

Accelerated Publications

Kinetics of Amide Proton Exchange in Helical Peptides of Varying Chain Lengths. Interpretation by the Lifson–Roig Equation[†]

Carol A. Rohl,[‡] J. Martin Scholtz,[‡] Eunice J. York,[§] John M. Stewart,[§] and Robert L. Baldwin^{*,‡}

Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305, and Department of Biochemistry, University of Colorado Health Sciences Center, Denver, Colorado 80262

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ABSTRACT: The kinetics of amide proton exchange ($^1\text{H} \rightarrow ^2\text{H}$) have been measured by proton nuclear magnetic resonance spectroscopy for a set of helical peptides with the generic formula $\text{Ac}-(\text{AAKAA})_m\text{Y-NH}_2$ and with chain lengths varying from 6 to 51 residues. The integrated intensity of the amide resonances has been measured as a function of time in $^2\text{H}_2\text{O}$ at $\text{pH}^* 2.50$. Exchange kinetics for these peptides can be modeled by applying the Lifson–Roig treatment for the helix-to-coil transition. The Lifson–Roig equation is used to compute the probability that each residue is helical, as defined by its backbone (ϕ, ψ) angles. A recursion formula then is used to find the probability that the backbone amide proton of each residue is hydrogen bonded. The peptide helix can be treated as a homopolymer, and direct exchange from the helix can be neglected. The expression for the exchange kinetics contains only three unknown parameters: the rate constant for exchange of a non-hydrogen-bonded (random coil) backbone amide proton and the nucleation (v^2) and propagation (w) parameters of the Lifson–Roig theory. The fit of the exchange curves to these three parameters is very good, and the values for v^2 and w agree with those derived from circular dichroism studies of the thermally-induced unfolding of related peptides [Scholtz, J. M., Qian, H., York, E. J., Stewart, J. M., & Baldwin, R. L. (1991) *Biopolymers* (in press)].

The amide proton exchange method of Linderstrøm-Lang (1955) is now an important tool in studying protein folding and dynamic processes in proteins in general (Englander & Kallenbach, 1984). The key development has been the use of two-dimensional proton NMR¹ (Wüthrich, 1986) to measure exchange rates of individual peptide amide protons in the polypeptide backbone. With rare exceptions, amide protons that are strongly protected against exchange in native proteins are found in α -helices and β -sheets [for reviews, see Wagner (1983), Englander and Kallenbach (1984), and Woodward et al. (1982)]. Structural hydrogen bonds must be broken before exchange occurs (Englander et al., 1972), and the hydrogen bonds in α -helices and β -sheets are formed coop-

eratively, resulting in strong protection against exchange. The patterns of protection found in α -helices and β -sheets are complex (Wagner, 1983; Englander & Kallenbach, 1984; Woodward et al., 1982), reflecting tertiary as well as secondary structure interactions. This complexity precludes a rigorous and definitive interpretation of exchange in proteins.

Describing the mechanism of exchange in monomeric helical peptides should be a simpler task and is very likely to provide insights into exchange from proteins. We have examined exchange in a series of peptides with the generic sequence $\text{Ac}-(\text{AAKAA})_m\text{Y-NH}_2$ and varying chain lengths. The peptides contain chiefly alanine because of its high helix-forming tendency (Marqusee et al., 1989) and because interpretation of the exchange kinetics is greatly simplified by using an amino acid sequence that is as uniform as possible. The framework for interpreting exchange in an α -helix is largely in place.

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* To whom correspondence should be addressed.

[‡] Stanford University School of Medicine.

[§] University of Colorado Health Sciences Center.

¹ Abbreviations: NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; FAB, fast atom bombardment; PD, plasma desorption; CD, circular dichroism; FID, free induction decay; NOE, nuclear Overhauser effect.

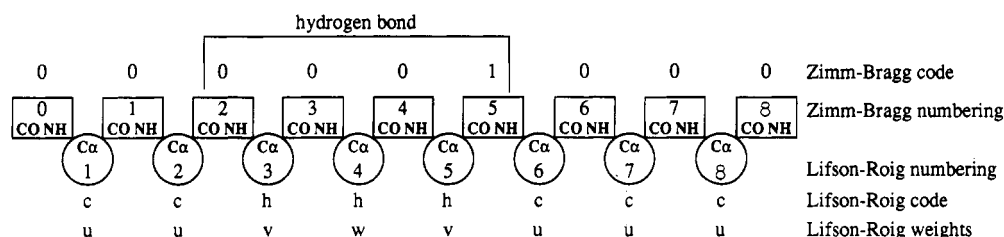


FIGURE 1: Diagram of a partially helical peptide with blocked N- and C-termini [adapted from Qian and Schellman (1991)]. Notation for both the Zimm-Bragg and the Lifson-Roig treatments is shown. Peptide linkages are shown as rectangles; α carbons and their associated side chains are shown as circles. The Zimm-Bragg helix and coil conformations 1 and 0 correspond respectively to bonded and nonbonded amide protons. The Lifson-Roig helix and coil conformations, as defined by (ϕ, ψ) angles, are denoted h and c. The Lifson-Roig statistical weights u, v, and w are given respectively to coiled, helical, and stabilized helical residues. A helical residue is considered to be stabilized only if both neighboring residues are also helical.

Exchange should occur by the acid- and base-catalyzed EX₂ mechanism (Hvidt, 1964; Wagner, 1983; Englander & Kalenbach, 1984; Woodward et al., 1982), with the exchange rate of proton *i*, $k_{\text{obs}}(i)$, being proportional to the probability $[1 - f_{\text{B}}(i)]$ that proton *i* is not hydrogen bonded in the helix:

$$k_{\text{obs}}(i) = k_{\text{c}}(i)[1 - f_{\text{B}}(i)] \quad (1)$$

The "chemical" rate constants, $k_{\text{c}}(i)$, can be estimated from data on model peptides (Molday et al., 1972). Due to their uniform sequence, these peptides are approximated here as homopolymers with respect to chemical exchange rate, and an average k_{c} is used.

The Lifson-Roig theory (Lifson & Roig, 1961) of the transition between α -helix and random coil is uniquely suited to analysis of the exchange kinetics because it gives information about the fractional helicity, $f_{\text{H}}(i)$, at each position. In addition, the theory correlates the helix conformation, which is defined by backbone (ϕ, ψ) angles, with hydrogen bonding. The probability that residue *i* is in a stabilized helical conformation, $f_{\text{H}}(i)$, is directly related to the hydrogen bonding between the backbone amide proton of residue *i* + 2 and the carbonyl oxygen of residue *i* - 2 (see Figure 1). Thus a recursion formula can be used to determine $f_{\text{B}}(i)$, the probability that the amide of residue *i* is hydrogen bonded; $f_{\text{B}}(i)$ is equal to the fractional helicity of residue *i* - 2. The terms "residue" and "amino acid" are used here in a strictly defined sense, as required by the Lifson-Roig theory; see the discussion by Qian and Schellman (1991). A residue in a peptide refers to an α -carbon flanked on both sides by peptide linkages, while an amino acid in a peptide refers to an α -carbon without regard to the neighboring linkages. A diagram of a partly helical conformation is shown in Figure 1 with the notation assigned to the residues for the Lifson-Roig (Lifson & Roig, 1961) and Zimm-Bragg (Zimm & Bragg, 1959) theories.

To use the Lifson-Roig theory to find values of $f_{\text{H}}(i)$ and $f_{\text{B}}(i + 2)$, it is necessary to know the values of the helix nucleation and propagation parameters v^2 and w for each type of residue in the peptides. The approximation is made here that, because of its simple sequence, the peptide can be treated as a homopolymer, and single average values of v^2 and w are used. Then the exchange kinetics depend only on three unknown parameters: v^2 , w , and k_{c} . Because the entire kinetic curve is measured for each peptide, the set of exchange curves provides a sensitive test of whether or not these three parameters fit all the data. The results show that the fit is surprisingly good and that amide proton exchange apparently provides a valuable new method for determining the parameters of the theory of the helix-coil transition.

MATERIALS AND METHODS

Peptide Synthesis and Purification. Peptides were synthesized with stepwise solid-phase procedures (Stewart &

Young, 1984) on a Bioscience 9500 automatic synthesizer. Syntheses were performed with 0.4–0.8 mequiv of *p*-methylbenzhydrylamine (polystyrene/1% divinylbenzene) resin using *tert*-butoxycarbonyl (BOC)-benzyl chemistry. After incorporation of the first six residues, double couplings and capping with acetylimidazole were employed routinely. Generally the first coupling used the standard Bioscience diisopropylcarbodiimide procedure and the second, the 1-hydroxybenzotriazole/(benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (HOBt/BOP) method (Hudson, 1988) in *N*-methylpyrrolidinone with addition of 20–25% of dimethyl sulfoxide after 0.5–1 h. The coupling time was extended if the qualitative Kaiser test did not show the second coupling to be complete. As units of AAKAA were added, aliquots of the resin (0.05–0.2 mequiv) were removed for N-terminal acetylation with acetic anhydride and/or acetylimidazole. The C-terminal amide was provided by cleavage with hydrofluoric acid from the resin. The crude peptides were purified by gel filtration on Sephadex G-15 in 50% acetic acid, followed by reverse-phase HPLC on either Vydac large-pore (300-Å) C₄ or YMC Pack D-ODS-5 resin with gradients of acetonitrile containing 0.1% trifluoroacetic acid. Peptide purity was greater than 95% as shown by reverse-phase HPLC on C₁₈ or diphenyl resin. Amino acid composition was confirmed by analysis on a Beckman 6300 amino acid analyzer after hydrolysis of 22 h at 110 °C in 6 N HCl. Molecular weights were determined by FAB or PD mass spectrometry.

Circular Dichroism Measurements. CD spectra were taken at 0 °C in 1 M potassium fluoride buffered with 3 mM potassium phosphate at pH 7.0. Data were collected at 0.2-nm increments from 300 to 190 nm. Thermal denaturation curves were constructed from data taken at 222 nm at 2 °C increments from 0 to 80 °C and 10 °C increments from 80 to 0 °C. Samples for thermal denaturation studies were prepared by diluting aqueous stock solutions into 1 M NaCl buffered with 1 mM sodium citrate, 1 mM sodium phosphate, and 1 mM sodium borate at pH 7.0. Stock peptide concentration was determined by measuring tyrosine absorbance at 275 nm in 6 M guanidine hydrochloride and 20 mM potassium phosphate, pH 6.5 (Brandts & Kaplan, 1973). An Aviv 60DS spectropolarimeter with a Hewlett-Packard 89100A temperature control unit and cuvettes with either 10- or 1-mm path lengths were used for all measurements. Ellipticity was calibrated with (+)-10-camphorsulfonic acid and is reported as mean molar residue ellipticity, $[\Theta]$ (deg cm² dmol⁻¹).

NMR Spectroscopy. Samples were prepared by dissolving peptide in water to a concentration of 1–2 mM and adjusting the pH to 2.50 with HCl. The samples were then lyophilized to dryness. At *t* = 0 min, the peptide was dissolved at 1–2 mM in ²H₂O at 5 °C containing 1 M NaCl and 5 mM sodium

phosphate, pH* 2.50. pH* represents the glass-electrode reading at room temperature, uncorrected for isotope effects. Samples for the pH profile of the six-residue peptide were prepared similarly, but were adjusted to the desired final pH* before lyophilization and dissolved in buffer at the desired final pH* at the beginning of the experiment. Exchange experiments at 0 M NaCl were carried out in unbuffered $^2\text{H}_2\text{O}$ adjusted to the desired pH* with ^2HCl . All reported pH* measurements were made after the acquisition of the NMR spectra. After the peptide was dissolved in buffer, multiple one-dimensional proton spectra were acquired during the exchange on a General Electric GN-Omega spectrometer at a proton frequency of 500.13 MHz. Data were collected using a 5000-Hz spectral width and a 60° pulse. The FID was the sum of 64 scans collected in 4096 complex points with a 5-ms recycle delay. The acquisition of each spectrum required approximately 1 min. Spectra were processed using FELIX (Hare Research, Inc.) on a Silicon Graphics Personal Iris computer. FID's were multiplied by an exponential decay function with line broadening of 1 Hz before Fourier transformation. Occupancy was determined by measuring the integral of all peaks in the amide region of each spectrum and normalizing to the area of a nonexchanging aromatic tyrosine resonance to correct for differences in spectrum plotting. Fractional occupancy was then determined by extrapolating the occupancy as a function of time back to zero time and setting this value equal to a fractional occupancy of 1. Fractional occupancy at infinite time is assumed to be 0. Under these experimental conditions, the amide protons of the C-terminal blocking group and the ϵ -amino protons of the lysine side chains have high intrinsic-exchange rates and are completely exchanged within the 3–4 min required for sample preparation.

For the pH profile of the six-residue peptide, exchange rate constants, k_{obs} , were determined by least-squares fit of the fractional occupancy data to a single exponential decay and plotted against the measured pH*. The pH dependence of the six-residue peptide at both 0 and 1 M NaCl was fitted as described previously (Robertson & Baldwin, 1991; Roder et al., 1985) to the equation:

$$k_{\text{obs}} = (k_{\text{min}}/2)[10^{(\text{pH}-\text{pH}_{\text{min}})} + 10^{(\text{pH}_{\text{min}}-\text{pH})}] \quad (2)$$

where k_{min} and pH_{min} are the exchange rate constant and pH, respectively, at the point of slowest exchange. This procedure assumes that there is no water-catalyzed exchange and that acid- and base-catalyzed exchange are both first order in the catalyst concentration. Nonlinear least-squares fitting was performed utilizing a program based on standard Gauss-Newton iteration, developed by Michael Johnson (Johnson et al., 1981) and modified for use on the IBM-PC (D. Whitman). Observed rate constants were weighted by the reciprocal of their magnitude so as not to favor points far from the pH_{min} (Robertson & Baldwin, 1991).

The final pH* values of the exchange reactions of the longer peptides showed small deviations, less than 0.07 pH unit from the reference pH* of 2.50. To facilitate comparison of the exchange behavior of these peptides, these small differences in pH* were corrected for by expanding or contracting the time axis according to the equation:

$$\text{time}^* = (\text{time})[10^{(2.50)}]/[10^{(\text{experimental pH}^*)}] \quad (3)$$

where time* is the corrected time. This correction is valid only for small interpolations in pH in the region where acid-catalyzed exchange predominates.

Application of Lifson-Roig Model. The Lifson-Roig model for the helix-coil transition uses (ϕ , ψ) angles to define helical

residues and describes fractional helicity of a peptide in terms of chain length, n , a propagation constant, w , and a nucleation constant, v^2 . The helix-coil constants w and v^2 are related to the Zimm-Bragg parameters s and σ through the expressions $s = w/(1 + v)$ and $\sigma = v^2/(1 + v)^4$ (Qian & Schellman, 1991). Although each residue in an amino acid sequence may be assigned individual w and v^2 constants, for the calculations in this paper, the peptides have been treated as homopolymers and average w and v^2 values have been assigned to all residues. In fitting exchange data to the Lifson-Roig model, peptides of different lengths must be fitted to different equations describing exchange (see eq 5 and Results). In order to fit all the data simultaneously, a modified least-squares procedure was used. Estimates of the three parameters, k_c , w , and v^2 , were used to generate an initial calculated curve for each peptide. The standard deviation of each experimental exchange curve from its corresponding calculated curve was determined and then totalled for all the peptides. This procedure was repeated for different values of the three parameters until the sum of the standard deviations reached a minimum.

RESULTS

Peptide Design. The sequences of the peptides in this study are based on those studied by Marqusee et al. (1989). Peptides contain repeats of the simple sequence AAKAA, yielding the sequences $\text{Ac}-(\text{AAKAA})_m\text{-Y-NH}_2$, $m = 1-10$. Alanine has been shown to have a strong helix propensity, and high alanine content is the primary factor contributing to the substantial helix formation of these peptides. Lysine residues are included to solubilize the peptides; the symmetric $i, i + 5$ spacing of the charged residues distributes the charge along the entire surface of the helix. The N-terminal acetyl and C-terminal amide capping groups eliminate unfavorable charge-helix dipole interactions, and a single tyrosine residue is included to facilitate accurate concentration determination (Brandts & Kaplan, 1973; Marqusee et al., 1989).

Circular Dichroism Studies. Under optimal helix-forming conditions, the CD spectra of the peptides 16 residues and longer show the characteristic minima at 222 and 208 nm and maximum around 190 nm which are typical of α -helical structure (data not shown). The presence of an apparent isodichroic point indicates that each residue exists only in either the helix or coil conformation, regardless of the peptide length. The thermally induced helix-coil transition for all the peptides was monitored by circular dichroism at 222 nm (data not shown). Superimposable curves are obtained for samples which are heated from 0 to 80 °C or cooled from 80 to 0 °C, indicating that the thermal transition is reversible. Identical curves are also obtained for samples containing different peptide concentrations. The independence of the CD signal on peptide concentration indicates that helix formation is monomolecular and does not involve aggregation. The results of these circular dichroism studies are very similar to the results obtained by Scholtz et al. (1991b) for a length-dependent series of peptides containing an AEAACA repeat.

pH-Dependent Exchange Behavior. The pH dependence of the exchange rate constant, k_{obs} , of the six-residue peptide at both 0 and 1 M NaCl is shown in Figure 2. The deviation of the observed exchange rates near pH_{min} from the behavior predicted by eq 2 may indicate a contribution from pH-independent exchange catalyzed by water (Englander et al., 1979; Gregory et al., 1983). The interpretation of the results discussed below, however, is unaffected by the possible presence of a water-catalyzed mechanism. At 1 M NaCl, pH* 2.50 is on the acidic limb of the profile and far enough from pH_{min}

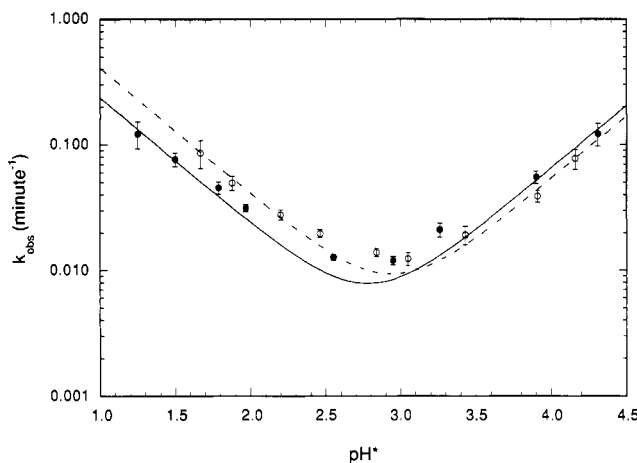


FIGURE 2: pH dependence of the exchange rate constant for the six-residue peptide. Data points are for exchange reactions in 0 M NaCl (●), and 1 M NaCl (○). Curves are the best fit of eq 1 to the data points at 0 M NaCl (solid line) and 1 M NaCl (dashed line).

that $\log k_{\text{obs}}$ is linear in pH^* with a slope of -1 . Small changes in pH^* found at the end of an experiment can therefore be corrected using eq 3. The pH profiles of the six-residue peptide at 0 and 1 M NaCl are slightly offset. At 1 M NaCl, the rate of acid-catalyzed exchange is slightly increased and the rate of base-catalyzed exchange is slightly decreased, resulting in an increase in both pH_{min} and k_{min} . pH_{min} is shifted from 2.78 to 2.94 with the addition of salt, while k_{min} increases from 0.008 to 0.009 min^{-1} . Kim and Baldwin (1982) observed similar effects of salt on the exchange behavior of charged polypeptides such as poly(DL-lysine). Much larger changes in the acid- and base-catalyzed rates of exchange were observed for poly(DL-lysine) than are seen here for the six-residue peptide; however, all exchange reactions for the longer peptides were carried out in 1 M NaCl to screen any possible charge-charge interactions.

Length-Dependent Exchange Behavior. To compare the exchange of the length-dependent series of peptides, fractional occupancy, f_o (see Materials and Methods), is plotted as a function of the pH-corrected time value (see eq 3 and Materials and Methods) in Figure 3. Each data set was fitted to the equation:

$$f_o(t) = \sum_{i=1}^n (1/n) \exp[-(k_c(i)[1 - f_B(i)] + k_h(i)[f_B(i)])t] \quad (4)$$

where n is the chain length, $k_c(i)$ is the exchange rate constant for residue i with an unbonded amide proton, $k_h(i)$ is the exchange rate constant for residue i with a hydrogen-bonded amide proton, and $f_B(i)$ is the fraction of time that residue i has a hydrogen-bonded amide proton. This equation assumes that every residue in the helix is in one of only two states: bonded or nonbonded. The isodichroic point in the CD spectra of the peptides indicates that the helix-coil transition is indeed two state on the level of individual residues. If we assume that amide proton exchange occurs only when a hydrogen bond is broken, then $k_h(i) = 0$ for all i . In addition, the peptides are treated as homopolymers with regard to intrinsic exchange rate, k_c , and an average value is used for all residues. Implementing these two assumptions, eq 4 reduces to

$$f_o(t) = \sum_{i=1}^n (1/n) \exp[-(k_c[1 - f_B(i)])t] \quad (5)$$

The fraction-bonded values for residue i , $f_B(i)$, can be determined directly from the helix probabilities, $f_H(i)$, calculated according to the Lifson-Roig theory. The Lifson-Roig theory calculates the α -helix to coil partition function for a peptide of n residues as a product of matrices, containing growth and

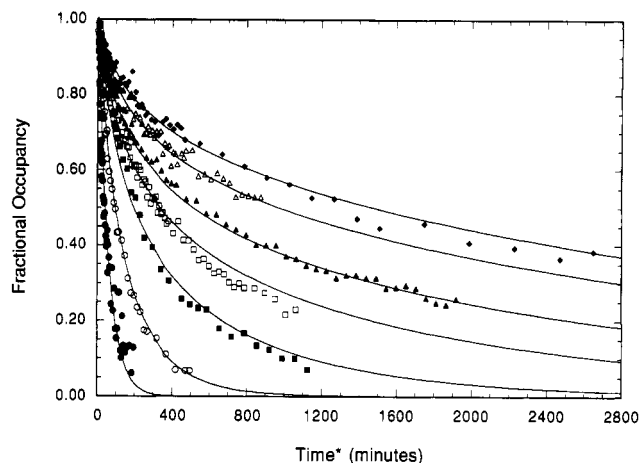


FIGURE 3: Amide proton exchange curves for the peptides as monitored by NMR. Fractional occupancy is determined as described under Materials and Methods. Time* is an adjusted time scale which corrects for small deviations, less than 0.07 pH unit, in the measured pH^* of different samples (see eq 3). Symbols are experimental points determined for peptides with chain lengths of 6 (●), 16 (○), 21 (■), 26 (□), 31 (▲), 41 (△), and 51 (◆) residues. Solid lines are curves calculated from eq 5 with $w = 1.58$, $v^2 = 0.0023$, and $k_c = 0.018 \text{ min}^{-1}$.

nucleation constants w and v^2 , corresponding to positions $1-n$. A residue is defined as a $\text{C}\alpha$ (with its corresponding side chain) with peptide bonds on both sides, while an amino acid is a $\text{C}\alpha$ with or without the neighboring amide linkages. For a peptide with unblocked N- and C-termini, the amino acid at each end of the chain is not a residue and does not contribute to the partition function because it cannot be confined to helical (ϕ , ψ) space. An unblocked peptide of chain length n therefore contains $n + 2$ amino acids numbered 0 to $n + 1$, but only the residues, in positions $1-n$, contribute to the partition function. When the N-terminal acetyl and C-terminal amide blocking groups are added, two additional peptide bonds are formed, and two additional hydrogen bonds are possible. As a result, the first and last amino acids can now be confined to helical (ϕ , ψ) space, and they become residues which contribute to the partition function. Therefore, all the amino acids in the blocked peptide are residues, and the chain length n is equal to the number of amino acids, while the blocking groups occupy the noncontributing positions 0 and $n + 1$.

The probability of the position i being a stabilized helical residue, $f_H(i)$, is the probability that residue i is given statistical weight w . According to the Lifson-Roig theory, a statistical weight of w can only be given to residue i if residues $i - 1$, i and $i + 1$ all have helical (ϕ , ψ) angles. Furthermore, residues $i - 1$, i , and $i + 1$ being in the helix conformation, as defined by (ϕ , ψ) angles, is a necessary and sufficient condition for the hydrogen bond between residues $i - 2$ and $i + 2$ to form (Lifson & Roig, 1961). Therefore, the probability that residue i has a hydrogen-bonded amide proton depends only on the statistical weight of residue $i - 2$:

$$f_B(i) = f_H(i - 2) \quad (6)$$

Fraction bonded is a function only of the growth and nucleation constants w and v^2 , so eq 4 has only three variables, w , v^2 , and k_c .

For a peptide which is completely coil, eq 5 reduces to a single exponential. The six-residue peptide is predicted by theory to be less than 5% helical and shows no significant helix formation by CD. The exchange behavior of this peptide, therefore, should accurately reflect the average exchange rate for the unstructured AAKAA repeat. A single-exponential fit of the exchange data for the six-residue peptide at 1.0 M

NaCl, measured at $\text{pH}^* 2.46$ and corrected to $\text{pH}^* 2.50$ (eq 3), gives $k_c = 0.018 \pm 0.001 \text{ min}^{-1}$. Information about the coil rate, however, is contained not only in the six-residue random coil peptide but also in the longer, more helical peptides, since we assume that $k_h = 0$. Equation 5, therefore, can be used to find the values of w , v^2 , and k_c which provide the best fit to all of the exchange data. Using the modified least-squares procedure described under Materials and Methods, the parameters which best describe the curves are $k_c = 0.018 \pm 0.003 \text{ min}^{-1}$, $w = 1.58 \pm 0.03$, and $v^2 = 0.0023 \pm 0.0007$. The curves corresponding to these parameters and eq 5 are shown in Figure 3.

The fit of all exchange data to the set of equations described by these parameters is relatively insensitive to the value used for k_c ; values between 0.015 and 0.021 min^{-1} do not significantly alter the fit and have only a small effect on the values determined for w and v^2 . The value of k_c can be determined with greater sensitivity from the six-residue random-coil peptide, however, and the two values are in agreement. As seen in eq 1, the exchange rate of a proton depends linearly on the chemical exchange rate constant, k_c . The dependence of exchange rate on w and v^2 is of a higher order, however, and the curves described by eq 5 are more sensitive to the values of w and v^2 . The effects of w and v^2 on fractional helicity and the calculated exchange curves are not completely independent. For any given w , the value of v^2 which best fits the data changes slightly. Changes in one constant, however, cannot completely compensate for changes in the other, because w and v^2 have different effects on helicity. The nucleation constant, v^2 , reflects both the ease of starting a helical segment and the cooperative length of the helical stretches. As a result, changing the value of v^2 can have opposite effects for long and short peptides. Since increasing v^2 makes it easier to nucleate a helix but decreases the cooperative length, long peptides have lower overall helicity as v^2 increases, thus increasing the predicted rate of exchange. Short peptides have only one helical segment, but can nucleate this segment with greater ease. Thus short peptides have a greater predicted helicity as v^2 increases, and exchange should occur at a decreased rate. Increasing the propagation parameter, w , in contrast, affects all chain lengths in the same manner. Peptides of all lengths are predicted to be more helical and exchange slower. Because w and v^2 affect predicted helicity differently, only one pair of helix-coil constants yields the best fit to all the data.

DISCUSSION

The systematic investigation of α -helix formation using peptides of de novo design provides interesting insights into the mechanism of α -helix formation. Substitution experiments made with short, alanine-based peptides (Padmanabhan et al., 1990; Padmanabhan & Baldwin, 1991; Chakrabartty et al., 1991; Merutka et al., 1990) and other monomeric peptides (Lyu et al., 1990) or dimeric coiled coils (O'Neil & DeGrado, 1990) are providing estimates of the helix propensities of the amino acids that are quite different from what was expected from host-guest studies (Wójcik et al., 1990). Other recent reports give the enthalpy of α -helix formation in water (Scholtz et al., 1991a), the effect of the α -helix macrodipole in a neutral peptide in shifting the equilibrium between helix and random coil as the ionic strength of the solution is varied (Scholtz et al., 1991c), and the stabilization energy of ion pair formation between side chains (Gans et al., 1991). These studies not only provide valuable information as to the mechanism of α -helix formation, they also illustrate the types of interactions that contribute to the stability of the α -helix in water.

Helix Fraying Confirmed by Amide Proton Exchange. The contributions of individual residues to the stability of an α -helical peptide can be determined by analyzing the helix-coil transition using the statistical mechanical models described by Zimm and Bragg (1959) or Lifson and Roig (1961). Scholtz et al. (1991b) have used these models to fit the thermal unfolding transitions, as monitored by CD, of a series of peptides of varying chain lengths and have found that the models adequately describe the observed transitions. A modification of the Zimm-Bragg model has also been employed successfully by Gans et al. (1991) in their studies of α -helix formation in short peptides. Although these formalisms use different reference states to define helical residues, the models are identical in their prediction that helicity is not distributed uniformly throughout the helix. The ends of a helix are predicted to show significant fraying while helicity increases toward the center of the chain. This prediction cannot be directly tested by circular dichroism, the method commonly employed to study helical peptides, because CD reports on a macroscopic property, the optical activity of the peptide group (Woody, 1988). Previous investigations of α -helical structure in peptides using NMR methods (Liff et al., 1991; Gans et al., 1991; Bradley et al., 1990; Pease et al., 1990; Osterhout et al., 1989) have demonstrated a nonuniform distribution of helical residues within a chain by measuring the chemical shifts of the CaH protons, coupling constants, or NOE's. These methods, which provide sequence-specific information and support the idea that α -helical peptides have strongly frayed ends, are not very amenable to quantitative analysis.

Amide proton exchange measured by NMR, however, provides an alternative method for studying the helix-coil transition which allows direct observation and quantitation of the distribution of helical residues in a chain. In this study, amide proton exchange is used to study the helix-coil transition of a series of peptides with defined lengths and sequences. When α -helical structure is formed by these peptides, the amide protons are protected from exchange both by the formation of the hydrogen bond itself and by the exclusion of solvent from the amide proton. Reduced rates of exchange with increasing peptide length can therefore be correlated directly to the helix-coil transition. As the peptide length increases, the effect of fraying of end residues should become proportionately smaller. Long peptides, therefore, are predicted to show greater protection from exchange than short peptides. Slower exchange is observed for peptides of longer chain lengths, and the length-dependent exchange behavior of this series of peptides can be predicted by the Lifson-Roig helix-coil theory.

Use of the Lifson-Roig Theory To Interpret Amide Proton Exchange in α -Helices. Although the Lifson-Roig theory defines helical and coiled residues by backbone (ϕ, ψ) angles, the theory does give information about the hydrogen-bonding pattern in the helix. Statistical weights are determined by correlating the $i - 1$, i , and $i + 1$ residues, but information about residues $i - 2$ and $i + 2$ is also included; if residue i is a stabilized helical residue with statistical weight w , then residues $i - 1$, i , and $i + 1$ all have helical (ϕ, ψ) angles and the backbone amide proton of residue $i + 2$ is hydrogen bonded to the carbonyl oxygen of residue $i - 2$. Figure 1 shows a diagram, adapted from Qian and Schellman (1991), of an eight-residue peptide in one partially helical conformation where a single hydrogen bond is formed. Residues 3, 4, and 5 all are in the h conformation, as defined by (ϕ, ψ) angles, resulting in a statistical weight of w for residue 4 and a hydrogen bond between the peptide amide proton of residue 6

and the carbonyl oxygen of residue 2. This hydrogen bond is formed even though residue 6 is in the random-coil state; only the conformations of residues 3, 4, and 5 are considered when determining whether the hydrogen bond is formed. The fractional helicities of individual residues given by the Lifson-Roig theory can therefore be converted into probabilities of hydrogen-bond formation using the recursion formula in eq 6. The Zimm-Bragg notation for the same peptide also indicates the presence of one hydrogen bond in the same location. The Zimm-Bragg treatment numbers peptide linkages rather than residues, and the hydrogen bond occurs between peptide linkages 2 and 5. Although these two theories use different reference states, they give very similar descriptions of a helical peptide.

No Direct Exchange from the Helix. In interpreting the exchange data, we assume that direct exchange from the helix does not occur. It is possible that direct exchange from the helix does occur at a very low rate by "cracking" a peptide hydrogen bond, but our results indicate that direct exchange does not contribute significantly in these experiments. Wagner and Wüthrich (1982) correlated the pattern of protection from amide proton exchange to the hydrogen-bonding network of bovine pancreatic trypsin inhibitor. They found that within a given β -structure or α -helix hydrogen-bonded amide protons exchanged approximately 10^3 -fold slower than amide protons not involved in hydrogen bonds. Although we have not measured the protection factor, our data indicate that hydrogen bonding provides substantial protection in helical peptides as well. The average exchange rate constant for an unstructured residue, k_c , was determined to be 0.018 min^{-1} . Good agreement is observed between the exchange rate constant of the six-residue peptide, which can be considered to be completely random coil, and the k_c required to fit all of the exchange data, providing further evidence that direct exchange from the helix is negligible. The chemical exchange rate constant determined here also agrees well with rates calibrated using model compounds. Using the exchange rates determined for poly(DL-alanine) (Englander & Poulsen, 1969) with corrections for side-chain effects (Molday et al., 1972) and modified as described previously (Roder et al., 1985; Robertson & Baldwin, 1991), an unstructured repeating sequence of $(\text{AAKAA})_m$ is expected to exchange with an average rate constant of 0.016 min^{-1} at 5°C , pH 2.5, and 0.1 M salt.

Use of Amide Proton Exchange To Measure Parameters of the Helix-Coil Transition. The average helix-coil constants determined for the peptides at 5°C are $w = 1.58$ and $v^2 = 0.0023$. The Lifson-Roig propagation and nucleation constants can be converted to the Zimm-Bragg parameters for comparison (see Materials and Methods), yielding $s = 1.51$ and $\sigma = 0.0019$. Parameters for the helix-coil transition in alanine-based peptides have been determined previously using CD to monitor helix formation. The values determined here by fitting amide proton exchange kinetics agree quite well with the nucleation and propagation parameters determined by Scholtz et al. (1991b) for a set of repeating-sequence peptides with the generic formula $\text{Ac-Y(AEAKA)}_m\text{F-NH}_2$, and $m = 2-8$. Values of $s = 1.35$ (at 0°C), $\sigma = 0.003$, and $\Delta H^\circ = -0.95 \text{ kcal/mol}$ of residue were determined from fitting the thermal transition curves monitored by CD. The value of ΔH° agreed with the one determined calorimetrically for the 50-residue peptide (Scholtz et al., 1991a). The values of v^2 and w found here also agree well with the parameters used by Chakrabartty et al. (1991) to fit the helix contents of a set of alanine-rich peptides that contained a single glycine at different positions.

Earlier Studies of Amide Proton Exchange in Helices. Experiments similar to those described here were conducted previously by Nakanishi et al. (1972). The amide proton exchange of poly(L-glutamic acid) with differing degrees of polymerization was monitored by infrared absorption. Samples with higher degrees of polymerization exchanged more slowly than samples with lower degrees of polymerization. From the results, they concluded that the rate of exchange decreases going from the termini to the central portions of the polymer chains. The Zimm-Bragg parameters determined for poly(L-glutamic acid) by the authors using optical rotary dispersion were not consistent with the observed exchange kinetics. The measured pH transitions of the polymers, however, do not approach 100% helix below the transition pH. As a result, the s values they determined are questionable.

Amide proton exchange as measured by NMR has been used to study helical regions in intact proteins and naturally occurring peptides. Kuwajima and Baldwin (1983) localized seven slowly exchanging amide protons of RNase S to the S-peptide helix. The protected helical residues were shown to be limited to the hydrogen-bonded residues at the C-terminal end of the helix. Wand et al. (1986) characterized the N-terminal helical segment, residues 4-15, in cytochrome *c* using amide proton exchange. The exchange rates of residues 7-10 were observed to decrease in a stepwise fashion with each amide exchanging more slowly than the previous one by a factor of 10 or more. From the results, they concluded that the N-terminus of the helix was frayed. The C-terminal portion of apamin has also been shown to be an α -helix frayed at the C-terminal end. Amide proton exchanges from the helical portion of the peptide is postulated to occur via cooperative "structure-opening" fluctuations involving the whole helix (Dempsey, 1986).

Amide proton exchange as measured by NMR has not been used previously to study de novo designed peptides of defined sequence and length. In this study, the length-dependent exchange behavior of a series of short peptides has been used to determine and quantitate the distribution of structure within the α -helix. Although the poor dispersion of amide proton peaks in the one-dimensional proton NMR spectra of these peptides precludes measurement of exchange rates of individual amide protons, two-dimensional NMR could be used to analyze helix formation by individual residues. Amide proton exchange shows great promise for studying the structures of short peptides. In addition, since amide proton exchange is becoming a popular method by which to investigate structure in proteins and folding intermediates, the study of simpler systems such as helical peptides will be useful in understanding these more complex systems.

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