Effects of Trifluoroethanol on the Conformations of Peptides Representing the Entire Sequence of Bovine Pancreatic Trypsin Inhibitor

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ABSTRACT: The effects of the cosolvent trifluoroethanol on the conformations of four peptides representing the entire sequence of bovine pancreatic trypsin inhibitor (BPTI) have been measured by CD and NMR. No substantial amounts of helical conformations were induced in one peptide with four proline residues dispersed throughout its sequence, and there were no substantial effects on its average conformational properties or on the interactions between neighboring residues that are normally evident. The other three peptides became helical, although not completely, over their entire lengths. There was a reasonable correlation between the induced content of α-helix and the predicted helical propensities of all four peptides. Only one of these peptides is helical in native BPTI; the other two are extended β-strands. The latter two have an intrinsic propensity for helix formation, but a greater propensity for β-sheet formation in folded proteins.

The intrinsic conformational tendencies of the linear polypeptide chain may well be very important for the process of protein folding to the native conformation. Certainly, these tendencies often appear to anticipate the conformation that residues will adopt in the native conformation (Segawa et al., 1991; Dyson et al., 1992a,b). As a consequence, it is possible to predict the secondary structure of the native conformation from just the sequence, not infallibly, but with greater accuracy than would otherwise be expected. Experimental observations of the folding process also suggest this: folding appears to occur rapidly because some unstable elements of local conformation in the unfolded protein tend to interact, and stabilize each other, to produce very unstable, transient intermediates that cannot be detected. Although unstable, these putative intermediates should be more stable than would be expected in the absence of such interactions. These intermediates can lead to structures that are sufficiently cooperative to generate net stability of the folded conformation (Creighton, 1995).

For these reasons, characterization of the conformational tendencies of unfolded proteins, and especially of the more amenable peptide fragments, is being pursued very actively (Dyson et al., 1988, 1992a,b; Sancho et al., 1992; Alexandrescu et al., 1994; Creighton & Shortle, 1994). One difficulty with these studies is that the tendency to adopt any particular conformation is often very weak, and any particular conformation is adopted in only a small fraction of the molecules at any time. A useful technique to magnify these tendencies, especially that to form α-helices, is to add trifluoroethanol (TFE[1]) as a cosolvent (Tamburro et al., 1968; Nelson & Kallenbach, 1986). This enhances the helicity of peptide segments, but apparently only if the residues have an intrinsic propensity to adopt that conformation (Nelson & Kallenbach, 1989; Lehrmann et al., 1990; Dyson et al., 1992a,b; Storrs et al., 1992; Sönnichsen et al., 1992; Jasanoff & Fersht, 1994; Jiménez et al., 1994). Consequently, it is a sensitive and useful method for measuring the α-helix tendency of polypeptide segments. The mechanism of action of TFE is not certain, however, and more data are required to assess its usefulness and mode of action.

The effects of TFE on the conformations of four peptides representing the entire sequence of BPTI are reported here. These peptides correspond to the four main structural elements of native BPTI; they have been characterized previously by CD and by NMR in aqueous solution (Kemmink & Creighton, 1993). They were shown to mimic the corresponding segments of intact reduced BPTI, which has no stable long-range structure, even under conditions normally favoring folding. The peptides were observed to have individual conformational tendencies, which only in some instances corresponded to their conformation in native BPTI. In addition, a number of unexpected local interactions involving amino acid side chains were detected. The roles of these interactions in folding of reduced BPTI could be determined, because the conformational properties of all the disulfide intermediates are known (van Mierlo et al., 1994). Some of the local interactions contribute to folding, whereas others do not, but are disrupted by the folding process. As the interactions must overcome the conformational entropy of the unfolded state, they are only weak in the unfolded protein, and their net energetic contributions are only small. The physical natures of the local interactions have been characterized (Kemmink & Creighton, 1995). All were weakened by increasing temperature, but only some by the denaturants urea and guanidinium chloride; therefore, some such interactions can also be present under unfolding conditions. The effects of TFE on these interactions are also pertinent to understanding the conformational tendencies of polypeptide chains and the effects of the cosolvent.

EXPERIMENTAL PROCEDURES

The peptides and the methods to characterize them have been described previously (Kemmink & Creighton, 1993),

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[1] Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; CD, circular dichroism; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, 2D NOE spectroscopy; ppm, parts per million; TFE, 2,2,2-trifluoroethanol; Θ_{222}, mean residue ellipticity at 222 nm; 2D, two-dimensional.

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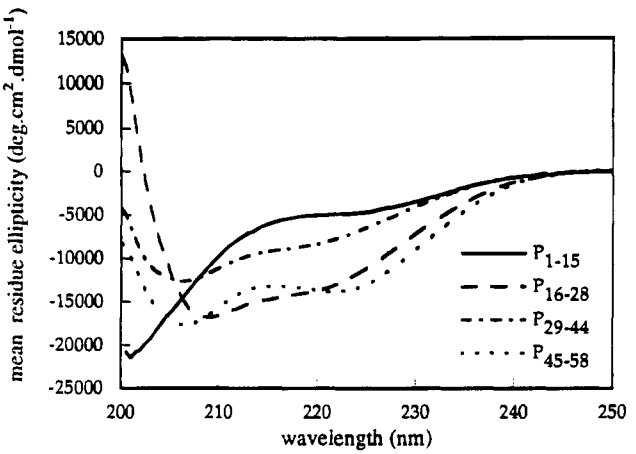

FIGURE 1: Far-UV CD spectra of the peptides in the presence of 50% (v/v) TFE. The buffer was 0.5 mM phosphate buffer, pH 7.0, and the temperature 273 K.

but peptide 29−44 was synthesized again to incorporate the N-terminal acetyl group that was missing previously. The primary difference in the present study was that the solvents contained 50% (v/v) TFE-d_3 (Cambridge Isotope Laboratories). NMR samples containing 4−10 mg of peptide were adjusted to pH 4.6 by adding small amounts of KOH or HCl solutions, before adding the TFE. The CD samples were buffered with 1 mM phosphate buffer at pH 7.0 prior to adding the TFE. CD measurements were made at 273 K and NMR measurements at 271 and 283 K.

RESULTS

The Peptides. The native structure of BPTI can be considered to consist of four roughly parallel segments of polypeptide chain, residues 1−15, 16−28, 29−44, and 45−58, packed together to form a very stable core of hydrophobic residues (Deisenhofer & Steigemann, 1975). Peptides corresponding to these four primary segments were studied; the residues are numbered as in intact BPTI. All cysteine residues were replaced by serine; the termini corresponding to internal positions were blocked with acetyl or amide groups at the N- and C-termini, respectively. The properties of these peptides were described previously in aqueous solution at low temperatures, to maximize the presence of any nonrandom conformations (Kemmink et al., 1993; Kemmink & Creighton, 1993).

TFE induces varying amounts of helicity in peptides by apparently increasing the equilibrium constant for helical content in particular residues; the maximum extent of helix is generally reached by 20−30% (v/v) TFE and complete by 50% (Jasanoff & Fersht, 1994). The CD and NMR spectra of the peptides in the presence of 50% TFE are reported here. The CD spectra (Figure 1) give quantitative estimates of the average content of helical conformation (Table 1), although other factors can influence these spectra (Manning & Woody, 1989; Chakrabartty et al., 1993b). All the NMR spectra were fully assigned (Table 2), so that the residues in helical conformations could be determined from the pattern of interresidue NOEs (Figure 2) and from changes in the chemical shifts of the backbone protons (Figure 3); the α-helical conformation causes the chemical shift of the $C^{\alpha}H$ protons to move upfield (Nelson & Kallenbach, 1989; Wishart et al., 1991; Herranz et al., 1992). The presence of TFE seemed to have very little effect on the chemical shifts of residues, unless the conformation was altered.

Table 1: α-Helix Content of the Peptides in the Presence and Absence of TFE Indicated by the Ellipticity at 222 nm

	no TFE		50% TFE	
peptide	$-\Theta_{222} \times 10^{-3}$	% α[a]	$-\Theta_{222} \times 10^{-3}$	% α[a]
1−15	0.09	<1	4.39	13
16−28	1.01	3	13.24	41
29−44	1.25	4	7.87	23
45−58	1.60	5	14.11	43

[a] Percent α-helical conformation indicated by the value of Θ_{222}, calculated as $100(\Theta_{222}/\Theta_{222}^{max})$, where $\Theta_{222}^{max} = -40000[1 - (2.5/n)]$ and n is the number of residues in the peptide (Forood et al., 1993).

Peptide 1−15. This segment is very irregular in native BPTI, but contains one turn of 3_{10}-like helix at residues 3−7. It also contains four proline residues at positions 2, 8, 9, and 13. The NMR and CD spectra of this peptide in aqueous solution indicated that it was largely disordered, with multiple conformations and the expected presence of *cis* isomers of the peptide bonds preceding the proline residues. There were, however, very distinctive nonrandom conformations involving Tyr10, which interacts with Gly12 or, when there is a *cis* peptide bond before Pro9, with Pro8 and Pro9 (Kemmink et al., 1993; Kemmink & Creighton, 1993, 1995). Residues 3−6 also appeared to adopt nonrandom conformations, but they could not be elucidated.

In the presence of TFE, this peptide adopted relatively little α-helical conformation: no more than 13% by CD (Figure 1, Table 1) and none by NMR (Figures 2 and 3). Although many of the expected helical NOE cross peaks would have been obscured by spectral overlap, there were only minor changes in the chemical shifts of the backbone protons (Figure 3). The absence of α-helix is not surprising in view of the presence of the four proline residues throughout this peptide. In addition to the absence of helix, the NMR spectra gave no evidence for changes in the conformation of this peptide produced by the TFE (Figure 2). Strikingly, the interaction between Tyr10 and Gly12 was still present, as apparent from the very anomalous chemical shift of the NH of Gly 12 (Table 2). There was no major change in the proportion of *cis* isomers of each of the peptide bonds preceding the four proline residues, but the multiplicity of such isomers, each of which can produce a unique spectrum, would make it impossible to detect any small changes.

Peptide 16−28. In native BPTI, this segment contains one β-strand of residues 18−24, with a type I β-turn at residues 25−28 leading to the second β-strand. Its CD and NMR spectra in aqueous solution indicated the presence of very many conformations, but with a tendency to remain extended (Kemmink & Creighton, 1993). There was also a distinct interaction between the side chains of Ile19 and Tyr 21 that caused the Ile19 $C^{\gamma}H_3$ resonance to be displaced upfield to 0.66 ppm. Likewise, an interaction between the Tyr23 side chain and the $C^{\alpha}H$ and $C^{\beta}H_3$ groups of Ala25 was apparent from the presence of NOEs between them and from an upfield displacement of the chemical shift of Ala25 $C^{\alpha}H$ resonance (Kemmink & Creighton, 1993).

In the presence of TFE, the CD spectra of this peptide indicated that it became largely helical (Figure 1, Table 1). This helix spanned the entire peptide, as evident from the NOEs (Figure 2) and the $C^{\alpha}H$ chemical shifts (Figure 3). The CD intensity indicated an average α-helix content of only 41%. This peptide has, however, three contiguous aromatic residues, Tyr21, Phe22, and Tyr23, which will

Table 2: ^1H Chemical Shifts of the Peptides in 50% ^1H_2O/50% TFE-d_3 at 271 K

residue	NH	$C^\alpha H$	$C^\beta H$	$C^\gamma H$	other
			Peptide 1−15		
R1		4.35	2.03, 1.76	1.76	3.26 ($C^\delta H_2$); 7.58 ($N^\epsilon H$)
P2		4.47	2.24, 1.70[a]	2.03[a]	3.76, 3.60 ($C^\delta H$)
D3	8.44	4.59	2.73, 2.69		
F4	8.00	4.67	3.24, 3.08		7.28 ($C^\delta H$); 7.36−7.28 ($C^\epsilon H$,$C^\zeta H$)
S5	8.15	4.42	3.92, 3.82		
L6	7.98	4.43	1.66	1.66	0.99, 0.93 ($C^\delta H_3$)
E7	8.04	4.68	2.11, 1.92	2.41	
P8		4.64	2.33, 1.82	2.07	3.87, 3.69 ($C^\delta H$)
P9		4.46	2.24, 1.91[a]	2.03[a]	3.77, 3.61 ($C^\delta H$)
Y10	7.94	4.64	3.16, 3.01		7.17 ($C^\delta H$); 6.85 ($^{C\epsilon}H$)
T11	7.99	4.39	4.39	1.16	
G12	6.82	4.09, 3.95			
P13		4.51	2.34, 2.02	2.07	3.71, 3.60 ($C^\delta H$)
S14	8.38	4.49	3.98, 3.91		
K15	8.31	4.35	1.94, 1.80	1.50	1.72 ($C^\delta H_2$); 3.00 ($C^\epsilon H_2$)
			Peptide 16−28		
A16	8.19	4.09	1.48		
R17	8.35	4.05	1.89[a]	1.80, 1.70[a]	3.23 ($C^\delta H_2$); 7.26 ($N^\epsilon H$)
I18	7.52	3.96	2.14	1.61, 1.21	1.05 ($C^\gamma H_3$); 0.95 ($C^\delta H_3$)
I19	7.92	3.88	1.97	1.73, 1.28	1.01 ($C^\gamma H_3$); 0.91 ($C^\delta H_3$)
R20	8.22	4.11	1.96, 1.82[a]	1.70[a]	3.20 ($C^\delta H_2$); 7.27 ($N^\epsilon H$)
Y21	7.94	4.23	3.26, 3.17		6.83 ($C^\delta H$); 6.72 ($C^\epsilon H$)
F22	8.52	4.29	3.25		7.33 ($C^\delta H$); 7.36−7.33 ($C^\epsilon H$,$C^\zeta H$)
Y23	8.95	4.15	3.17		7.19 ($C^\delta H$); 6.86 ($C^\epsilon H$)
N24	8.35	4.43	2.89, 2.78		7.75, 6.82 ($N^\delta H$)
A25	8.17	4.07	1.35		
K26	8.05	4.16	1.81	1.42	1.65 ($C^\delta H_2$); 2.95 ($C^\epsilon H_2$)
A27	8.05	4.22	1.33		
G28	7.99	3.90			
			Peptide 29−44		
L29	8.04	4.28	1.65	1.67	0.96, 0.90 ($C^\delta H_3$)
S30	8.20	4.31	4.04, 3.92		
Q31	8.66	4.19	2.18	2.47	7.44, 6.76 ($N^\epsilon H$)
T32	8.04	4.11	4.24	1.26	
F33	7.96	4.48	3.25		7.28 ($C^\delta H$); 7.34−7.32 ($C^\epsilon H$,$C^\zeta H$)
V34	7.99	3.79	2.06	1.05, 0.83	
Y35	8.33	4.42	3.15, 3.09		7.16 ($C^\delta H$); 6.85 ($C^\epsilon H$)
G36	8.39	3.98,3.89			
G37	8.23	3.96,3.90			
S38	8.18	4.35	4.04, 3.95		
R39	8.07	4.20	1.94, 1.81	1.69, 1.63	3.15 ($C^\delta H_2$); 7.22 ($N^\epsilon H$)
A40	8.01	4.24	1.47		
K41	8.03	4.27	1.94, 1.87	1.56, 1.48	1.74 ($C^\delta H_2$); 3.02 ($C^\epsilon H_2$)
R42	8.16	4.32	1.94, 1.86	1.73, 1.68	3.23 ($C^\delta H_2$); 7.27 ($N^\epsilon H$)
N43	8.38	4.73	2.91, 2.83		7.67, 6.87 ($N^\delta H$)
N44	8.33	4.73	2.84		7.67, 6.87 ($N^\delta H$)
			Peptide 45−58		
F45	8.03	4.55	3.14, 3.03		7.27 ($C^\delta H$); 7.37, 7.32 ($C^\epsilon H$,$C^\zeta H$)
K46	8.36	4.34	1.85, 1.78	1.47	1.69 ($C^\delta H_2$); 3.01 ($C^\epsilon H_2$)
S47	8.44	4.46	4.15, 4.00		
A48	8.61	4.21	1.50		
E49	8.57	4.08	2.09	2.43	
D50	8.31	4.53	2.87, 2.79		
S51	8.26	4.21	4.05, 3.97		
M52	8.27	4.37	2.21	2.71, 2.60	2.09 ($C^\epsilon H_3$)
R53	8.25	4.28	2.00	1.86, 1.72	3.26 ($C^\delta H_2$); 7.41 ($N^\epsilon H$)
T54	8.08	4.41	4.44	1.32	
S55	8.14	4.53	4.06		
G56	8.40	4.07			
G57	8.26	4.00			
A58	7.92	4.27	1.42		

[a] Assignments of $C^\beta H$ and $C^\gamma H$ are uncertain due to spectral overlap.

affect both the CD and the NMR, so quantitative comparison of the two measures of helicity is not possible. The interaction between Ile19 and Tyr21 present in aqueous solution was disrupted, and the chemical shift of Ile19 $C^\gamma H_3$ assumed a normal value (Figure 4). Likewise, there were no indications of the interaction between Tyr23 and Ala25 (data not shown). In both cases, these interactions are not possible in the helical conformation; their disruption in TFE was presumably a result of the change in the conformation, and it is not possible to determine whether TFE disrupted the interaction itself.

Peptide 29−44. In native BPTI, this segment is largely a β-strand comprising residues 29−35, followed by an irregular loop. The peptide in aqueous solution was very flexible, with multiple conformations including turns and nascent helices, but with no marked preference for one type of

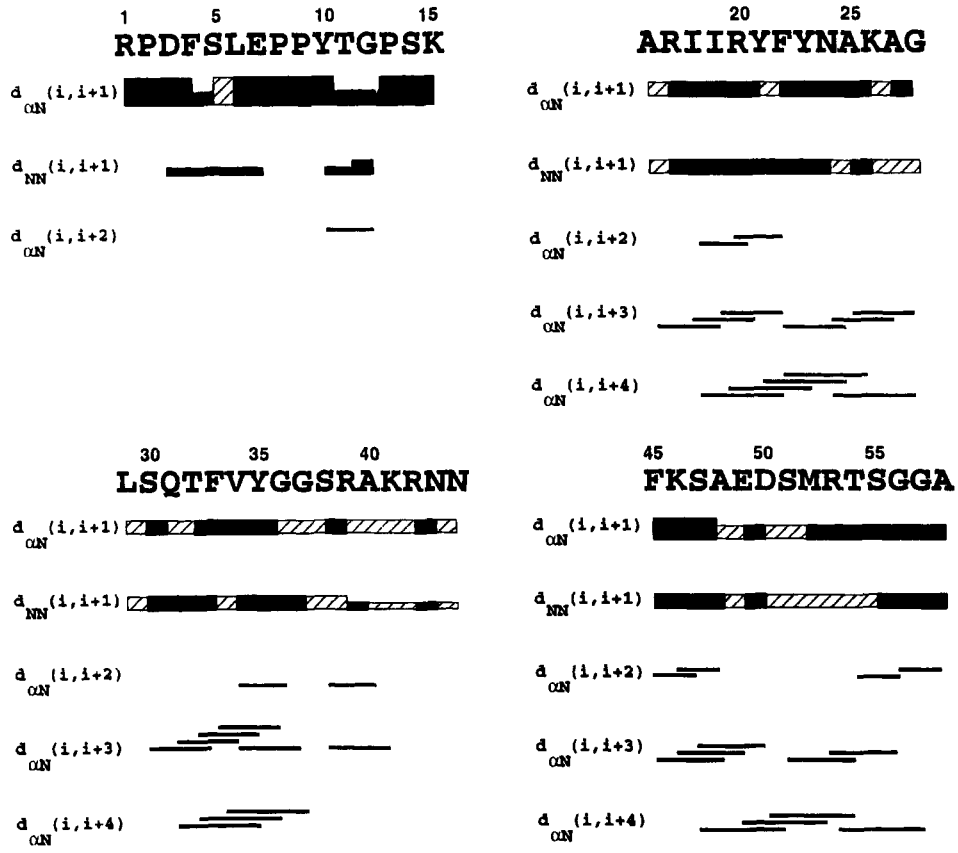

FIGURE 2: Schematic diagram summarizing the relative intensities of the NOE connectivities between the backbone NH (or $C^{\delta}H$ in the case of proline residues) and $C^{\alpha}H$ protons for the peptides in the presence of TFE. The data are from NOESY spectra recorded at 283 K with a mixing time of 150 ms. The standard nomenclature of Wüthrich (1986) is used. Hatched areas indicate spectral overlap for the sequential NOEs, but medium-range NOEs that would be obscured for this reason are not indicated.

conformation. There was, however, an interaction between the side chain of Tyr35 and Gly37, like that observed between Tyr10 and Gly12, which was evident from the very anomalous chemical shift of the NH of Gly37.

In the presence of TFE, this peptide adopted a largely helical conformation, extending over nearly the entire length of the peptide (Figure 2), although the helix appeared to predominate in the N-terminal half. The CD at 222 nm indicated an α-helical content of only about 23% (Table 1), but the shape of the spectrum, particularly the substantial positive ellipticity at 200 nm, is not consistent with solely α-helical conformations and suggests that a positive signal is also contributing in the region of 220 nm. This is probably due to the presence of the aromatic residues Phe33 and Tyr 35 (Chakrabartty et al., 1993b). The interaction between Tyr35 and Gly37 observed in the absence of TFE was not apparent, presumably as a result of the change to a helical conformation.

Peptide 45−58. In native BPTI, this segment contains the major α-helix of residues 47−56. The peptide in aqueous solution had a weak tendency to adopt the same α-helix, but this helix was present to only a very small extent. There were also indications of a nativelike "capping" interaction of the amide proton of Ser47 with the $C^{\beta}H_2$ protons of Asp50 (Kemmink & Creighton, 1993).

In the presence of TFE, this peptide became largely helical over nearly its entire length (Figure 2). The CD intensity indicated a helical content of only 43% (Table 1). This peptide contains only a single, N-terminal aromatic residue, which would have only a small effect on the CD spectrum (Chakrabartty et al., 1993b), so the α-helix was not present all of the time. Detailed characterization of this peptide in TFE, using an algorithm to take into account the presence of multiple conformations and the effects of spin diffusion on NOESY spectra, confirmed that the α-helical conformation of the entire peptide was in equilibrium with other conformations (Kemmink & Scheek, 1995). The $C^{\alpha}H$ chemical shifts suggest, however, that the helix predominates between residues 48 and 55. The reasons for this discrepancy are not clear, although NOEs and chemical shifts are averaged differently when there are multiple conformations in rapid equilibrium.

DISCUSSION

The cosolvent TFE induced substantial helicity in the three peptides studied here that do not contain proline residues. The fourth peptide contains four proline residues distributed throughout its length, so the absence of the helical conformation in it is not surprising. TFE had no substantial effect on the average conformations of this peptide, nor did it alter the local interactions between neighboring residues that are evident in aqueous solution. The other three peptides had N-terminal acetyl groups, which tend to increase the local helicity at the N-terminus (Chakrabartty et al., 1993a).

In each of the three peptides that became helical, the presence of NOEs between residues i, $i + 3$ and i, $i + 4$ indicated that the helical conformation extended over the entire length of the peptide. The maximum amount of helical conformation is usually present with 50% TFE, yet the CD spectra indicated α-helical contents of only 23−43%. There was no apparent gradation of the intensities of the α-helical i, $i + 3$ and i, $i + 4$ NOEs from the middle of the peptides

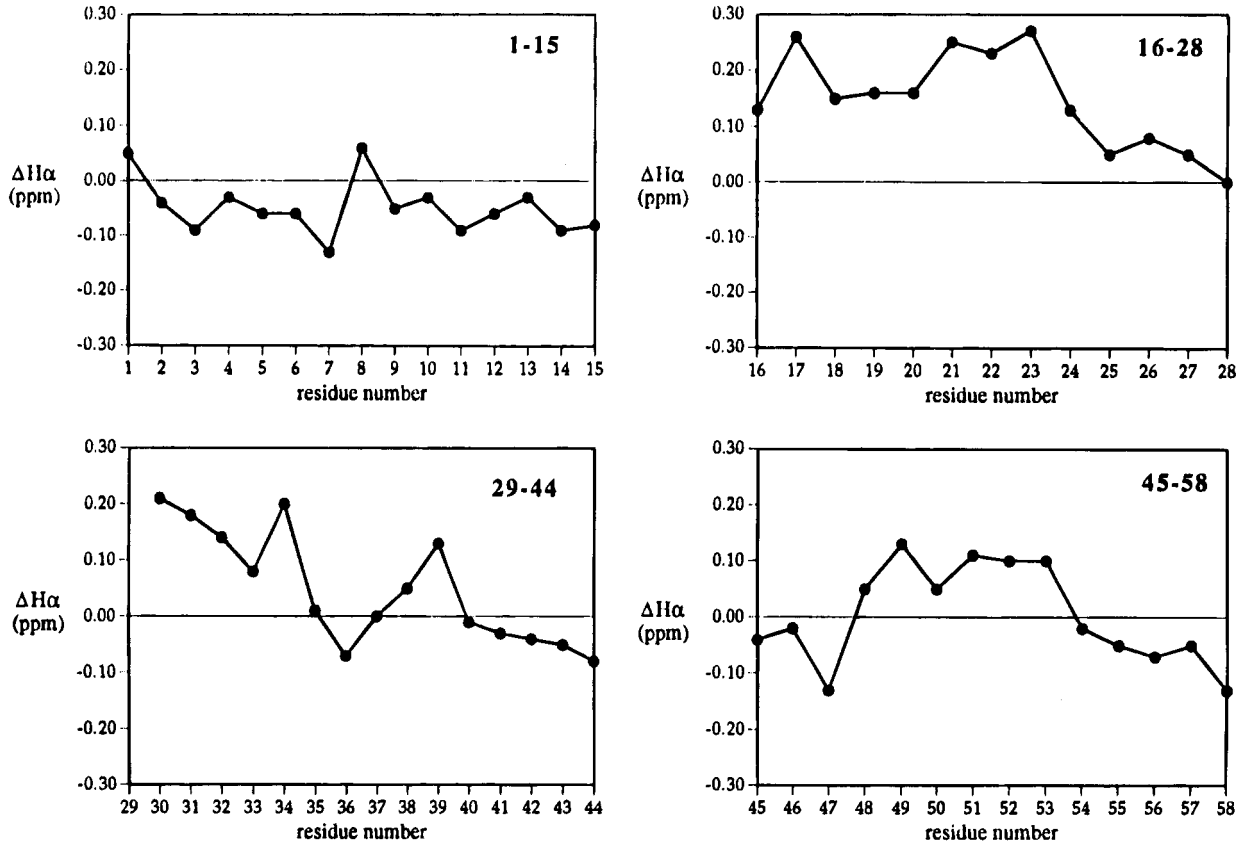

FIGURE 3: Differences between the chemical shifts of the $C^{\alpha}H$ protons of the peptides dissolved in 50% H_2O/50% TFE-d_3 (v/v) (Table 1) and dissolved in 90% H_2O/10% D_2O (v/v) [values taken from Kemmink et al. (1993) and Kemmink and Creighton (1993)]. The L29 $C^{\alpha}H$ chemical shift is omitted because the peptide studied in water was lacking the N-terminal acetyl group (Kemmink & Creighton, 1993).

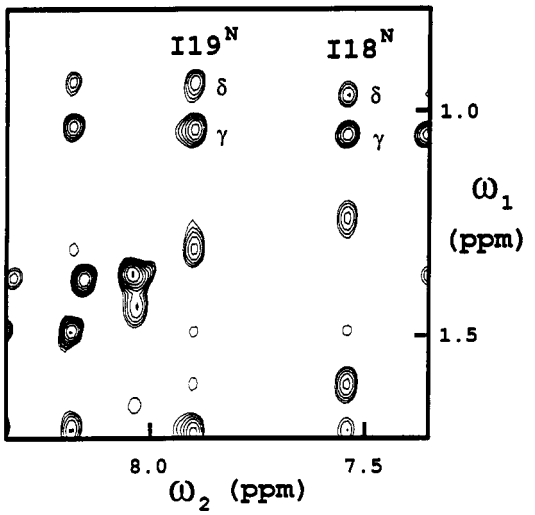

FIGURE 4: Portion of 500 MHz NOESY spectra of peptide 16−28 in the presence of 50% TFE recorded at 271 K. The positions of the I18 and I19 NH protons are indicated with the symbols $I18^N$ (7.52 ppm) and $I19^N$ (7.92 ppm). The positions of the I18 and I19 methyl groups are indicated with the symbols γ and δ (I18: $C^{\gamma}H_3$ 1.05 ppm, $C^{\delta}H_3$ 0.95 ppm. I19: $C^{\gamma}H_3$ 1.01 ppm, $C^{\delta}H_3$ 0.91 ppm).

to the ends, as would be expected if there were simply an equilibrium between only α-helix and random coil conformations at each residue. On the other hand, the quantification of α-helix content is complicated by the presence of aromatic residues, and varying effects on the $C^{\alpha}H$ chemical shifts (Figure 3) suggest that the various residues did have different helicities. Such discrepancies between content of α-helix measured by CD and NMR have also been observed by others (Dyson et al., 1988; Bradley et al., 1990; Sönnichsen et al., 1992). One possible explanation is that other conformations were present at the same time, in equilibrium with the α-helix. This is suggested by the presence of substantial $i, i + 2$ NOEs in the peptides studied here (Figure 2). Also, detailed structure calculations on peptide 45−58 confirmed the presence of other conformations (Kemmink & Scheek, 1995). Such $i, i + 2$ cross peaks have been observed in other peptides (Dyson et al., 1988; Storrs et al., 1992); they are believed to arise from turnlike conformations or incomplete helices.

Only one of the three peptides that became helical in TFE has this conformation in native BPTI, between residues 47 and 56. The other two peptides exist primarily as extended β-strands of residues 18−24 and 29−35 in native BPTI. Consequently, TFE cannot be assumed to induce only nativelike α-helices.

TFE is thought to induce the α-helical conformation only when there is a substantial tendency for the peptide to adopt that conformation (Nelson & Kallenbach, 1989; Lehrmann et al., 1990; Dyson et al., 1992a,b; Storrs et al., 1992; Sönnichsen et al., 1992; Jasanoff & Fersht, 1994; Jiménez et al., 1994). Indeed, in other cases it can induce other conformations, such as β-hairpins (Blanco et al., 1994). In the present case, there was reasonable agreement between the amount of α-helix measured by CD (Table 1) and the α-helix propensity in aqueous solution predicted by the empirical procedure of Muñoz and Serrano (1994) (Figure 5). Peptide 1−15 is predicted to have negligible α-helix in aqueous solution, while peptides 16−28 and 45−58 are predicted to have the most. The expected helical content of peptide 16−28 was not observed, however, in aqueous solution, while that of peptide 45−58 was (Kemmink & Creighton, 1993). This may be due to peptide 16−28 having

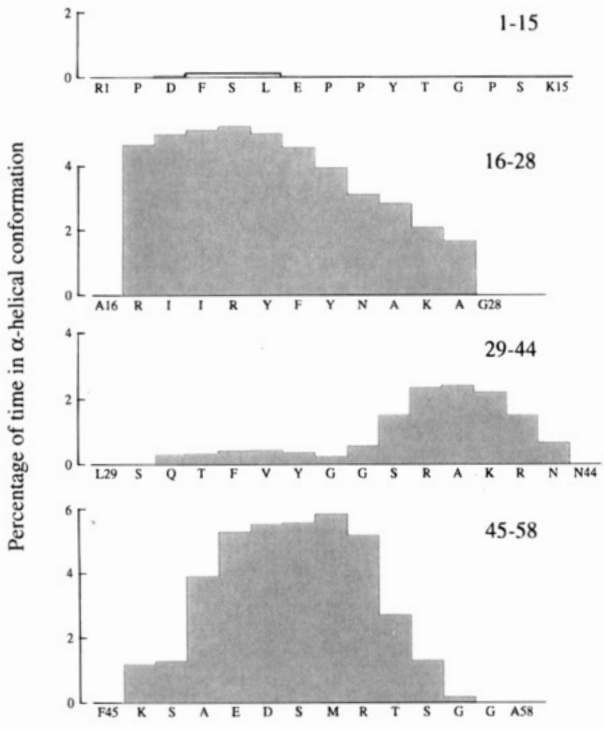

FIGURE 5: Predicted α-helical propensities of individual residues of the four peptides of BPTI calculated by the procedure of Muñoz and Serrano (1994). The conditions were taken to be pH 4.6 and 273 K.

a much greater tendency to adopt extended conformations (Chou & Fasman, 1974), which will compete with α-helix formation. In each of the three peptides that became helical in TFE, the α-helical conformation appeared from the NOEs to extend approximately uniformly over the length of the peptide, rather than being limited to the residues with the greatest helical propensity. The chemical shifts of the $C^{\alpha}H$ protons did indicate varying degrees of helicity (Figure 3), and in peptides 16−28 and 45−58 the shifts matched the predicted helical propensities (Figure 5). In the case of peptide 29−44, the helix appeared from the chemical shifts to be greater in the N-terminal half than in the predicted C-terminal half, but this could be due to the presence of two aromatic residues. Overall, there was a reasonable agreement between the predicted and observed α-helices in the peptides.

There is no contradiction in the fact that TFE induced the α-helical conformation in two peptides that are β-strands in native BPTI. These two peptides have substantial α-helical propensity, but their propensities to form β-strands are even greater (Chou & Fasman, 1974). TFE enhances intramolecular α-helix and β-hairpin formation, but not β-strand (Sönnichsen et al., 1992).

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