appl. Math.* **1963**, *11*, 431–441.
(11) Rogers, M. T.; Woodbrey, J. C. *J. Phys. Chem.* **1962**, *66*, 540–546.
(12) (a) Bai, Y.; Milne, J. S.; Mayne, L.; Englander, S. W. *Proteins: Struct., Funct., Genet.* **1993**, *17*, 75–86. (b) Connelly, G. P.; Bai, Y.; Jeng, M.-F.; Englander, S. W. *Ibid*, 87–92.
(13) Chakrabartty, A.; Kortemme, T.; Baldwin, R. L. *Protein Sci.* **1994**, *3*, 843–852.

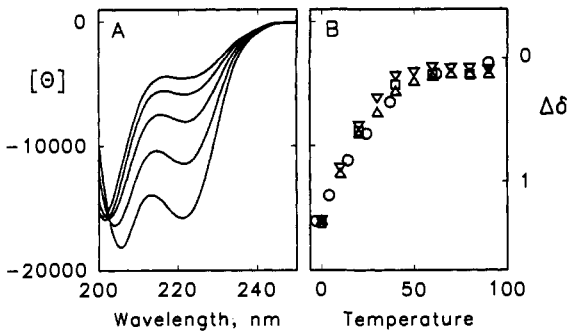

Figure 1. Circular dichroic measurements. The mean residue ellipticity, [Θ], has the units deg cm^2 (dmol res)$^{-1}$. Panel A illustrates the thermal dependence of the dichroic spectrum of peptide acetyl(AAQAA)$_3$amide in water at pH 7. The spectra were obtained, at 0, 10, 20, 30, and 40 °C, reading upward. Panel B illustrates the effect of peptide concentration on the observed thermal transitions. The upright and inverted triangles indicate the thermal dependence of the mean residue ellipticity for peptide acetyl(AAQAA)$_3$amide measured at 222 nm in water at peptide concentrations of 0.023 and 0.23 mM, respectively. The circles indicate the thermal dependence of the mean change in chemical shift, $\delta - \delta_c$, measured in water at a peptide concentration of 3 mM. For purposes of comparison, the squares indicate the thermal dependence of the mean residue ellipticity of peptide acetyl(AAQAA)$_3$Yamide measured[6] at 222 nm in 0.1 M KF.

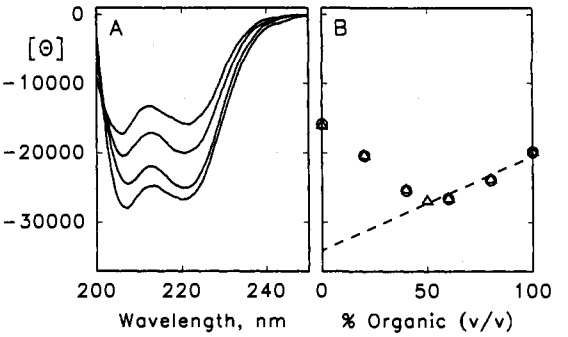

Figure 2. Effect of mixed solvents on the dichroic properties of peptide acetyl(AAQAA)$_3$amide at 0 °C and pH 7. Panel A illustrates the effect of methanol on the circular dichroic spectrum of the peptide. The spectra were obtained in 0%, 20%, 40%, and 60% (v/v) methanol, reading downward. Panel B compares the effects of methanol, circles, and trifluoroethanol, triangles, on the mean residue ellipticity of the peptide at 222 nm having the units deg cm^2 (dmol res)$^{-1}$.

modification includes contributions from defined specific side-chain/side-chain and side-chain/backbone interactions expressed as a free energy contribution. Both modifications have been incorporated into a menu-driven FORTRAN program which calculates the helical content for each residue given a peptide sequence, σ and s parameters for each residue, the free energy for each interaction, and the residues participating in each interaction. Values for these parameters can be refined using the Brent algorithm[14] to fit either the helical content measured for each residue in a peptide or the mean residue helical content for the entire peptide.

Results

Circular Dichroic Measurements. The effect of temperature on the far ultraviolet dichroic spectrum of the unenriched peptide acetyl(AAQAA)$_3$amide in water at pH 7 is illustrated in Figure 1A. The dichroic spectrum at 0 °C exhibits negative minima at 206 and 221 nm, characteristic of a peptide having a significant content of helical residues. As the temperature of the peptide solution is raised, these minima gradually disappear, generating a rather featureless dichroic spectrum characteristic of a peptide having a statistical random coil as its dominant conformation. The spectra obtained at temperatures between 0 and 30 °C, inclusively, appear to exhibit an isodichroic point at 202 nm, characteristic for a two-state helix/coil transition. This apparent isodichroic point is lost at temperatures greater than 30 °C, due to the thermal dependence of the dichroic spectrum of the randomly coiled conformation of the peptide which predominates in this temperature range.

The mean residue ellipticity at 222 nm exhibits a curvilinear dependence on temperature, as illustrated by the triangles in Figure 1B. This thermal dependence is comparable with that reported[6] for the related peptide acetyl(AAQAA)$_3$Yamide, illustrated by the squares in Figure 1B. The mean residue ellipticity of peptide acetyl(AAQAA)$_3$amide appears to reach a limiting value of about −3400 deg cm^2 (dmol res)$^{-1}$, a value characteristic for randomly coiled peptides at elevated temperatures.[2b,15]

The mean residue ellipticity for the maximal accessible helix conformation was experimentally determined by addition of an organic solvent, trifluoroethanol or methanol,[16] to solutions of the peptide at 0 °C, as illustrated in Figure 2. Addition of an organic solvent first decreases the mean residue ellipticity and then increases it, as shown explicitly in Figure 2B. The decrease in ellipticity likely reflects the preferential stabilization of the helical conformation, while the increase in ellipticity likely reflects the lyotropic effect of the organic solvent. The linear extrapolation indicated by the dashed line in Figure 2B predicts that the helix conformation of the peptide has a mean residue ellipticity, $[\Theta]_\alpha$, of −34 000 deg cm^2 (dmol res)$^{-1}$ at 222 nm in water at 0 °C and neutral pH. This value is appropriate[17] for a 15-residue peptide in an α-helical conformation.

Chemical Shift Measurements. Each peptide containing a single enriched alanine residue exhibits a dominant singlet in the carbonyl carbon region of the ^{13}C NMR spectrum. By contrast, each peptide containing a single enriched glutamine residue exhibits a dominant doublet in this region, owing to the coupling between the carbonyl carbon and the α-carbon, which are equally enriched in the starting material. The chemical shifts of the carbonyl carbon resonances of the glutamine residues reported here represent the average of the chemical shifts of the individual components in each doublet, which are separated by 0.35(0.02) ppm.

The thermal dependence of the carbonyl carbon chemical shift of representative enriched alanine residues is illustrated in Figure 3A. The chemical shift of alanine 9 decreases with increasing temperature, as expected for a central residue in a peptide helix.[3e,18] The thermal dependence of the chemical shifts of alanine 4 and 9 are coincident, as expected for central residues having the same sequential environment, Q*A*A. The chemical shift of alanine 15 increases linearly with temperature, characteristic of the thermal dependence of a carbonyl carbon resonance of a residue in the coil conformation.[3e] Such a dependence is expected for the terminal residue on a frayed end using the traditional Lifson–Roig model.[1] However, the thermal dependence of the chemical shift of alanine 1 resembles that of the central alanine 9, suggesting that the N-terminus is considerably less frayed. This distinction is also evident in the thermal dependence of the carbonyl carbon resonances of the penultimate residues 2 and 14, as illustrated in Figure 3A.

(14) Brent, R. P. *Algorithms for Minimization Without Derivatives*; Prentice Hall: Englewood Cliffs, NJ, 1974; Chapter 5.

(15) (a) Merutka, G.; Lipton, W.; Shalongo, W.; Park, S.-H.; Stellwagen, E. *Biochemistry* **1990**, *29*, 7511–7515. (b) Lyu, P. C.; Wang, P. C.; Liff, M. I.; Kallenbach, N. R. *J. Am. Chem. Soc.* **1991**, *113*, 3568–3572. (c) Scholtz, J. M.; Marqusee, S.; Baldwin, R. L.; York, E. J.; Stewart, M. S.; Santoro, M.; Bolen, D. W. *Proc. Natl. Acad. Sci.* **1991**, *88*, 2854–2858.

(16) Methanol is the solvent of choice for NMR measurements since it contributes less to solvent viscosity.

(17) Chen, Y.-H.; Yang, J. T.; Chau, K. H. *Biochemistry* **1974**, *13*, 3350–3359.

(18) Wishart, D. S.; Sykes, B. D.; Richards, F. M. *J. Mol. Biol.* **1991**, *222*, 311–333.

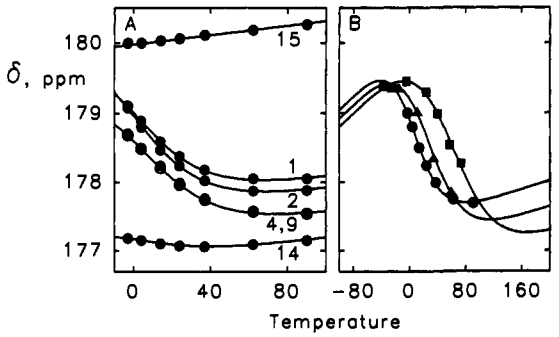

Figure 3. Carbonyl carbon chemical shift measurements. Panel A illustrates the thermal dependence of the carbonyl carbon resonance of representative alanine residues in peptide acetyl(AAQAA)$_3$amide in D_2O at pH 7. Each line represents the best fit two-state analysis using eq 1 and the values listed in Table 1. The sequence position of each illustrated alanine carbonyl resonance is indicated. Panel B illustrates the analysis of the thermal dependence of the carbonyl carbon resonance of alanine 6. The circles indicate measurements in D_2O, the triangles measurements in 25% methanol, and the squares measurements in 50% methanol. The lines represent a simultaneous fitting of all the experimental values to the chemical shift version of eq 1. The fitted values for the transition in the absence of methanol are listed in Table 1.

Table 1. Analysis of the Thermal Transitions of the Carbonyl Resonances of Peptide Acetyl(AAQAA)$_3$amide at pH 7.0

		chemical shift, ppm at 0 °C			
residue	position	δ_{coil}	$\Delta\delta_{helix-coil}$	T_m, °C	ΔT, °C
alanine	1	177.75	2.53	0	80
alanine	2	177.58	2.94	-2	81
glutamine	3	175.11	2.93	7	89
alanine	4	177.23	2.54	3	85
alanine	5	177.36	2.83	6	90
alanine	6	177.36	2.75	5	90
alanine	7	177.60	2.71	6	84
glutamine	8	175.15	2.96	9	92
alanine	9	177.24	2.46	5	84
alanine	10	177.32	2.64	3	89
alanine	11	177.29	2.48	-5	94
alanine	12	177.49	2.42	-6	84
glutamine	13	175.07	2.38	-2	86
alanine	14	176.86	0.78	-8	84
alanine	15	179.98	0.00		
		Standard Deviation			
		0.06	0.07	1	5

The effect of methanol on the thermal dependence of the carbonyl carbon chemical shift of a representative central residue, alanine 6, is illustrated in Figure 3B. Methanol appears to stabilize the helical conformation of the peptide, in agreement with the dichroic measurements described above. The thermal dependence of the carbonyl resonance of alanine 6 in each solvent was fit simultaneously to a common two-state helix/coil transition using eq 1. Such fitting predicts that the carbonyl carbon resonance of alanine 6 would have values of 180.11 and 177.36 ppm in the helix and coil conformations, respectively, in water at 0 °C. The difference in these chemical shift values, 2.75 ppm, and the width of the helix/coil transition, 90°, are characteristic for an alanine residue in the central region of a helix.[3e]

The carbonyl carbon resonance of each enriched residue in peptide acetyl(AAQAA)$_3$amide was measured in D_2O and in an equivolume mixture of D_2O and methanol at a series of temperatures. The thermal dependence of each residue in both solvents was fit simultaneously to a two-state helix/coil equilibrium. The fitting parameters which simulate the thermal transitions observed for each residue in D_2O are presented in Table 1.

The percent helicity of each residue in peptide acetyl-(AAQAA)$_3$amide can be calculated at any temperature using the parameters listed in Table 1 and eq 2. The distribution of

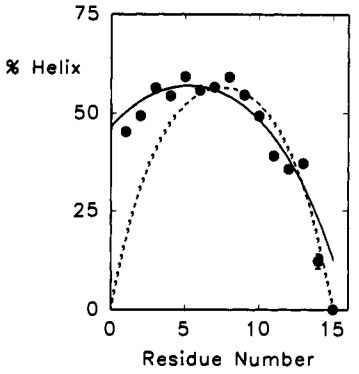

Figure 4. Analysis of the distribution of residue helicity. The filled circles indicate the percent helicity of each residue in water at 1 °C obtained from two-state analysis of the thermal dependence of carbonyl carbon chemical shift measurements using eq 2. The dashed line represents the distribution of residue helicity predicted using the traditional Lifson–Roig algorithm and a best fit propagation parameter, s, for all residues of 1.36. The solid line represents the smoothed distribution of residue helicity predicted using the modified Lifson–Roig algorithm, best fit propagation parameters for alanine and glutamine of 1.47 and 0.50, respectively, and a best fit interaction parameter for the acetyl group of -1.22 kcal/mol. All Lifson–Roig calculations were made assuming alanine and glutamine residues have a common nucleation parameter of 0.0019.[13]

the residue percent helicity values within the peptide sequence at 0 °C is illustrated by the filled circles in Figure 4. This distribution is clearly skewed toward the N-terminus.

Changes in the solution conditions at 0 °C have little effect on residue carbonyl chemical shift values. Addition of 1 M NaCl increases the chemical shift by 0.10(0.05) ppm. Lowering the pH from 7.0 to 2.0 in the absence of NaCl decreases the chemical shift by 0.04(0.01) ppm. These results suggest that the helical conformation of peptide acetyl(AAQAA)$_3$amide is not stabilized by electrostatic interactions. Dilution of the peptide solution with D_2O at pH 7 in the absence of NaCl to a peptide concentration of 0.1 mM does not change the chemical shift values beyond experimental variation. The thermal dependence of the mean residue helicity calculated from chemical shift measurements using 3 mM peptide is coincident with the thermal dependence of the mean residue ellipticity measured using 0.023 and 0.23 mM peptide solutions, as shown in Figure 1B. Such coincidence is as reliable a test of the absence of aggregation as is sedimentation equilibrium.[6]

Amide Exchange Measurements. The chemical shift of a carbonyl carbon resonance in peptide acetyl(AAQAA)$_3$amide is increased about 0.1 ppm by replacement of D_2O with H_2O. In an equivolume mixture of H_2O and D_2O, the profile of a carbonyl carbon resonance will be dependent upon the exchange time of its adjacent backbone amide. If the exchange time is slow, the carbonyl resonance will be resolved into two narrow peaks of equal area whose chemical shifts are separated by about 0.1 ppm. If the exchange time is fast, the carbonyl carbon resonance will appear as a single narrow peak whose chemical shift will be midway between that of the resonance in H_2O and in D_2O. If the exchange time is intermediate, the carbonyl carbon resonance will appear as a broadened peak which may display two poorly resolved maxima. The exchange time for the hydrogen/deuterium exchange of an amide group at equilibrium may be estimated by fitting the profile of its adjacent carbonyl carbon resonance using eq 6. Comparison of the estimated rate constant with that reported for an unstructured dipeptide of the same sequence[12] facilitates calculation of a protection factor and the percent amide which is hydrogen bonded using eqs 11 and 12, respectively. Protection factors increase from 1 to infinity as the amide hydrogen bonding content increases at equilibrium.

The reliability of the amide exchange analysis was tested using a solution of the unstructured peptide acetylAAAamide in an

Table 2. Analysis of Amide Exchange Measurements[a]

carbonyl amide		rate constant, s^{-1}		protection	% hydrogen
observed	reported	observed	model	factor	bonded
		Peptide AcetylAAAamide			
Ala 2	Ala 3	50.3(0.9)	49.9	0.99(0.02)	0(2)
		Peptide Acetyl(AAQAA)$_3$amide			
Ala 1	Ala 2	33.1(2.6)	32.0	0.97(0.08)	0(8)
Ala 2	Gln 3	40.0(0.4)	47.7	1.19(0.01)	16(1)
Ala 5	Ala 6	31.2(0.5)	24.8	1.79(0.04)	44(2)
Ala 6	Ala 7	18.9(0.4)	38.2	2.02(0.04)	51(1)
Ala 7	Gln 8	16.2(2.7)	38.0	2.35(0.40)	57(7)
Ala 9	Ala 10	13.2(0.2)	31.1	2.36(0.04)	58(1)
Ala 10	Ala 11	16.8(2.2)	30.3	1.80(0.24)	45(7)
Ala 11	Ala 12	14.6(0.2)	34.6	2.37(0.03)	58(1)
Ala 12	Gln 13	18.5(0.5)	47.9	2.59(0.07)	61(1)
Ala 14	Ala 15	18.9(0.4)	30.5	1.61(0.03)	38(1)

[a] Peptide acetylAAAamide was measured at 3 °C in 50% D_2O at pH 9.05. Enriched peptides acetyl(AAQAA)$_3$amide were measured at 1 °C in solvents whose D_2O volume concentration ranged from 44% to 57% and whose pH meter readings ranged from 8.68 to 8.86.

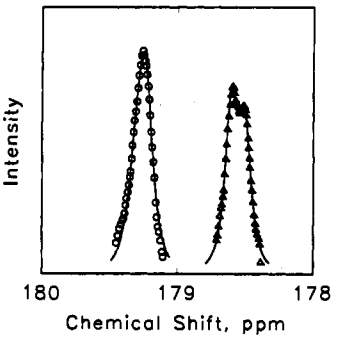

Figure 5. Analysis of carbonyl carbon line shape in terms of amide exchange. The circles describe the carbonyl carbon resonance of enriched alanine 2 observed in 56% (v/v) D_2O at pH 8.86 and 1 °C. The line describes the profile simulated using eq 5, peak half-widths of 12 Hz, a peak separation of 19.5 Hz, and the exchange rate listed in Table 2. The triangles describe the carbonyl carbon resonance of enriched alanine 6 observed in 47% D_2O at pH 8.85 and 1 °C. The line describes the profile simulated using peak half-widths of 12 Hz, a peak separation of 18.6 Hz, and the exchange rate listed in Table 2.

equivolume mixture of D_2O and H_2O at pH 9.03 and 3 °C. The fast exchange profile for the carbonyl carbon resonance of enriched alanine 2 was fit to a D → H rate constant of 50.3(0.9) s^{-1}. This value corresponds to a protection factor of 0.99(0.02) and an amide hydrogen bonding content of 0(2)%, as shown in Table 2.

Two representative carbonyl carbon resonances observed for peptide acetyl(AAQAA)$_3$amide are illustrated in Figure 5. The fast exchange profile observed for the carbonyl carbon resonance of alanine 2 can be simulated using a D → H rate constant of 40.0(0.4) s^{-1} for the amide of glutamine 3, corresponding to a protection factor of 1.19(0.01) and an amide hydrogen bonding content of 16(1)%. The intermediate exchange profile for the carbonyl carbon resonance of the central alanine 6, also illustrated in Figure 5, can be simulated by a rate constant of 18.9 s^{-1} for the amide exchange of alanine 7, corresponding to a protection factor of 2.02(0.04) and an amide hydrogen bond content of 51-(1)%.

The amide exchange rate, protection factor, and percent amide hydrogen bonded calculated for selected residues in peptide acetyl-(AAQAA)$_3$amide are listed in Table 2. Unfortunately, the carbonyl carbon resonance profiles of the glutamine residues cannot be analyzed in terms of amide exchange because these resonances are already doublets in the absence of exchange, owing to the coupling between the enriched carbonyl carbon and α-carbons. Accordingly, the amide exchange rates of alanines 4, 9, and 14 could not be measured.

The solubility of the peptide having enriched alanine 4 was strangely inadequate to obtain a carbonyl resonance profile suitable for measurement of the amide exchange rate of alanine 5. At the beginning of this investigation, the peptide having enriched alanine 4 was soluble and well-behaved, as indicated by its comparative thermal dependence in Figure 3A. However, as this peptide was placed in and subsequently separated from the various solvents used in this study, it became uniquely less soluble. This lowered solubility, however, does not necessarily indicate the population of a distinct polymeric form such as a helix bundle.

Table 3. Potential *i*,*i*+4 Backbone Hydrogen Bonds[a]

carbonyl acceptor		amide donor		
residue	% helicity	residue	% H bonded	difference %
		1		
		2	0(8)	
		3	16(1)	
		4		
1	45	5		
2	49	6	44(2)	5
3	56	7	51(1)	5
4	54	8	57(7)	-3
5	59	9		
6	56	10	58(1)	-2
7	57	11	45(7)	12
8	59	12	58(1)	1
9	55	13	61(1)	-6
10	49	14		
11	39	15	38(1)	1
12	35			
13	37			
14	12			
15	0			

[a] The percent helicity of the carbonyl acceptor was calculated at 1 °C using eq 2. The $\delta_\alpha - \delta_c$ for alanine and glutamine residues was assumed to be 2.76 and 2.95 ppm, respectively. The percent of each amide which is hydrogen bonded was obtained from Table 2. The difference percent is the percent helicity of the *i*th carbonyl acceptor minus the percent hydrogen bonded of the *i*+4th amide donor.

The sequence distributions of the residue percent helicity values obtained from carbonyl carbon chemical shift measurements and the residue percent backbone hydrogen bonding values obtained from amide exchange measurements are compared in Table 3. These distributions generate a reasonable correlation when arranged as an α-helix containing *i*,*i*+4 backbone hydrogen bonds. The apparent 16% helicity calculated for the amide exchange of glutamine 3 may result from hydrogen bonding between the backbone amide and the side-chain carbonyl of this residue, an interaction observed[19] for glutamate residues in unstructured peptides and in proteins.

Discussion

The carbonyl carbon resonance of each residue in peptide acetyl-(AAQAA)$_3$amide except alanine 15 exhibits a nonlinear downfield shift as an aqueous solution of the peptide is cooled from 90 to 0 °C. Such a shift indicates an increase in the fractional population of the helical conformation of a residue as the solution is cooled.[3e,18] Unfortunately, the fractional population of the helical residues in peptide acetyl(AAQAA)$_3$amide in an aqueous solvent at 0 °C is insufficient to predict the carbonyl carbon chemical shift for each residue in its helical conformation. Accordingly, methanol was added to the peptide solvent to increase the fractional population of the helical conformation of each residue. Such enhanced stability facilitated prediction of a carbonyl carbon chemical shift value for the helix and the coil conformations of each residue at 0 °C using a two-state approximation. While methanol/water solvents have not been used previously to establish the chemical shift for a carbonyl carbon resonance in the helical conformation, the results obtained using these mixed solvents are reasonable. The mean $\delta_\alpha - \delta_c$

(19) (a) Bundi, A.; Wüthrich, K. *Biopolymers* **1979**, *18*, 299–311. (b) Ebina, S.; Wüthrich, K. *J. Mol. Biol.* **1984**, *179*, 283–288.

Table 4. Comparison of Peptide Parameters[a]

parameter	acetyl-(AAQAA)$_3$-amide	acetylW-(EAAAR)$_3$-Aamide[3e]
transition width, ΔT, °C	88(4)	86(4)
melting temperature, T_m, °C	4(4)	41(3)
ΔH, kcal (mol res)$^{-1}$	0.80(0.25)	0.86(0.17)
ΔS, cal K^{-1} (mol res)$^{-1}$	2.86(0.87)	2.86(0.47)
ΔC_p, cal K^{-1} (mol res)$^{-1}$	2.45(2.61)	4.46(1.08)

[a] The first two comparisons involve residues not expected to be involved in the frayed ends, namely, residues 5–11 in acetyl(AAQAA)$_3$amide and residues 5–13 in acetylW(EAAAR)$_3$Aamide. The thermodynamic comparisons involve all the residues. The values for ΔH and ΔS were calculated at the protein convergence temperatures, 100 and 112 °C, respectively.

values at 0 °C for the alanine and the glutamine residues in the central portion of the sequence of peptide acetyl(AAQAA)$_3$amide are 2.63(0.14) and 2.95(0.02) ppm, respectively. These values compare favorably with the $\delta_\alpha - \delta_c$ values at 0 °C for the alanine and glutamate residues in the central portion of the sequence of peptide acetylW(EAAAR)$_3$Aamide, 2.68(0.17) and 2.89(0.13) ppm, which were measured without the use of methanol/water solvents.

Establishment of a $\delta_\alpha - \delta_c$ value for each residue facilitated calculation of the distribution of the helicity among the residues in peptide acetyl(AAQAA)$_3$amide at 0 °C, which is illustrated in Figure 4. This distribution is at variance with that predicted by a traditional Lifson–Roig model, illustrated by the dashed line in Figure 4. The variance is greatest in the N-terminal frayed end, where the helical content is greater than expected. The amide exchange measurements presented in Table 3 suggest that the $i,i+4$ backbone hydrogen bonding pattern characteristic for the central region of the helix is extended into the N-terminal frayed end. The stabilization of helical structure in this frayed end is an emerging recognition based on NMR, ESR, and circular dichroic measurements of a variety of monomeric model peptide solutions.[3] Such stabilization is often provided by capping interactions, most commonly polar side chains which hydrogen bond with the unpaired amide backbone groups in the frayed ends. It has been proposed[3c] that an N-terminal acetyl blocking group can also provide interactions which stabilize the helical conformation of an N-terminal frayed end. The observed distribution of helicity in peptide acetyl(AAQAA)$_3$amide can be simulated using a modified Lifson–Roig model which accommodates side-chain interactions, as illustrated by the solid line in Figure 4. This simulation was achieved using a ΔG value for the acetyl interaction and propagation, s, values for the alanine and glutamine residues which are within 94% of the corresponding values obtained by two-state analysis of dichroic measurements of a series of host/guest peptides using an alternative modified Lifson–Roig model.[13] The correlation of these values suggests that the modified Lifson–Roig model, the refined residue s values, and the evaluated interactions can be combined to predict the helical content of peptides with significantly greater precision.

Establishment of the thermal dependence of the $\delta_\alpha - \delta_c$ for each residue facilitates analysis of some thermodynamic parameters associated with the helix/coil transition of each residue in the model peptide. Since the variations in the ΔC_p, ΔH, and ΔS values calculated for each residue are within the cumulative errors of the analysis, these quantities are presented as mean residue values in Table 4 and compared with similar values previously calculated for peptide acetylW(EAAAR)$_3$Aamide. The mean residue values for both peptides are smaller than corresponding values for representative globular proteins.[20] The smaller values for the peptides likely reflect the diminished opportunity for buried surface area and noncovalent interactions in an isolated helix compared with a globular protein. The larger mean ΔC_p value for peptide acetylW(EAAAR)$_3$Aamide suggests that about 70 Å^2 more surface area is buried[20] by the helical conformation of this peptide. This surface area could easily be contributed by portions of the side chains of the three arginine residues, the tryptophan residue, and the extra two residues uniquely present in this peptide. The larger buried surface area and the opportunity for complementary electrostatic interactions in the helical conformation of peptide acetylW(EAAAR)$_3$Aamide likely account for the enhanced thermostability of the helical conformation of this peptide.

The analysis of the thermal dependence of the carbonyl chemical shift measurements of the model peptide in terms of a two-state helix/coil transition has been done with some concern. Since the residues in the frayed ends of a peptide helix are often less thermostable than those in the central helix, the helical conformation which is stabilized at one temperature is not necessarily the same helical conformation which is stabilized at a different temperature. Accordingly, two-state analysis of the thermal dependence of measurements which examine the peptide as a whole, such as mean residue ellipticity measurements, is not strictly valid and must be treated as an approximation. By contrast, two-state analysis of the thermal dependence of an individual residue within a peptide, such as carbonyl chemical shift measurements, would seem to be valid. A given residue is considered to populate either a helix conformation or a nonhelix (coil) conformation. The helix conformation of a residue, in contrast to the helix conformation of the whole peptide, remains independent of temperature. While the conformational states of adjacent residues may influence both the midpoint, T_m, and the cooperativity, ΔT, of the thermal transition of an observed residue, they would appear to do so by altering the helix/coil equilibrium of the observed residue and not by stabilization of alternative residue helical conformations.

Several features of peptide acetyl(AAQAA)$_3$amide suggest that two-state analysis of the thermal transition of the carbonyl carbon chemical shift measurements is valid. (i) The observed thermal dependence of the carbonyl carbon chemical shift of each residue can be nicely fit by a two-state transition, as illustrated in Figure 3. (ii) The T_m and $\delta_\alpha - \delta_c$ values for the residues in the C-terminal region are appropriate to those expected for a frayed end, as shown in Table 1 and Figure 4. (iii) The residue fractional helical contents and amide hydrogen bonding contents are consistent with those expected for an α-helix, as shown in Table 3. (iv) The mean residue ΔH, ΔS, and ΔC_p values listed in Table 4 appear appropriate for the helix/coil transition of an isolated monomeric peptide. (v) The ΔG value for the acetyl interaction and the s values for alanine and glutamine are very similar to the corresponding values obtained from isothermal dichroic measurements of host/guest peptides.[13] While an invalid two-state analysis could by coincidence generate one of these features, it would seem unlikely to coincidentally generate all five features. We suggest that two-state analysis of the thermal dependence of chemical shift measurements of individual residues is a valid undertaking.

Acknowledgment. This investigation was supported by U.S. Public Health Service Research Grant GM22109 and National Research Service Award GM15759 (to W.S.) from the National Institute of General Medical Sciences. The authors are indebted to Mr. Soon-Ho Park for productive discussions.

(20) Murphy, K. P.; Bhakuni, V.; Xie, D.; Freire, E. *J. Mol. Biol.* **1992**, *227*, 293–306.