Chapter I

Effects of cosolvent on peptide conformation
The propensity of aqueous-alcohol mixtures to influence structure and dynamics of peptide/protein has been extremely useful in studies of protein folding, sub-zero temperature enzymatic reactions and in methods of crystallographic cryoenzymology (Virden et al, 1990; Ding et al, 1994; Travers & Barman, 1995). In addition, cosolvents also endow selectivity and/or specificity to reactions and modulate the equilibrium of enzyme-catalyzed reactions. The restriction of proteolysis as well as shifting of the equilibrium of a protease-catalyzed reaction to peptide bond synthesis represent notable examples (Homandberg et al, 1978; Roy et al, 1992; De Laureto et al, 1995).

In recent years, fluoro alcohols, especially trifluoroethanol (TFE) and to a lesser extent, hexafluoroisopropanol (HFIP) and hexafluoroacetone (HFA) have been used to enhance the helical conformation of peptides (Sonnichsen et al, 1992; Jackson et al, 1991; Kemmink & Creighton, 1995; Arvidsson et al, 1993; Bhattacharjya & Balaram, 1997; Bhattacharjya et al, 1998; Anderson et al, 1996). The helix-enhancing property of fluoroalcohols is also shared by aliphatic alcohols (alkyl alcohols) despite their disparate physicochemical properties. Thus, the quantitative analyses of the effect of fluoro and alkyl alcohols displaying varied properties on peptide sequences that are endowed with intrinsic helical propensity, under different environmental conditions of pH, temperature and solvent concentration has the potential of elucidating the mechanism of peptide helix-enhancement due to TFE or other alcohols. Furthermore, this would be useful in optimizing the use of cosolvents in cryoenzymology or modulation of equilibrium of enzymatic reactions such as shifting the protease-catalyzed hydrolysis equilibria towards peptide bond synthesis.

Materials and Methods

Materials

F-moc protected amino acids and other chemicals used in peptide synthesis were obtained from Novabiochem (Switzerland). 1-Propanol, 2-propanol, TFE, 2,2,3,3-tetrafluoro-1-propanol (TFP) and HFIP were procured from sigma Chemical Co (St.Louis, MO, USA) or Fluka Biochemika (Fluka Chemie, AG,
Switzerland). All other chemicals were of reagent grade and procured from standard commercial sources.

**Synthesis of peptides and purification**

The peptides were synthesized on a semi-automated peptide synthesizer (Model 90, Advanced Chemtech, Louisville, KY, USA) employing standard solid phase synthesis protocols and using F-moc chemistry. N, N'-diisopropylcarbodiimide (DIPCDI) was used as coupling reagent and 1-hydroxybenzotriazole (HOBr) was used as an additive. After each coupling reaction, coupling efficiency was monitored by Kaiser test (Kaiser et al., 1970) and subsequently the F-moc group was removed by treatment with 20% piperidine (v/v) in dimethylformamide (DMF). The peptides were cleaved from the resin and deprotected using appropriate amounts of a mixture of phenol-water-ethanedithiol-thioanisole-trifluoroacetic acid (5: 5: 5: 5: 80, by volume) at room temperature with constant stirring for 2-3 hrs. The resin was removed by filtration and the crude peptides precipitated in cold anhydrous ether. The precipitate was dissolved in water and lyophilized. The peptides were purified by reverse-phase high performance liquid chromatography (RPHPLC) on a semi-preparative aquapore RP300 (250 x 7mm, 7 μm; Applied Biosystems) column using a linear gradient of 5-70% solvent B (solvent B: 80% acetonitrile containing 0.1% trifluoroacetic acid (TFA), solvent A: 0.1% TFA in water) in 130 min. The flow rate was maintained at 2 mL/min throughout the run. The identity of peptides was established by sequencing and mass spectrometry.

**Electrospray mass spectrometry (ESMS)**

The RPHPLC purified peptides were taken in 50% acetonitrile containing 1% formic acid and analyzed by positive ion electrospray mass spectrometry (VG Platform, Fisons). The instrument calibration was usually done with standard gramicidin S or myoglobin solutions.
Circular dichroism analysis

Circular dichroism (CD) spectra were recorded on a Jasco-710 spectropolarimeter (Jasco, Tokyo, Japan) equipped with a Peltier type temperature controller (PTC-348W). The instrument was calibrated with (+)-10-camphorsulfonic acid. The spectra were an average of 10 scans. The respective buffer baselines were subtracted from the sample CD data. The ellipticity of peptides were measured at 222 nm and are reported as mean residue ellipticity (MRE) in deg/cm²/dmol units. It is assumed that helical content of peptide is directly proportional to the Θ222 (Holzwarth & Doty, 1965).

Thermodynamic analysis

The free energy ΔG of helix formation in a two-state helix/coil transition was calculated from the observed mean residue ellipticity at 222 nm using the equation, ΔG = -RT ln K. K = Θ_{observed} - Θ_{coil} / Θ_{helix} - Θ_{observed}. Θ_{helix} is the maximum Θ222 that a peptide would give in fully helical form (Scholtz et al, 1991). The Θ_{helix} for TC-peptide (22 residues) is estimated as -35,600 and for 20 residues S-peptide and Sa-peptide, the calculated value is -35,000. The baseline value for random coil for all the three peptides, Θ_{coil}, was taken as +300 (Merutka, et al, 1990; Park et al, 1993). Based on the crystal structure of ribonuclease A and thermolysin as well as NMR data available for S-peptide and TC-peptide, 9 and 10 helical peptide units were assumed in S-peptide and TC-peptide, respectively, and Θ222 values were revised to account for the helix fraying ends (Kim et al, 1982).

pH-dependent studies

The peptide stock solutions were usually made in water to which an appropriate volume of the desired buffer and cosolvent was added. The buffers consisting of 1mM sodium phosphate, 1mM sodium borate, 1mM sodium citrate were prepared in the pH range 2-9 by addition of HCL or NaOH solution. The concentration of each peptide was estimated spectrophotometrically using the
molar extinction coefficient of a single tyrosine residue, $E_{280} = 1280$ L/mol/cm (Edelhoch, 1967).

RESULTS

Choice and design of peptide fragments

The peptides with known helical disposition from the helical portion of native proteins were selected with the assumption that the peptides would emulate the structure found in the native protein itself. The N-terminal helical fragment (S-peptide, residues 1-20) of RNase A and the carboxy terminal fragment of thermolysin spanning residues 295-316 (TC-peptide) were chosen as prototype peptides for studying their conformational behavior in organic cosolvents. The S-peptide has been studied extensively and shown to acquire significant helical conformation in aqueous solutions (Brown & Klee, 1971). The earlier studies of conformation of the S-peptide by NMR have suggested that most of the conformational features of S-peptide in aqueous solution are retained in aqueous-TFE mixtures. The crystal structure of thermolysin, along with CD and NMR data in aqueous and aqueous-organic solutions of the fragment 299-316 of

S-peptide 1 K E T A A A K F E R Q H M D S S T S Y A 20
Sa-peptide 1 K E T A A A K F E R A A A D S S T S Y A 20
TC-peptide 295 L Y G S T S Q E V A S V K Q A F D A V G V K 316

Fig 1. Sequence of peptides. Sequences corresponding to S-peptide of RNase A, an analog of S-peptide (Sa-peptide) and TC-peptide (residues 295-316) of thermolysin are shown. The Ala at position 19 in S-peptide and Sa-peptide sequence has been substituted by a Tyr residue to facilitate the concentration determination of the peptides. Tyr at position 295 serves the same purpose in TC-peptide.
thermolysin indicate helical structure for residues 301-311 (Jimenez et al, 1993). An analog of S-peptide (Fig 1) was designed to destabilize the S-peptide helix.

**Choice of cosolvents**

To compare the helix-enhancing ability of fluoro versus alkyl alcohols, two fluoro alcohols (TFE and HFIP) and two alkyl alcohols (1-Propanol and 2-Propanol) were chosen as these are known to stabilize secondary structures. The physicochemical properties of these alcohols are quite different from each other and they are totally miscible with water and are stable within the pH range of 2-9. TFE and HFIP have been used extensively to study the helical propensity of peptides in solution (Storss et al., 1992; Kuroda et al, 1996; Wei & Fasman, 1995). 1-Propanol and 2-propanol, although used to a lesser extent, are known to induce helix formation in peptides and proteins. Moreover, 1-propanol has been used as a cosolvent in protease-mediated ligation reactions (Shibata et al, 1992; Sahni et al, 1989; Roy et al, 1992).

**pH-dependence of cosolvent-mediated helix formation**

The isolated S-peptide of RNAse A forms significant helix in aqueous solutions at low temperatures and has been investigated extensively (Kim et al, 1982; Rico et al, 1986). The helix formation of S-peptide is pH-dependent and the helix is localized to the same residues as in the native RNAse A suggesting that a functional helix termination signal exists in the isolated peptide (Kim & Baldwin, 1984). NMR studies of S-peptide in TFE solution have suggested that most of the conformational features of S-peptide including the pH-dependence and the 'helix stop signal' are retained in TFE solutions (Nelson & Kallenbach, 1989). Thus, S-peptide represents an ideal system with which to probe and compare the effects of various cosolvents.

The failure of TFE to alter the pH-dependent helix formation of S-peptide has been interpreted to mean that the helix-inducing effect of TFE is not due to dielectric properties of aqueous TFE solutions. If this were true then pH-dependence of S-peptide helix formation should be retained in the presence of
alkyl alcohols as well. To test this, the helix formation of S-peptide in aqueous solution of 40% TFE (v/v) and of 1-propanol in the pH range of 2-7 at low temperature was studied. In aqueous solutions, at 1°C, the θ_{222} value increased from pH 2 to pH 4, but decreased between pH 4 and 7 (Fig. 2A). This was in agreement with the earlier report (Kim & Baldwin, 1984) suggesting the presence of maximum helicity in S-peptide at pH 3.8. The overall nature of the pH titration curve was similar to that reported previously and was consistent with the ionization behavior of carboxylate and imidazole side chains. A similar trend was maintained in the presence of 40% TFE solutions. Thus, consistent with earlier studies (Nelson & Kallenbach, 1986), S-peptide exhibited a pH-dependence of θ_{222} in aqueous solutions as well as in the presence of 40% TFE solution. The pH titration curve of S-peptide in the presence of 40% 1-propanol was similar to that obtained in the presence of 40% TFE. However, the θ_{222} values in 40% 1-propanol at any given pH were about 5-10% lower than that observed in the presence of 40% TFE solution. The preserved nature of the pH titration curve of aqueous solution in the presence of both 40% TFE as well as 40% 1-propanol (Fig. 2A) indicate that the ionization behavior of carboxylate and imidazole side chains are similar in these two solvents.

To further explore the pH effect, an analogue of S-peptide (Sa-peptide) was designed in which residues 11-13 (QHM) were changed to Ala (AAA) residues (Fig. 1). This design was based on the knowledge that Arg10 and His12 are critical residues contributing to the stability of the S-peptide helix through charge interaction to Glu2 and stacking interaction to Phe8, respectively (Rico et al, 1986; Shoemaker et al, 1985). In addition, Gln at position 10 is known to destabilize the helix (Shoemaker et al, 1985). Thus the substitution QHM→AAA would lead to abrogation of Phe8-His12 interactions and may also partially influence the Glu2-Arg10 interaction by interfering with the stereochemistry of the salt bridge formation. The overall effect is likely to manifest itself as pH-dependence of helix formation and destabilization of Sa-peptide helix as compared with S-peptide.
As expected, the Far-UV CD spectrum of Sa-peptide was characterized by very small negative minimum at 222 nm and a large negative minimum at 204 nm, suggesting that the peptide was predominantly unstructured in aqueous solutions between pH 2 and 7 at 1°C (Fig. 2B). Although QHM→AAA substitution exerted a profound effect on the stability of helix, Sa-peptide retained its helical propensity as significant enhancement of helix formation in the presence of 40% TFE or 40% 1-propanol could be achieved. Importantly, Θ_{222} values were more or less insensitive to pH changes (Fig. 2B) supporting the rationale of the peptide design as Sa-peptide was designed to interfere with the

Fig 2. Effect of pH on cosolvent-mediated helix enhancement. The spectra of peptides were recorded at 1°C in aqueous solution as well as in the presence of TFE or 1-propanol at various pH. The Θ_{222} values are plotted as a function of pH: A: S-peptide, B: Sa-peptide, and C: TC-peptide. O—O: aqueous buffer, Δ—Δ: 40% TFE, and □—□: 40% 1-propanol.
pH dependence of helix formation. Taken together, the results suggest that the 'message' of elimination of the Phe8-His12 interaction and likely perturbation of the Glu2-Arg10 interaction by QHM→AAA sequence changes have been conserved in the presence of fluoro as well as alkyl alcohols.

The generality of these results were further tested by studying the conformational features of TC-peptide (residues 295-316 of thermolysin). The crystal structure of the protein show that residues 301-311 form the terminal helix of thermolysin (Holmes & Matthews, 1982). NMR data have also indicated helical conformation for residues 302-311 in an isolated fragment corresponding to the sequence 296-316 of thermolysin (Jimenez, et al, 1993). In order to see that the conformation of TC-peptide also exhibited a similar trend as that found in S-peptide and Sa-peptide in the presence of aqueous and organic solvents, the pH-dependent conformational behavior of TC-peptide was investigated. Under conditions similar to that of S-peptide and Sa-peptide, the values associated with TC-peptide varied as a function of pH in much the same way as S-peptide in both aqueous as well as organic cosolvents (Fig 2C). Taken together the results indicate that both TFE and 1-propanol effect the pH-dependent conformation of the peptide in a similar fashion.

**Cosolvent concentration dependence of helix formation**

The above pH-dependence studies showed that S-peptide and TC-peptide exhibited maximum helix enhancement around pH 4, while the helix formation of Sa-peptide was insensitive to changes in the pH between 2 and 7. The titration experiments of S-peptide, Sa-peptide and TC-peptide against a range of concentrations of 1-propanol, 2-propanol, TFE and HFIP respectively, at 1°C and pH 4 generated an array of spectra which showed minima at 222 nm and 206-208 nm which is highly characteristic of α-helical conformation. The cosolvent concentration dependence of individual peptides on the CD spectra in each of the above cosolvents exhibited an isodichroic point at 202-204 nm (Fig. 3) which suggested a two-state helix/coil transition (Brown & Klee, 1971). All three peptides showed saturable helix formation in all the four cosolvents. The concentration of a cosolvent needed to achieve maximum helix formation was in
the range of 5-5.5 M (= 40%, v/v) for 1-propanol, 2-propanol or TFE and about 3 M (27.4%, v/v) for HFIP which is about two-fold less compared to the aforementioned solvents (Fig. 4). Also the maximum enhancement of helical

![Graph](image)

**Fig 3.** Concentration dependence of 2-propanol on the CD spectra of S-peptide. The spectra of the peptide (approximately 25 μM) was recorded at pH 4 and 1 °C in the presence of various concentrations of 2-propanol. The spectra are depicted in the order of increasing concentration of 2-propanol (v/v): no 2-propanol, 10%, 20%, 30% and 40%. Note the presence of an isodichroic point at 203 nm.

conformation achieved in the presence of the three cosolvents, 1-propanol, 2-propanol or TFE were similar. The C_{1/2} values (mid-point concentration of transition in molar units) for 1-propanol, 2-propanol, and TFE for S-peptide were 2.2, 2.4 and 2.3, respectively. Likewise for Sa-peptide and TC-peptide, the C_{1/2} values were 3.8, 3.7, 3.8 and 3.2, 3.1, 3.0 respectively. However, HFIP elicited significantly much higher helix formation compared to the other cosolvents with C_{1/2} values of 1.2, 1.1 and 1.0 for S-peptide, Sa-peptide and TC-peptide respectively.

The titration data for the helix stabilization effect of each cosolvent for all the three peptides were transformed into plots of free energy (ΔG) versus
cosolvent concentration and analyzed using the linear free energy model employing the equation, \( \Delta G_{\text{helix}} = \Delta G_{\text{water}} - m[\text{cosolvent}] \), where \( \Delta G_{\text{helix}} \) is the free energy of helix formation in water and \( m \) is the constant of proportionality.

Fig 4. Cosolvent concentration dependent helix enhancement. The spectra of each peptide were recorded in the presence of various cosolvents as described in Fig 3. The \( \Theta_{222} \) values are plotted as a function of cosolvent concentration. The \( C_{1/2} \) values (cosolvent concentration at half-maximal helix-induction) were calculated from each curve A: S-peptide, B: Sa-peptide, and C: TC-peptide. Empty triangle (△—△): 1-propanol, filled triangle (▲—▲): 2-Propanol, filled circle (○—○): TFE, and empty circle (●—●): HFIP.

(m-value). The free energy decreased linearly in the cosolvent range of 0-5 M for all the three peptides (Fig. 5). The \( m \)-value of any given peptide for 1-propanol, 2-propanol or TFE was similar since the data for these three solvents could be fitted in a single line. The \( m \)-values (kcal/mol) for S-peptide, Sa-peptide and TC-peptide were 0.3875, 0.4381 and 0.3768, respectively. The dependence of the \( m \)-value on the nature of peptide suggests that cosolvents act within the pre-existing helix-coil equilibrium characteristic of each peptide while the independence of the \( m \)-value with respect to 1-propanol, 2-propanol and TFE indicate similar helix-enhancing propensities of the above alcohols.

The \( m \)-value of HFIP for all three peptides was different (0.7863, 1.575 and 1.708 for S-peptide, Sa-peptide and TC-peptide, respectively) and dramatically different from the \( m \)-values of other cosolvents. However, the linear
extrapolation of the HFIP-induced transition and 1-propanol, 2-propanol or TFE-induced transitions of each peptide exhibited a common intersection point at the Y-axis. This suggests that HFIP also induces helix formation by a mechanism similar to that of other cosolvents (albeit with higher efficiency) and that linear dependency of free energy at low alcohol concentration may be an useful approximation for comparing the helix-inducing effects of various cosolvents (Hirota et al, 1997 & 1998; Jasanoff & Fresht, 1994).

**Additivity of cosolvent-induced helix formation**

The additive effect of cosolvents, if any, on helix-enhancement was tested. The helix forming ability of the cosolvents individually and in a 1:1 (v/v) mixture was assessed by CD spectroscopy. This is illustrated in Fig. 6 which shows almost identical spectra of S-peptide recorded in the presence of 40% TFE or 40% 1-propanol or a mixture consisting of 20% (v/v) of 1-propanol and 20%. (v/v) TFE. The additive effect was observed for all the three peptides of all tested blend of cosolvents (Table. 1). These results show that the presence of the
trifluoro group in TFE or tetrafluoro group in tetrafluoro-1-propanol (TFP) do not endow any helix-enhancing prowess to the alcohols. These results rule out the possibility of any strong interaction of the fluorine atoms of the alcohol with the peptide.

Fig 6. Additive effect of cosolvent in helix-enhancement. The spectra of S-peptide was recorded at pH 4 and 1 °C in the presence of individual as well as mixture of cosolvents. The peptide concentration was approximately 25 μM.

<table>
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<th>Alcohol combinations (alcohol I, alcohol II)</th>
<th>Mean Residue Ellipticity (-θ&lt;sub&gt;222&lt;/sub&gt;)</th>
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<td></td>
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<td>TFE, 2-P</td>
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Table 1. Additive helix-enhancing effect of alcohols. The mean residue ellipticities (-θ<sub>222</sub>) were obtained for S-peptide at pH 4 and 1 °C in the presence of 40% (v/v) of various alcohols, alone or in combinations except for the TFE and TFP combination where the individual or combined alcohol concentration was 30% (v/v).
Fig 7. Temperature dependence of helix formation as a function of cosolvent concentration. The $\Theta_{222}$ values obtained from the thermal melting curves of each peptide in the presence of a given concentration of the cosolvent are plotted as a function of cosolvent concentration. The data represented in panels A, B, C are for S-peptide in the presence of TFE, 1-propanol and 2-propanol respectively. Likewise panels D, E, F and G, H, I represent respective data for Sa-peptide and TC-peptide. The curves are plotted at 10 °C intervals starting at 0 °C. The plateauing $\Theta_{222}$ value decreases with increase in temperature.

Temperature dependence of cosolvent helix induction

The thermal transition curve for each peptide was measured at a given concentration of 1-propanol, 2-propanol and TFE and the data obtained were used to generate cosolvent helix induction curves at various temperatures (Fig. 7). The salient features of these curves are as follows: (a) the curves exhibit saturable helix formation independent of the cosolvent and peptide; (b) the $\Theta_{222}$
value saturates or plateaus at a similar concentration of all three cosolvents; and
(c) the plateauing value of $\Theta_{222}$ decreases with increasing temperature, but
pleatuing occurs at the same concentration of cosolvents. In addition, the shape
of curves display features characteristic of individual peptides that are preserved
in all the three cosolvents. In the case of TC-peptide, the $\Theta_{222}$ value, at low
alcohol concentration was seen to increase with increasing temperature and
isodichroic points between 0 and 30° C were present in all the cosolvents (Fig.
7G, H, and I). Thus increase in the amplitude of $\Theta_{222}$ with temperature at lower
cosolvent concentration may be intrinsic to the nature of the peptide. This type of
behavior of a peptide has also been noticed earlier in aqueous TFE solutions
(Jasanoff & Fersht, 1994).

DISCUSSION
The cooperative cosolvent concentration-dependent helix stabilization of
peptides of similar length (20-22 residues) at low temperature showed similar
transition mid-points ($C_{1/2}$ values) for TFE, 1-propanol or 2-propanol as well as
similar m values were obtained for these cosolvents for any given peptide
suggesting that TFE and the two alkyl alcohols have similar helix-enhancing
propensities. These results are discrepant with a recent study of alcohol effects
on helix formation in melittin (Hirota et al, 1998) that suggest significantly
higher m value for TFE compared to 1-propanol and 2-propanol. Hirota et al,
(1998) have estimated effectiveness of various alcohols in inducing helical
conformation in melittin using an empirical equation that generates m value as
the additive contribution of the accessible surface area (ASA) of each group (CH,
OH, F etc.). The authors attribute the enhancement in m value of TFE relative to
1-propanol and 2-propanol in melittin to the additive contribution of F atoms to
the ASA. However, our results of the comparative analysis of 1-propanol, 2-
propanol and TFE, suggest that helix-enhancing propensities of alcohols are
better correlated with the total ASA (1-propanol, 2-propanol and TFE display
similar ASA; 234, 230 and 231 Å$^2$ respectively) that is independent of the
chemical nature of the individual contributing groups. Thus F atoms in TFE do not play a significant role in enhancing helix formation in S-peptide, Sa-peptide and TC-peptide. The higher m values of melittin in TFE may be intrinsic to the nature of the peptide.

The \( C_{1/2} \) values of peptides for HFIP were 2 to 3 times lower than the other cosolvents. This is consistent with the known ability of HFIP to effect saturation of helix formation at a much lower concentration compared to TFE (Hirota et al, 1997; Wei & Fasman, 1995; Anderson et al, 1996). The m values obtained with HFIP were 2-4 times higher than the other cosolvents, however the extrapolation of HFIP titration curves yielded the same value of \( \Delta G_{\text{water}} \) as other cosolvents suggesting a common mechanism of helix formation. In addition, the helix-enhancing propensity of HFIP may also be related to the fact that HFIP-water mixtures display a large negative excess molar enthalpy of mixing at all mole fractions relative to TFE-water, 1-propanol-water or 2-propanol-water mixtures (Denda et al, 1987). The increase in negative enthalpy of HFIP may be a manifestation of strong ordering of water molecules in water-HFIP mixtures. The water-HFIP mixtures have been shown to exhibit strong X-ray scattering suggesting HFIP cluster or micelle formation which may have a role in the facilitation of peptide helix enhancement (Kuprin et al, 1995). This observation is supported by Hong et al, (1999) who found correlation between clustering of alcohol molecules and helix formation in the low alcohol concentration range. Thus it is conceivable that steric properties of the alcohol (size) as well as structuring of water/alcohol in the cosolvent are important factors contributing to helix stability.

The cosolvent concentration-dependent saturation of helix-induction as well as plateauing of \( \Theta_{222} \) in cosolvent helix-induction curves at various temperatures seem to be a common attribute of all cosolvents. In the case of TFE, the plateauing effect has been correlated to the plateauing of helix propensity with TFE concentration. This is further corroborated with the observation that the hydrogen bonding strength of a model compound (salicylic acid) increased with TFE concentration but exhibited saturation behavior (Luo &
Baldwin, 1997). It has also been noted that decrease in Θ_{222} with temperature emanates from strong temperature dependence of Θ_{helix} while apparent loss of cooperativity in thermal unfolding at high concentration is due to decrease in enthalpy. The thermal transition curves of any given peptide (S-peptide, Sa-peptide and TC-peptide) in all the three cosolvents (TFE, 1-propanol and 2-propanol) displayed similar shape and character. Thus, it is reasonable to conclude that alkyl alcohols exert their influence on peptide helix in the same way as TFE.

The presence of -CF₃ group in TFE enhances the acidity compared with the alkyl alcohols. These differences in physical property of TFE relative to water presumably alter the hydrogen bonding pattern such that bonding of amide protons to the solvent is decreased and intramolecular hydrogen bonds are strengthened favoring the helical state (Nelson & Kallenbach, 1986; Storrs et al, 1992). However, the failure of TFE to alter the pH-dependence of helix formation of S-peptide of RNAse A suggest that helix induction due to TFE is not a manifestation of its dielectric properties (Nelson & Kallenbach, 1986). The independence of the nature of the cosolvent (fluoro or alkyl) on the pH dependence of the three peptides (S-peptide, Sa-peptide and TC-peptide) rule out any significant role of dielectric properties of the cosolvent towards helix enhancement mechanism. The results also suggest that a peptide retains a sort of 'pH memory' when it is transferred from water to aqueous-alcohol mixtures. The 'pH memory' of a peptide may not be possible if the first hydration shell of the peptide were to be completely dried off or exchanged by the cosolvent. The results indicate the presence of water molecules in the interface between the peptide and the bulk solvent. The change in the structure of the bulk solvent could be a consequence of the ordering of water molecules around the hydrophobic groups in the alcohol (-CF₃/-CH₃ groups). The association of water molecules would be stronger at lower temperature as indicated by large increase in negative enthalpy (Denda et al, 1987; Westh & Hvidt, 1993) and is likely to be more for fluoro alcohols such as HFIP where formation of clusters or micelles have been observed (Hirota et al, 1997 & 1998; Anderson et al, 1996).
The additive effect of helix-induction of cosolvents consisting of fluoro alcohol and alkyl alcohol, two alkyl alcohols or two fluoro alcohols points to a common mechanism of helix-induction by fluoro and alkyl cosolvents and also rules out the possibility that enhanced alcohol acidity of TFE manifest into strengthening of the peptide hydrogen bond. The acidity of the solvent has been shown to be related to its hydrogen bonding ability (α). 1-Propanol and 2-propanol show similar effects as TFE though their α values (0.78 and 0.76 respectively) are about half compared with TFE (Kamlet et al, 1983). In contrast methanol and ethanol with higher α values compared to 1-propanol or 2-propanol, exert considerably less effect on helix enhancement.

Although the ability of aqueous-TFE mixtures to magnify the intrinsic helix forming tendencies of amino acid sequences is known for quite sometimes (Sonnichsen et al, 1992; Jackson et al, 1991; Kemmink & Creighton, 1995; Wei & Fasman, 1995; Tamburro et al, 1968; Conio et al, 1970; Shiraki et al, 1995; Goodwin et al, 1996), the mode of TFE-peptide interaction is not fully understood. Whether TFE induces helix formation by interacting with the peptide backbone (Jasanoff & Fersht, 1994; Guo & Karplus, 1994; Rajan & Balaram, 1997) or indirectly influences the peptide conformation through the medium or thermodynamic effect remains a contentious issue (Nelson & Kallenbach, 1986; Westh & Hvidt, 1993; Goodwin et al, 1996). The mechanism of alcohol-mediated helix enhancement was first studied by Conio et al, (1970) who proposed that helix enhancement in TFE solution resulted from selective destabilization of the coil state due to decreased solvation of the exposed amide groups. The helix enhancement in peptides is due to this thermodynamic or medium effect of the cosolvent was reiterated by Storss et al, (1992).

Recently, Goodwin et al, (1996) demonstrated significant rate enhancement in the interconversion of S-cis/S-trans amide conformers of acetyl-Pro-NH-CH₃ supporting the above proposal. However, Jasanoff and Fersht (1994) suggested a helix-induction mechanism that involved direct binding of TFE to the peptide. A more recent study has suggested that TFE stabilizes the helix by strengthening the intramolecular hydrogen bonds (Luo & Baldwin, 1997). Thus the current status of our knowledge of the process of TFE-mediated
helix formation suggest that facilitation of intramolecular hydrogen bonding of peptide amides is the principal cause of helix enhancement of peptides but how this is accomplished in the presence of TFE or other cosolvents is not clearly understood.

The retention of pH-dependence of helix formation in TFE and 1-propanol, additivity effect of alkyl alcohols and TFE and similar thermal profiles of both fluoro as well as alkyl cosolvent helix-induction curves suggest that the direct binding of the cosolvents to the peptide is unlikely and thus TFE or other cosolvents may perturb the conformational state of the peptide only in an indirect fashion by altering the structure of the water in the peptide hydration shell. In this connection, it is instructive to note that the \( \alpha \) helices in protein crystal structures have been found to be hydrated either externally through hydrogen bonding of water molecule to the backbone carbonyl oxygen atom or internally by forming a bridge of hydrogen bonds between the backbone carbonyl oxygen and amide nitrogen atoms (Satyshur et al, 1988; Sundarlingam & Sekharudu, 1989). The gamut of water associated protein segments have been suggested as possible "conformational reaction coordinates" in the unfolding of helices. For example, insertion of an externally hydrogen bonded water molecule between the backbone carbonyl oxygen atom and amide nitrogen atom would open up the helix leading to unfolding. The reverse of this would be realized in the process of helix formation. It is conceivable that alcohol-water mixtures facilitate the rupture of hydrogen bond between the water molecule and amide nitrogen atom while retaining the water molecule externally hydrogen bonded to the backbone carbonyl oxygen atom (the carbonyl oxygen atom has the capacity to form bifurcated hydrogen bonds) thereby destabilizing the coil state and favoring helix formation.

The results emanating from the comparative analyses of the effects of fluoro- and alkyl alcohols on helix formation in protein segments suggest that both fluoro as well as alkyl cosolvents act by a similar mechanism. The study reveals similar peptide helix-enhancing propensity of TFE, 1-propanol and 2-propanol precluding a significant role for fluoro group in the helix-enhancement
mechanism. The additivity effect of comixtures of both fluoro and alkyl cosolvents as well as their similar abilities to perturb helical conformation as a function of temperature and pH is likely to expand the choice and utility of cosolvents in carrying out many biochemical transformations. The helix-inducing property of 1-propanol has been already exploited in protease-catalyzed peptide ligation reaction that do not occur in the presence of glycerol as a cosolvent (Sahni et al, 1989; Roy et al, 1992).