behind the scatter in the TR plot.

Considering the uncertainty in k_{ion} and the different solvents, the data from this work fall quite close to the TR curve (Figure 4). They do, however, indicate that region b is not real. This was a possibility admitted by TR, who proposed this region essentially to fit the points for **10** (T^+), **19** (4,4'-$(MeO)_2D^+$), and **30** (4-$Me_2NC_6H_4CHCH_3^+$) (see Figure 4). The more extensive data from this study, particularly for the triarylmethyl cations, show that there is a single break from the constant selectivity region a to region c. Our data also suggest that there is not a single curve describing the behavior of all cations but there are separate families, for example, triarylmethyl, diarylmethyl, and xanthylium ions. These families are also apparent from the curves in Figure 1. This dependency on the type of cation is undoubtedly another factor in the scatter of the TR plot. A recent example shows how large the deviations can be. The 1-(*p*-methoxyphenyl)-2,2,2-trifluoroethyl cation has a similar k_{Az}/k_S ratio to its nonfluorinated analogue, the 1-*p*-methoxyphenethyl cation, and yet k_{ion} for the formation of the former is ~6 orders of magnitude smaller.[32]

The "Azide" Clock. As a general conclusion, the assumption of diffusion control in the "azide" clock method is valid but only for cations with k_S greater than 10^6 s^{-1}. For cations that are slightly longer lived, k_{Az} will be reasonably close to that for diffusion control, but when k_S drops to 10^3-10^4 s^{-1}, the difference will be large, and values of k_S calculated from selectivity ratios with the assumption will be overestimates. The value of 5×10^9 M^{-1} s^{-1} taken for k_d(azide), and admittedly an approximation,[7f,g] is a good choice. The actual numbers are probably slightly larger than this. For example, 7×10^9 M^{-1} s^{-1} would be an appropriate number for a diarylmethyl cation in 0-50% acetonitrile-water. However, as shown by the results of this study, there are effects of solvent and cation structure, and these are difficult to predict at the theoretical level, particularly the latter effect. The conclusion, however, is that rate constants calculated from selectivity ratios using $k_{Az} = 5 \times 10^9$ M^{-1} s^{-1} will be within a factor of 4 of the true value, providing the cation is short-lived. In highly aqueous solutions the agreement may even be better.

Acknowledgment. R.A.M. acknowledges the donors of the Petroleum Research Fund, administered by the American Chemical Society, and the Natural Sciences and Engineering Research Council of Canada for support of this research.

Supplementary Material Available: Tables of rate constants k_{Az} and k_S for the reactions of triarylmethyl and diarylmethyl cations in acetonitrile-water solutions of varying composition (3 pages). Ordering information is given on any current masthead page.

(32) Richard, J. P. *J. Am. Chem. Soc.* **1989**, *111*, 1455-1465.

Analysis of Asymmetry in the Distribution of Helical Residues in Peptides by ^1H Nuclear Magnetic Resonance

M. I. Liff, P. C. Lyu, and N. R. Kallenbach*

Contribution from the Department of Chemistry, New York University, 4 Washington Place, New York, New York 10003. *Received June 8, 1990*

Abstract: Peptides that assume full or partial helical structure in aqueous solution have provided useful models for investigating the determinants of α-helical structure. Circular dichroism (CD) spectroscopy, the usual measure of helicity, affords an estimate of the mean helix content when calibrated against suitable standards. Analysis of these systems by means of ^1H NMR makes it possible to determine precisely the location and extent of helix structure in a chain. NMR criteria for identifying helical domains include the following: NOE's between adjacent NH protons and between an NH proton at position *i* and the Cα proton at *i* + 3; values of the three-bond coupling constants $^3J_{\alpha N}$; and the relative chemical shift of the Cα protons. Application of these criteria to members of the series of partially helical synthetic peptides, succinylTyrSer$Glu_4Lys_4XXXGlu_4Lys_4NH_2$, in which sets of three amino acids are inserted between blocks of glutamic acid and lysine side chains, shows that the helix is located preferentially near the N terminus in chains with the central substitution Ala_3,Leu_3, as well as in the parent species lacking any substitution. The degree of helicity rises sharply at the N terminus to a maximum near residue 8 and diminishes gradually from Glu14 to the C terminus. Application of the NMR criteria to the peptide containing Gly_3 reveals very little helical structure in this peptide. These results suggest that helix formation in short chains does not conform to an all-or-none reaction.

Introduction

The α-helix constitutes one of the dominant structural motifs in proteins.[1] Models of the process of forming an α-helix more from initially disordered residues suggest that nucleating a helix is intrinsically more difficult than its propagation;[2] theoretical descriptions introduce two sets of equilibrium constants to take this into account.[2,3] In the notation introduced by Zimm and Bragg,[3] nucleation of a helix by forming the initial peptide hydrogen bond has equilibrium constant $\sigma s \ll 1$, while the propagation of a nucleated helix has a stability constant, *s*. The fact that certain amino acids tend to occur frequently in the helical regions within proteins of known structure, while others do so rarely,[4] raises the possibility that the sequence of amino acid side chains governs the stability or lack of stability of α-helical structure. The availability of synthetic polypeptides has made it possible to test this proposition experimentally. In an extended investigation, Scheraga's group determined experimental values of σ and *s* for each of the 20 natural amino acids, by incorporating them at low concentration as "guests" into synthetic copolymers

(1) Creighton, T. E. *Proteins*; Freeman: New York, 1984.
(2) (a) Schellman, J. A. *C. R. Trav. Lab. Carlsberg, Ser. Chim.* **1955**, *29*, 230. (b) Schellman, J. A. *J. Phys. Chem.* **1958**, *62*, 1485. (c) Gibbs, J. H.; DiMarzio, E. A. *J. Chem. Phys.* **1958**, *28*, 1247. (d) Peller, L. *J. Phys. Chem.* **1959**, *63*, 1194. (e) Lifson, S.; Roig, A. *J. Chem. Phys.* **1961**, *34*, 1963.
(3) Zimm, B. H.; Bragg, W. K. *J. Chem. Phys.* **1959**, *31*, 526.
(4) Fasman, G. D. *Prediction of Protein Structure and the Principles of Protein Conformation*; Fasman, G. D., Ed.; Plenum Press: New York, 1989; p 193.

0002-7863/91/1513-1014$02.50/0

containing hydroxyalkylated glutamine "host" side chains.[5] The results indicate values of σ from ca. 10^{-2} to 10^{-5}, and s between 0.4 and about 1.4.[5] The s values derived from host–guest experiments make it improbable that short-chain peptides (20 residues or so) will be helical. These values further suggest that temperature has only a minor influence on helix formation, since the enthalpies are relatively small and of different sign for different side chains.

Since this pioneering study, numerous examples of natural and synthetic short peptides that contain temperature-dependent α-helical structure in aqueous solution have been reported.[6-10] Besides temperature, factors that influence the extent of helical structure in short peptides include the chain length, the nature of the side chains present, and the position and arrangement of charged groups.[6-10] Individual amino acid substitutions appear to exert strong effects on the helical structure in short peptides[6-10] as in the copolymers. Several groups[7-9] have determined values of the helix-forming propensities for individual amino acid side chains on the basis of substituting them into background peptides of natural amino acids; these tend to correlate imperfectly with the host–guest copolymer results[5] as well as with scales based on the relative frequencies of occurrence of amino acids in helical sequences within globular proteins.[4,11]

One fundamental requirement in studying helical structure in isolated short peptides is precise information concerning the extent and location of helical segment(s) within a chain. The most commonly used measure of helix content is the value of the molar residue ellipticity $[\theta]$ at 222 or 208 nm in the CD spectrum.[12] This provides an estimate of the mean helix content in a protein, on the basis of calibration of the rotatory power of peptide absorbance bands in the UV near 200 nm[13] against appropriate standards. This measure cannot address a number of important issues: a $[\theta]_{222}$ or $[\theta]_{208}$ value corresponding to 50% helix and 50% coil, for example, does not distinguish between the possibilities that half the chains in solution are fully helical and the remainder fully coil or that each chain in the solution is 50% helical and 50% coil. Any intermediate situation between these extremes is also possible in principle. This uncertainty poses a major obstacle to establishing accurate scales of helical propensities or to deciphering how helical structure is determined. The recent study by O'Neil and DeGrado[10] of helix formation in a series of coiled-coil dimers couples the process of helix formation to a well-defined dimerization equilibrium between the two chains, and their scale of s values is the only one that avoids this problem because the system is essentially fully cooperative.

We present here complete assignments of the ^{1}H NMR spectra and a comparative analysis of the helical structure of four peptides in the series of 21-mers that we designate EXK

succinylTyrSerGlu$_4$Lys$_4$XXXGlu$_4$Lys$_4$NH$_2$

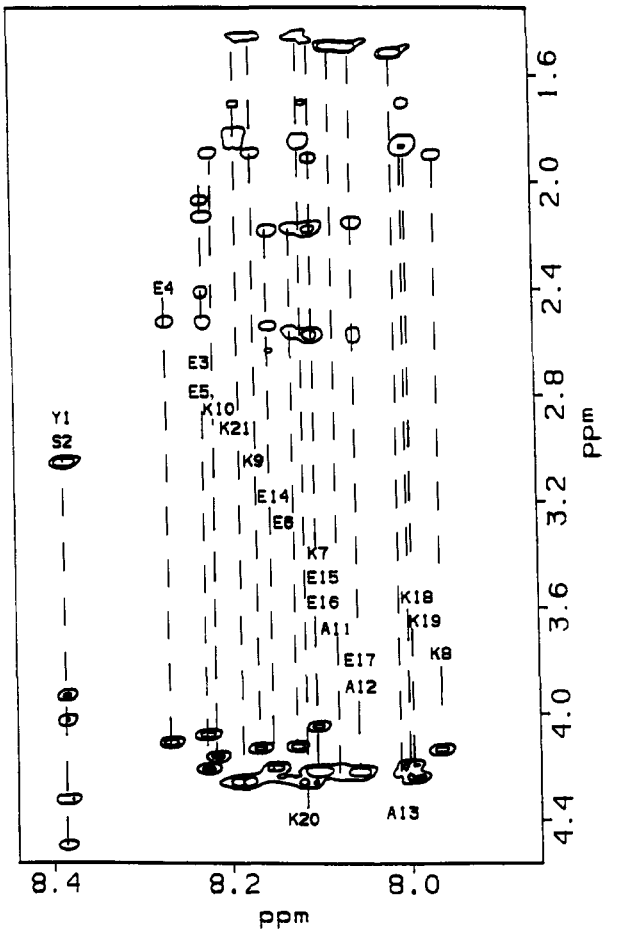

Figure 1. Fingerprint region of HOHAHA spectrum of EAK peptide at 5 mM concentration in H_2O/D_2O (90%/10%) at 25 °C; mixing time 48.8 ms.

in which the central trimers are Ala$_3$ (EAK), Leu$_3$ (ELK), and Gly$_3$ (EGK) as well as the parent species lacking the central substitution (EK). CD analysis of this series has been used to define the relative helix stabilizing propensities of 10 neutral amino acids.[9] The peptide EAK has been reported to contain a high degree of helical structure at low T and neutral pH,[9] whereas EGK contains little helix on the basis of CD spectral data. The two other chains, ELK and EK, have intermediate helix content in terms of CD data. The NMR assignments make it possible to identify residues in the chain with helical structure by using a combination of four ^{1}H NMR criteria: (i) strong NOEs between next-neighbor peptide NH protons;[14] (ii) NOEs between a peptide NH and CαH three residues away;[15] (iii) values of the three-bond $^3J_{\alpha N}$ coupling constants;[16] and (iv) values of the CαH chemical shifts.[17] We show here that each of these peptides is partially helical, with the helical residues asymmetrically distributed within each chain, being localized toward the N terminus in each helical species. EGK contains very little helix on the basis of CD and NMR criteria. Thus, helix formation in these and other short peptides is poorly approximated as an all-or-none reaction. Instead, these data can be used to construct helix probability profiles for each residue in a chain, from which it should be possible to determine more accurate s values than are now available.

(5) (a) Sueki, M.; Lee, S.; Powers, S. P.; Denton, J. B.; Konishi, Y.; Scheraga, H. A. *Macromolecules* **1984**, *17*, 148. (b) Scheraga, H. A. *Pure Appl. Chem.* **1978**, *50*, 315.

(6) (a) Brown, J. E.; Klee, W. A. *Biochemistry* **1971**, *10*, 470. (b) Bierzynski, A.; Kim, P. S.; Baldwin, R. L. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 2470. (c) Kim, P. S.; Bierzynski, A.; Baldwin, R. L. *J. Mol. Biol.* **1982**, *162*, 187. (d) Shoemaker, K. R.; Kim, P. S.; Brems, D. N.; Marqusee, S.; York, E. J.; Chaiken, I. M.; Stewart, J. M.; Baldwin, R. L. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 2549. (e) Shoemaker, K. R.; Kim, P. S.; York, E. J.; Stewart, J. M.; Baldwin, R. L. *Nature* **1987**, *326*, 563. (f) Strehlow, K. G.; Baldwin, R. L. *Biochemistry* **1989**, *28*, 2130.

(7) (a) Marqusee, S.; Baldwin, R. L. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 8988. (b) Marqusee, S.; Robbins, V.; Baldwin, R. L. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *86*, 5286. (c) Padmanabhan, S.; Marqusee, S.; Ridgeway, T.; Lane, T. M.; Baldwin, R. L. *Nature* **1990**, *344*, 268.

(8) (a) Merutka, G.; Stellwagen, E. *Biochemistry* **1989**, *28*, 352. (b) Merutka, G.; Stellwagen, E. *Biochemistry* **1990**, *29*, 894.

(9) (a) Lyu, P. C.; Marky, L. A.; Kallenbach, N. R. *J. Am. Chem. Soc.* **1989**, *111*, 2733. (b) Lyu, P. C.; Marky, L. A.; Kallenbach, N. R. *Peptides, Proceedings of the Eleventh American Peptide Symposium*; Rivier, J. E., Marshall, G. R., Eds.; ESCOM: Leiden, 1990; p 632. (c) Lyu, P. C.; Liff, M. I.; Marky, L. A.; Kallenbach, N. R. *Science* **1990**, *250*, 669.

(10) O'Neil, K. T.; DeGrado, W. F. *Science* **1990**, *250*, 646.

(11) Levitt, M. *Biochemistry* **1978**, *17*, 4277.

(12) (a) Greenfield, N.; Fasman, G. D. *Biochemistry* **1969**, *8*, 4108. (b) Johnson, W. C., Jr. *Annu. Rev. Biophys. Biophys. Chem.* **1988**, *17*, 145.

(13) Woody, R. W. *J. Polym. Sci., Macromol. Rev.* **1977**, *12*, 181.

(14) (a) Billeter, M.; Braun, W.; Wuthrich, K. *J. Mol. Biol.* **1982**, *155*, 321. (b) Wuthrich, K.; Wider, G.; Wagner, G.; Braun, W. *J. Mol. Biol.* **1982**, *155*, 311.

(15) Wuthrich, K.; Billeter, M.; Braun, W. *J. Mol. Biol.* **1984**, *180*, 715.

(16) (a) Bystrov, V. F. *Prog. Nucl. Magn. Reson. Spectrosc.* **1976**, *10*, 41. (b) Bystrov, V. F.; Arseniev, A. S.; Gavrilov, Yu. D. *J. Magn. Reson.* **1978**, *30*, 151. (c) Pardi, A.; Billeter, M.; Wuthrich, K. *J. Mol. Biol.* **1984**, *180*, 741.

(17) Pardi, A.; Wanger, G.; Wuthrich, K. *Eur. J. Biochem.* **1983**, *137*, 445.

Table I. ^{1}H Chemical Shifts in EAK Peptide in H_2O/D_2O (90%/10%) at 25 °C[a]

	NH	α	β	γ	δ	ε	others
succinyl		2.51/2.6					
Y1	8.35	4.42	3.02				7.12 (2,6H)
							6.8 (3,5H)
S2	8.35	4.36	3.90/3.99				
E3	8.19	4.18	2.1	2.38/2.39			
E4	8.23	4.08	2.07/2.11	2.50			
E5	8.19	4.05	2.04	2.50/2.51			
E6	8.09	4.09	2.15	2.54			
K7	8.07	4.01	1.88	1.44	1.65/1.60	2.97	
K8	7.93	4.10	1.87	1.38	1.65/1.58	2.96	
K9	8.13	4.10	1.86	1.43	1.64/1.55	2.93	
K10	8.18	4.13	1.86	1.42	1.67/1.53	2.97	
A11	8.05	4.17	1.46				
A12	8.03	4.19	1.47				
A13	7.98	4.19	1.49				
E14	8.12	4.17	2.15	2.51			
E15	8.02	4.18	2.15	2.54			
E16	8.07	4.18	2.15	2.54			
E17	8.02	4.19	2.13	2.55			
K18	7.97	4.17	1.84	1.52/1.42	1.68	2.9	
K19	7.96	4.21	1.84	1.52/1.42	1.68	2.9	
K20	8.08	4.23	1.82	1.50/1.45	1.67	2.98	
K21	8.15	4.23	1.79/1.83	1.48	1.68	2.99	
terminal NH_2	7.63/7.5						

[a] Chemical shifts are measured relative to internal DSP.

Results and Discussion

Assignments. The standard sequential assignment procedure[18] was applied to three peptides EAK, ELK, and EK that show helicity in CD spectra. Intraresidue spin systems are identified by means of the HOHAHA experiment.[19] Figure 1 shows the fingerprint region of the HOHAHA spectrum of EAK as an example. Each line corresponds to one residue, and each cross peak on the line belongs to the same residue. Varying the mixing time from about 25 to 90 ms allows one to locate all the protons belonging to a given spin system apart from NH_3^+ of Lys, OH of Glu or Ser, and the aromatics of Tyr. The shifts of aromatics and NH_3 groups can be located and measured elsewhere in the spectrum.

Sequential assignments in these peptides are not straightforward because of the frequent occurrence of identical residues in similar chemical environments. This problem is aggravated by the absence of unique residues within each block of four identical residues. Even if there is no overlap of the signals of two residues in the center of a block, they can still impede tracing connectivities through them because they lie close to the diagonal in the amide–amide region. Cross peaks obtained on the basis of α-proton signals tend not to be useful for sequential assignment, because a substantial number of these signals overlap. This problem becomes still more severe with β, γ, and other cross peaks. Nevertheless, peptides with higher helical content, such as EAK and ELK, turn out to have well-resolved spectra due to the difference between helical conformations in the two repeats. Figure 2 shows an example of tracing amide–amide connectivities in the NOESY spectrum of ELK.

Following identification of the spin systems, the next step in the sequential assignment strategy for these chains is to identify two EK cross peaks and two boundary SE and KX cross peaks and then test all the different pathways for connecting these. While laborious, a unique, self-consistent path emerges for all peptides, except EGK. For the latter (which has no significant helicity as shown below) very few NOE peaks are observable in the amide–amide region, while the degeneracy in the amide–α region is too strong. A ROESY experiment[20] using spin locking

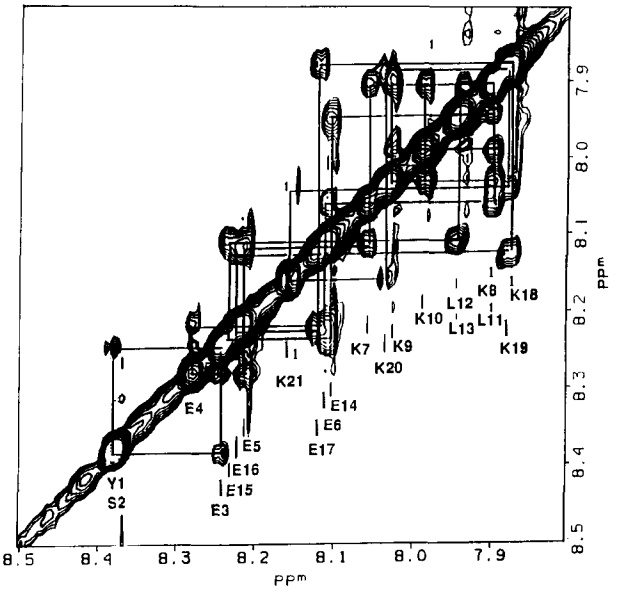

Figure 2. Amide–amide region of NOESY spectrum of ELK peptide in at 5 mM concentration at 25 °C; mixing time 250 ms.

times up to 600 ms fails to provide new peaks in the amide–amide region and thus does not help to resolve overlapping peaks in the α-amide region. Since the assignment in analogous peptides with greater helical structure is feasible, we induced helicity in EGK artificially by adding 10 mol % TFE to the water solution. This amount of TFE shifts the equilibrium between helix and coil to favor helix.[21,22] In the presence of TFE, the problem of assignment of EGK becomes comparable in difficulty to that shown in Figure 2. Once this is done, assignment of the spectrum of EGK in water is achieved by monitoring gradual changes in peak positions in spectra as the fraction of TFE is progressively reduced. As an example of a complete assignment, the chemical shifts for EAK are presented in Table I.

A characteristic feature in all these peptides is the coincidence or near coincidence in chemical shift of the two β-protons for every side chain except those at the ends. This would occur if, in each chain, the helical conformation is in fast exchange with other states

(18) Wuthrich, K. *NMR of Proteins and Nucleic Acids*; Wiley-Interscience: New York, 1986.
(19) (a) Braunschweiler, L. R.; Ernst, R. R. *J. Magn. Reson.* **1983**, *53*, 521. (b) Bax, A.; Davis, D. G. *J. Magn. Reson.* **1985**, *65*, 355. (c) Davis, D. G.; Bax, A. *J. Am. Chem. Soc.* **1985**, *107*, 2821.
(20) (a) Stephene, A. A.; Lee, R. L.; Warren, E. D.; Jeanloz, R. W. *J. Am. Chem. Soc.* **1984**, *106*, 811. (b) Bax, A.; Davis, D. G. *J. Magn. Reson.* **1985**, *63*, 207.
(21) Nelson, J. W.; Kallenbach, N. R. *Proteins* **1986**, *1*, 211.
(22) Nelson, J. W.; Kallenbach, N. R. *Biochemistry* **1989**, *28*, 5256.

Figure 3. Diagram of sequential amide–amide NOEs for EAK, ELK, EK, and EGK peptide, for which central block XXX is equal to, respectively, AAA, LLL, absent, and GGG. Normalization between the rows is relative to the signal of the standard DSP, which was kept at constant concentration through all the peptide solutions of this series. The lower case x in the diagram indicates spectral overlap.

on the NMR time scale. The alternative requires that each side chain be sufficiently mobile in the helix to average the chemical shifts of the β-protons.

Secondary Structure. To compare different peptides in their ability to form helical structures, we apply four criteria of helicity: sequential and medium-range NOEs; values of $^3J_{\alpha N}$ coupling constants; and values of chemical shifts of α-protons. Taken together, these provide a reliable means of characterizing the local secondary structure in these molecules.

(i) **Sequential NOEs.** A strong statistical correlation has been demonstrated between sets of short distances (<2.8 Å) connecting adjacent peptide NH protons and α-helical secondary structure in globular proteins.[14,18] Figure 3 summarizes the relative intensities of the sequential NH–NH NOE cross peaks corresponding to these distances that are observed in NOESY experiments on these peptides. Normalization within each row corresponding to a given peptide is done relative to the most intense peak in that row. The normalization between rows is tentative, based on the peak of the standard DSP, which was kept at the same concentration in each solution. This normalization is performed only to show that EAK and ELK have strong sequential NOE peaks relative to the parent peptide, while EGK has almost no peaks. The diagram shows that NOE peaks are strongest near the center of a molecule, while their intensity decays toward either terminus, more abruptly for the C terminus than for the N terminus. It should be noted that the presence of sequential amide–amide NOEs is a necessary but insufficient criterion for helical structure.

(ii) **Medium-Range NOEs.** A more stringent criterion for helical structure is the presence of NOESY cross peaks connecting α-protons with amide NH protons in the chain three residues away.[15,18] Such $i, i + 3$ NOEs define helical conformation with a very high degree of reliability when they occur in sequential series. Figure 4 shows the α-amide region of the NOESY spectrum of ELK. It is possible to locate at least five $i, i + 3$ peaks in ELK:

E6K9, K7K10, K9L12, K10L13, E15K18

Additional cross peaks may be present but are obscured by α-amide peaks belonging to the same residue or to the adjacent $i + 1$ residue. Medium-range NOEs involving β-protons[18] were not used for analysis here due to severe overlapping in this region. No $i, i + 3$ peaks could be located for EK and EGK. The NOE patterns thus indicate significant α-helical structure in EAK and ELK, some amount in EK, and essentially no helical structure in EGK. This is consistent with the helical propensities of these amino acids deduced from CD spectroscopy.[9] However, it is difficult to distinguish between the relative efficiency of Ala and Leu from the NOE data exclusively because there is no mutual yardstick to compare the intensities of $i, i + 1$ or $i, i + 3$ NOE cross peaks for the two systems. NOE intensity measurements are not easy to quantitate since this requires distinguishing differences between sets of peaks that are very close in values.

(iii) **$^3J_{\alpha N}$ Couplings.** These coupling constants are known to give unambiguous information on conformational state of the peptide backbone.[16] It is difficult in practice to measure them accurately in helical regions without isotopic labeling.[23] To avoid collecting FIDs with over 4K points and difficulties in simulating overlapped split peaks, we determined only approximate values of spin couplings. The measured couplings were divided into four groups: those with $J < 4.8$ Hz; $5 < J < 6$ Hz; $6 < J < 6.8$ Hz; and $6.8 < J < 7.3$ Hz. Figure 5 summarizes the values of the $^3J_{\alpha N}$ coupling constants determined for EAK, ELK, and EK. Residues in the center of EAK and ELK and near the N terminus have values of J close to those estimated for helix, about 3.9 Hz.[16] EGK shows J-coupling values that are remote from helix values or those for any other secondary structures. The helical values for EAK and ELK approach random coil values near the C terminus. These observations are in complete agreement with the results obtained from NOE measurements.

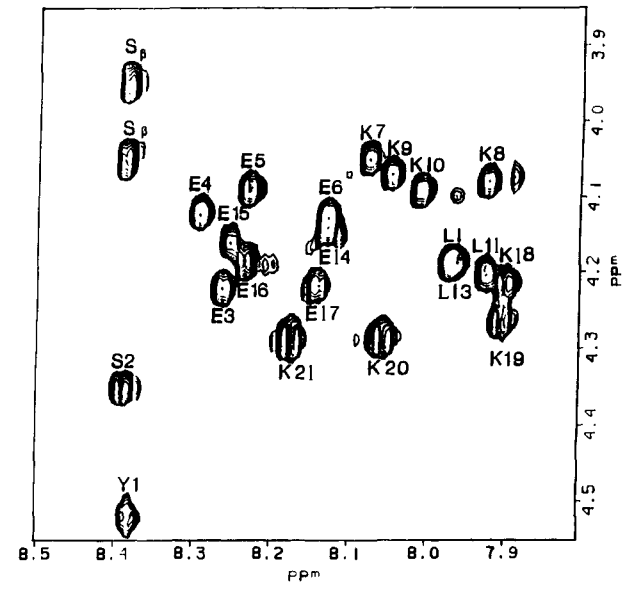

Figure 4. α-Amide region of the NOESY spectrum of the ELK peptide under the conditions given in the legend to Figure 2.

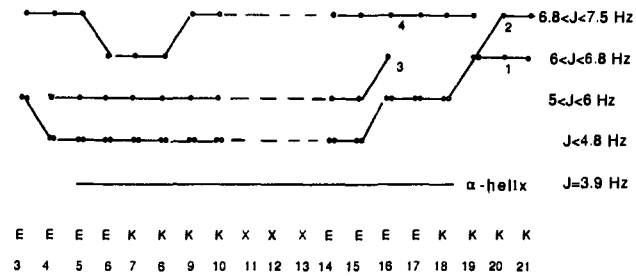

Figure 5. Diagram of spin-coupling constants $^3J_{\alpha N}$ for EAK (1), ELK (2), EK (3), and EGK (4) peptides.

Even these J-coupling measurements fail to distinguish between the effect of the two residues Ala and Leu and thus cannot confirm or refute the order of stabilizing helix, Ala > Leu, suggested by circular dichroism measurements.[9] A difference in the J values for K19 tends to support the CD order but is too small given the accuracy of measurement to warrant such a conclusion. There thus remains a need for an additional criterion to solve this problem.

(iv) **α-Proton Chemical Shifts.** It has been repeatedly observed that formation of helical structure tends to be associated with upfield shifts in the α-proton resonances of the residues involved,[17,22,24] although shifts per se are rarely used for secondary structure elucidation because so many factors can also affect chemical shifts. However, in cases where the dependence of helical structure on solvent or pH is to be studied, the CαH shifts provide

(23) Neri, D.; Otting, G.; Wuthrich, K. *J. Am. Chem. Soc.* **1990**, *112*, 3663.
(24) Szilagyi, L.; Jardetzky, O. *J. Magn. Reson.* **1989**, *83*, 441.

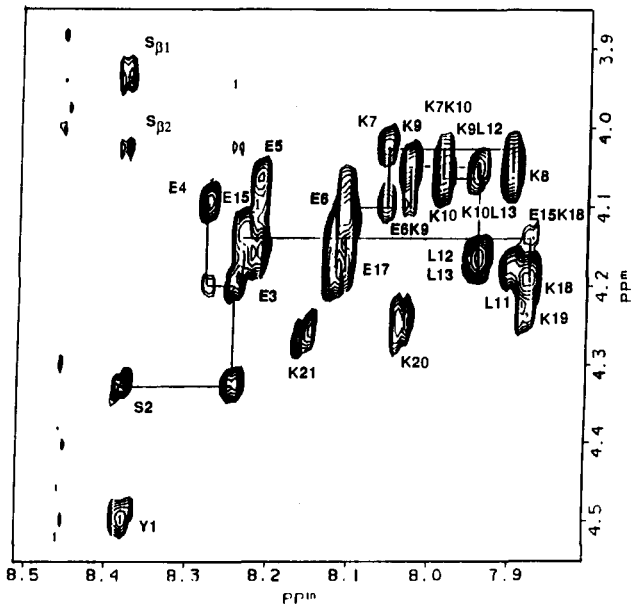

Figure 6. α-Amide region of the HOHAHA spectrum of ELK peptide at 5 mM concentration in H_2O/D_2O (90%/10%) at 25 °C; mixing time 49.2 ms.

a practical guide to defining helical conformation.[22] In the present series of peptides, the sequences are homologous over most of the chain, making it reasonable to attribute differences in chemical shifts of the homologous residues to differences in secondary structure. Figure 6 shows the α-amide region of the HOHAHA spectrum of ELK as an example. It can be seen that the CαH of residues K7, K8, K9, and K10 located in the central helical block resonate at significantly higher field than those in less structured C-terminal block, K18, K19, K20, and K21. Of these, K8 and K19, for example, lie in identical next-neighbor sequences. But if we compare the chemical shift of residue K21 in EAK and in ELK, any difference in the chemical shifts between the CαH of K21 in these two chains is unlikely to reflect a sequence effect. The NOE and coupling constant measurements described above provide independent, direct evidence for a difference in the extent of helical structure between peptides. Hence, the difference in shifts can be assigned with confidence to a difference in helicity or, more properly, the local equilibrium between helical and random coil conformations at this site. Comparison of chemical shifts in identical positions among peptides shows that apart from residues located in the immediate neighborhood of the variable XXX block the remaining residues in these chains exhibit the following order with respect to the upfield shift of CαH: EAK > ELK > EGK. This is the order observed by CD, in which Ala stabilizes helix significantly more than does Leu.

Thus, while the factors that control chemical shifts are complex, the particular character of these peptides in which guest residues cluster in one part of the molecule, flanked by extended and invariant host sequences, makes it possible to use the CαH shifts as an effective probe for helical structure.

Effect of Trifluoroethanol. The helix-forming solvent TFE[21,22] has been used to induce helicity in EGK to remove degeneracy in its spectra. In addition, TFE permits a direct test of the hypothesis that the CαH chemical shifts reflect secondary structural differences among chains. The difference between the chemical shifts of α-protons in water and following addition of 10 mol % TFE is shown in Figure 8. In the case of EGK, signals of different residues shift markedly on addition of TFE, reducing the overlap. The shifts most affected correspond to residues near the N terminus, and they almost reach values seen in ELK, which is helical in this region as revealed by NOE experiments and coupling constant values. Acquisition of helical structure in this segment of EGK when TFE is added is confirmed by $^3J_{\alpha N}$ measurements (Figure 9), together with observation of a helical S2, E5 cross peak in the α-amide region of the corresponding NOESY

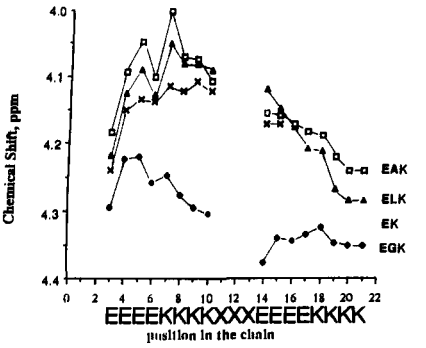

Figure 7. Diagram of α-proton chemical shifts for EAK, ELK, EK, and EGK peptides. Shifts were measured from HOHAHA at identical conditions at 25 °C.

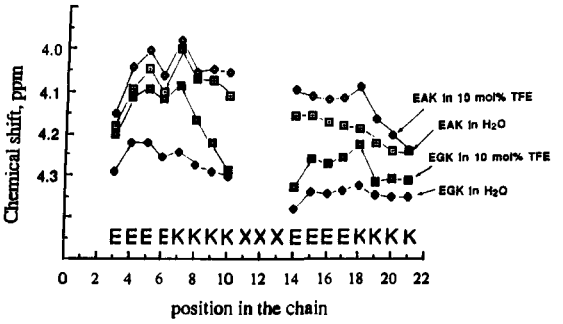

Figure 8. Influence of TFE on chemical shifts of α-protons for EAK and EGK peptides.

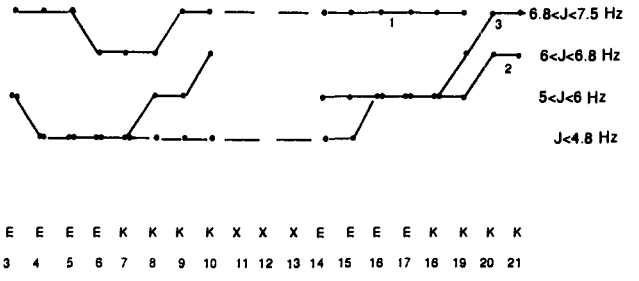

Figure 9. Comparison of $^3J_{\alpha N}$ couplings for EGK in water (1) and in 10 mol % TFE (2). Couplings for ELK in water (3) are given for comparison.

spectrum. The coupling constants of several residues in EGK (Figure 9) near the N terminus fall below 4.8 Hz in the presence of 10 mol % TFE, consistent with significant helical structure.

In the vicinity of the Gly residues, the J values increase and chemical shifts of α-protons in the adjacent K block move sharply downfield, both indicative of loss in helical structure. This is an interesting result. Helix formation (or loss) has been thought to be a cooperative process, involving interaction among all the residues within a short chain; very short chains should tend to approximate all-or-none systems if the host–guest parameters apply.[5] While a global effect of glycine is reflected in the loss of overall helicity in the molecule, the extent of this loss differs at each position in the chain, reaching a maximum in the vicinity of the GlyGlyGly block. Thus, there appears to be a significant local component of the destabilizing effect on Gly on the helix.

A second interesting effect of TFE in this series of peptides can be seen in EAK and ELK (Figure 9). Titration of either of these peptides with 10 mol % TFE produces an extreme in the value of $[\theta]_{222}$, which we have taken to represent complete helix formation in these chains.[9] The chemical shifts of the CαH of residues in the region near the H terminus move further upfield on addition of TFE. While the α-protons of residues near the C terminus also move upfield, the final state of the chain achieved is not fully helical over the entire chain. That is, the helix in these peptides remains nonuniformly distributed even under the strong

stabilizing influence of TFE. It has previously been noted that the propagation of the α-helix in the S peptide of ribonuclease A beyond Met13 is inhibited in TFE. This effect has been attributed to some helical termination signal in the sequence of this peptide.[25] The present observations suggest that some such effect might be operative in the EXK peptides also.

Conclusions

A number of peptides that form α-helical structures in aqueous solution have now been investigated by ^{1}H NMR, including the neurotoxin apamin, from the venom of the honeybee,[26] the S and C peptides of bovine ribonuclease A,[6,27] and a variety of synthetic molecules such as the chains investigated here. In apamin, which consists of 18 amino acids, the helix occupies the 9 residues at the C terminus of the chain. The three residues at the C terminus appear to fray, since the peptide NH groups exchange rapidly with water. Replacement of the helical sequence of apamin with that of the C or S peptides shows that the helix still terminates well before the C terminus, despite the stabilization due to the disulfide bridges in the molecule.[28] Thus, loss of helicity near the C terminus observed in the series of peptides we have studied might reflect some intrinsic asymmetry due to the sequence present or promotion of N-terminal nucleation by the succinyl group and/or the Ser residue, which we included as a capping group.[29]

The precise definition of helix structure within this series of peptides has implications both for our own scale of s values and for those determined by other groups.[7-9] Analysis of CD data on partially helical members of these series has been based on assuming that helix formation in such short chains is fully cooperative, the all-or-none model. Instead, these systems seem to require analysis in terms of multistate models, in which one or more helical sequences can occupy various positions in the chain. The present information can be used to recalibrate the CD results, which assume that a value of $-32\,000$ deg·cm^2·dmol^{-1} corresponds to 100% helicity in chains such as these.[7,9] Calculations based on the latter kind of model suggest that presence of one helical sequence fraying at the ends accounts for the structure in the EXK peptides quite well. This picture seems consistent with the thermal transition behavior of these peptides also. The transition profiles monitored by CD show weak cooperativity, revealing a gradual loss of helicity with temperature from 0 to 90 °C, in contrast to the strongly cooperative unfolding transitions in globular proteins.[1] To understand the temperature dependence of the helical structure in these peptides will require determination of σ and the set of s values as a function of temperature.

At present the actual mechanism by which a specific side chain exerts its effect on helical structure is uncertain. In a number of peptides,[7,8] substitution of single residues has been found to produce a roughly additive effect on the helical structure present (at least to a first approximation). Thus, a side chain seems to exert its effect individually rather than via strong neighbor or next-neighbor interactions. In the EXK system, we have verified that single substitution of amino acids in the central block is also nearly additive in its effect. Current thinking tends to implicate the differences in the rotamer population of each side chain between the helical state relative to the population it can access in the coil form as a factor.[10,30] The conformational freedom of Gly in the coil is greater than for other amino acids, and this may explain the destabilizing effect of Gly on α-helix formation.[30] On the other hand, the restriction in the rotamer population of Ala in the helix state is probably less than for bulkier side chains and hence leads to its stabilizing helix relative to Leu or Met. Whether or not these considerations play a role in helix stabilization needs to be addressed both experimentally and theoretically. Consistent and accurate information regarding the helical propensities of different side chains is fundamental to this effort, and application of NMR criteria should prove essential in generating this information.

Experimental Section

Peptide Synthesis. The synthesis of parent peptide and succinylation procedures are as described previously.[9] All other peptides were synthesized by solid-phase peptide synthesis on a Milligen/Biosearch 9600 synthesizer using Fmoc chemistry.[31] Following treatment with TFA, peptides were purified on a preparative reverse-phase C_{18} HPLC column using a gradient of 0–30% acetonitrile in 0.1% trifluooroacetic acid and subsequently desalted on Sephadex G-10 columns.

Characterization of Peptides. Purity of each peptide was verified by analytic HPLC following purification and determination of the correct primary ion molecular weight by fast atom bombardment mass spectrometry (MScan, Inc., West Chester, PA).

^{1}H NMR Spectroscopy. NMR spectra were obtained on a Bruker AM-500 spectrometer. Samples consisted of 5 mM peptide in 10% D_2O/90% H_2O. TPPI[32] was used to obtain phase-sensitive DQF COSY,[33] NOESY,[34] and HOHAHA[19] spectra. All pulses in HOHAHA experiments were generated via the high-power decoupler channel (90° pulse = 22–26 μs). HOHAHA spectra were recorded with different mixing times (ca. 25, 50, and 90 ms) to trace direct and relayed through-bond connectivities. A mixing time of 250 ms was used in the NOESY spectra. From 512 to 1024 FIDs were collected for each spectrum, using 2K complex points; 4K points were used for determination of coupling constants in DQF COSY spectra. Zero filling was done in the t_1 dimension to obtain a final square matrix of 2048 by 2048 real points. In DQF COSY a matrix of 4K points in the t_2 dimension was collected and reduced to 2K points following the first Fourier transformation to save memory space; further processing involved only half of the spectrum in the t_2 dimension, which is sufficient to measure $J_{\alpha N}$. In all spectra the first row of the (t_1,ω_2) matrix was divided by 2 before the second Fourier transform to suppress ridges in the t_1 dimension.[35] Sine windows were used in both dimensions with phase shifts between 0 and 60 °C. All data processing was done with FTNMR software (Hare Research, Inc). The temperature was controlled at 25 °C. The water signal was suppressed by presaturation during the delay, t_1, and also during the mixing period in NOESY experiments.

Acknowledgment. This research was supported by a grant from NIH (GM 40746). The peptide synthesizer was acquired with support from NSF (Grant DIR 87-22895). The Bruker AM 500 NMR spectrometer was purchased with grants from NIH (RR02497) and NSF (DMB 84-13986).

(25) Kim, P. S.; Baldwin, R. L. *Nature* **1984**, *307*, 329.
(26) Wemmer, D. E.; Kallenbach, N. R. *Biochemistry* **1983**, *22*, 1901.
(27) Rico, M.; Santoro, J.; Bermejo, F. J.; Herranz, J.; Nieto, J. L.; Gallego, E.; Jimenez, M. A. *Biopolymers* **1986**, *25*, 1031.
(28) Wemmer, D. E. Private communication.
(29) Richardson, J.; Richardson, D. *Science* **1988**, *240*, 1648.
(30) Hermans, J.; Yun, R.-H.; Anderson, A. G. Private communication. (b) Piela, L.; Nemethy, G.; Scheraga, H. A. *Biopolymers* **1987**, *26*, 1273.
(31) Bodanszky, M. *Principles of Peptide Synthesis*; Springer-Verlag: New York, 1984.
(32) Marion, D.; Wuthrich, K. *Biochem. Biophys. Res. Commun.* **1983**, *113*, 967.
(33) Rance, M.; Sorensen, O. W.; Bodenhausen, G.; Wagner, G.; Ernst, R. R.; Wuthrich, K. *Biochem. Biophys. Res. Commun.* **1983**, *117*, 479.
(34) (a) Jeener, J.; Meier, B. H.; Bachmann, P.; Ernst, R. R. *J. Chem. Phys.* **1979**, *71*, 4546. (b) Bodenhausen, G.; Kogler, H.; Ernst, R. R. *J. Magn. Reson.* **1984**, *58*, 370.
(35) Otting, G.; Widmer, H.; Wagner, G.; Wuthrich, K. *J. Magn. Reson.* **1986**, *66*, 187.