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Chetal, P., Chauhan, V. S., Sahal, D.: Poster titled “A meccano set approach of joining Trpzip a water soluble β-hairpin peptide with a didehydrophenylalanine containing hydrophobic helical peptide” was presented at the 15th IUPAB and EBSA International Biophysics Congress held at Montpellier, France (Aug – Sep 2005).
A Meccano set approach of joining trpzip a water soluble β-hairpin peptide with a didehydrophenylalanine containing hydrophobic helical peptide

Key words: ββ2 motif; circular dichroism; fluorescence; long-range interactions; peptide design via Meccano approach

Abstract: A 16 residues long, water soluble, monomeric β-hairpin peptide 'trpzip' (Cochran et al. Proc. Natl. Acad. Sci. U.S.A., 98 (2001), 5578), stabilized by tryptophan zipper has been linked via a tetraglycyl linker to a hydrophobic didehydrophenylalanine (ΔF) containing helical octapeptide. Circular dichroism studies of this 28 residues long peptide, 'trpzipalpha' (Ac-GEWTWDATKTWTWTEGGG-ΔFALΔFALΔFAL-NH2) in water have revealed the presence of both the β-hairpin and the helical conformations. This is the first instance where a ΔF containing peptide has been found to display a helical fold in water. The fluorescence emission wavelengths of tryptophan in Ac-G-W-G-NH2, trpzip and trpzipalpha were 341.5, 332.8 and 332.6 nm, respectively. The fluorescence quantum yield of trpzip was 2.6-fold higher than trpzipalpha suggesting that proximal interactions between the β-hairpin and the helix caused the quenching of tryptophan fluorescence in the former by the ΔFs in the latter. The molar ellipticity of the far UV couplet characteristic of trpzip was reduced in trpzipalpha and the CD based thermal melting temperatures at 228 nm were 62 °C (trpzip) and 57 °C (trpzipalpha). A concentration-dependent variable temperature CD study in water showed that in trpzipalpha, increasing temperature is detrimental to the β-hairpin, but it augments the helical motif, perhaps by intermolecular oligomerization. Our results show that in water, trpzipalpha exhibits long-range interactions between two different secondary structures. In contrast to trpzip, trpzipalpha has shown a greater tendency to oligomerize in water.

Introduction

Every globular protein that remains folded in water has a hydrophobic, buried interior around which the polar,
charged residues exposed to water are organized in three-dimensional space (1). Water is both a threat and an opportunity for a polypeptide in its fast journey to a folded state. If the hydrophobic core is missing or insufficient, the protein is threatened by water that forces it to unfold. On the other hand, if this hydrophobic core is disproportionately large, the protein finds itself unable to dissolve in water. Water, if it must act as a catalyst of protein folding, requires a judicious apportioning of the hydrophobic and the hydrophilic elements in the sequence of a polypeptide. Peptide design is the arena of testing the level of our understanding of the principles that operate behind the design of natural biosynthetic proteins. In this context, organization of amino acid sequences in a binary code of hydrophobic and hydrophilic residues is known to cause the collapse of polypeptide chains into globular helical folds (2-3). Designed sequences (23-28 residues) that display ββz ‘zinc-finger’ fold are among the smallest protein domains, which fold cooperatively without assistance from metal ions or disulfide links and are the icons of the promise that this field holds (3-5). More recently, it was discovered that a structural motif called the tryptophan zipper (trpzip) greatly stabilizes the β-hairpin conformation in short peptides. Such trpzip peptides (12 or 16 residues long) are the smallest to adopt a unique tertiary fold in water, unaided by metal ions or disulfide links (6). Using hydrophobic and conformationally constrained non-protein amino acids, there have been notable successes in the design of super secondary structural protein motifs (7-10). However, a major shortcoming of such hydrophobic peptides has been their poor solubility in water. As a result, such peptides have up until now been studied either by solid-state crystallographic studies or by solution studies in organic solvents only. In a significant development, making use of Cα-tetra substituted α-amino acids and an extremely water-solubilizing azacrown-functionalized α-amino acid, the formation of stable 310 helices in bulk water has been reported (11). In the present study, we have synthesized and examined the conformational features of a peptide [Ac-GEWTWDATKTWTE-GGGG-ΔAFALAFALFA-NH2] in which trpzip, a 16 residues long bonafide water soluble β-hairpin peptide [6] has been linked to a hydrophobic, helical didehydrophenylalanine containing octapeptide via a tetra glycy1 linker. The helical sequence has been taken from a helical hairpin designated at the indi-
Results

Characterization by RPHPLC and mass spectrometry

Synthetic trpzip, trpzipalpha, ‘KΔF’ and GWG peptides were characterized by RPHPLC and mass spectrometry. Trpzipalpha showed a longer retention time at 53 min (58% acetonitrile) than trpzip at 37 min (42% acetonitrile), Fig. 1. This is consistent with the fact that in trpzipalpha, a hydrophobic octapeptide has been linked to trpzip via a tetraglycyl linker. The ESMS values observed for synthetic trpzip, trpzipalpha, ‘KAF’ and GWG peptide were 2054, 3161.2, 1173.4 and 360.3 Da, respectively. These values are consistent with the mass values of 2054, 3158, 1173.4 and 360.3 Da expected from the four peptides, respectively.

Circular dichroism studies of trpzip vs. trpzipalpha

Figure 2 shows the CD spectra of trpzip and trpzipalpha in water at pH 8. The two spectra have characteristic similarities and differences. Thus, both peptides show a couplet of strong exciton split bands in the far UV. This couplet, which originates from tryptophan–tryptophan interactions in the β-hairpin of trpzip, is stronger in intensity for trpzip than for trpzipalpha (Fig. 2A). The decrease in total molar ellipticity (deg cm²/dmol) is from ~700 000 [trpzip] to ~400 000 [trpzipalpha]. In the near UV segment of the CD spectrum, while trpzip shows no exciton-split couplet, the trpzipalpha shows a strong couplet of exciton split bands with a positive ellipticity at 271 nm, a negative ellipticity at 302 nm and a crossover point at 292 nm (Fig. 2B). The presence of the two characteristic exciton split couplets in the near and the far UV regions of the spectrum confirms the presence of a β-hairpin and a helix in trpzipalpha.

Figure 1. RPHPLC profiles of trpzip and trpzipalpha. Column Ultrapac TSK-ODS C18, flow rate: 2 ml/min, 0.1%TFA/H₂O-0.1%TFA/Acetonitrile gradient [5-75%Acetonitrile/70 min] was used. Trpzip (dashed line) and trpzipalpha (solid line) elute at 37 and 53 min, respectively.

Figure 2. Circular dichroism spectra of trpzip and trpzipalpha. The peptides (20 μM) were dissolved in 10 mM phosphate buffer pH 8 and spectra recorded at 298 K [Fig. 2A]. Note the decrease in [θ]T (total molar ellipticity) in the far UV region for trpzipalpha (solid line) in comparison with trpzip (dashed line). The near UV region shows a strong exciton couplet uniquely in trpzipalpha. This region is shown in full details in Fig. 2B.
Fluorescence studies of trpzip vs. trpzipalpha

Trpzip with its four tryptophan residues is inherently fluorescent. Any changes in the microenvironment of these tryptophans upon linkage of an additional sequence that is not fluorogenic, can be monitored by changes in the fluorescence characteristics of trpzip. Trpzipalpha represents such a peptide in which while trpzip is fluorescent, the di-dehydrophenylalanine containing helix is not. We measured the emission characteristics of trpzip vs. trpzipalpha taking Ac-Gly-Trp-Gly-NH₂ as a control for a peptide whose tryptophan is fully exposed to the solvent. As shown in Fig. 3 and Table 1, the emission wavelengths of Ac-Gly-Trp-Gly-NH₂, trpzip and trpzipalpha in aqueous buffer were 341.4, 332.8 and 332.6 nm, respectively. An emission at 341.4 nm for Ac-Gly-Trp-Gly-NH₂ is suggestive of a fully exposed tryptophan. A large blue shift of 8.6 nm for trpzip suggests considerable burial of tryptophans in trpzip. The nearly identical value of emission wavelength (~332 nm) for trpzip and trpzipalpha suggests the integrity of the trpzip β-hairpin in both the peptides. In going from aqueous buffer to 8 M guanidine hydrochloride solution, while the emission wavelength of the tryptophan of the tripeptide changed (from 341.4 to 342.2 nm) by only 0.8 nm, trpzip and trpzipalpha emissions showed large red shifts of 7.7 and 8.3 nm, respectively. In comparison with trpzip, trpzipalpha displayed a 2.6-fold reduction in the intensity of fluorescence emission (Fig. 3). This suggested that the di-dehydrophenylalanine containing helix by virtue of compaction with the tryptophan containing β-hairpin might be quenching the fluorescence of the latter.

An overlay of the absorption spectrum of ΔF as in 'KΔF' peptide and the emission spectrum of tryptophan as in trpzipalpha (Fig. 3, inset) revealed a region of overlap. The observed quenching could thus be caused by fluorescence resonance energy transfer between tryptophans from the β-hairpin and ΔFs in the helix. However, it was important to confirm that the observed quenching was not a consequence of the strong molar extinction coefficient of di-dehydrophenylalanine (~19 000/cm, λ 280 nm), which is approximately 4-fold more than the molar extinction of tryptophan. Indeed the ratio of absorption of light by tryptophans vs. di-dehydrophenylalanine in trpzipalpha [4W + 3ΔF] is ~3-fold more in favor of the non-fluorescent ΔF. Therefore, we measured the emission intensity of a mixture of trpzip with a water-soluble ΔF containing peptide [′KΔF' peptide] in a stoichiometry to have the same number of ΔFs, as is the case with trpzipalpha. In this situation, the degree of quenching observed was barely 0.8-fold in comparison to the 2.6-fold quenching seen in trpzipalpha (Fig. 3).

Water solubility of trpzip vs. trpzipalpha

Unlike trpzip, which is quite soluble (5 mM) in water, the aqueous solubility of trpzipalpha in phosphate buffer pH 8 was less by approximately 10-fold. Interestingly, the otherwise insoluble ΔF segment in trpzipalpha had gained considerable water solubility (0.5 mM) in the engineered peptide.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Emission wavelength (nm) in 10 mM phosphate buffer pH 8 (A)</th>
<th>Emission wavelength (nm) in 8 M Guanidine HCl/10 mM phosphate buffer pH 8 (B)</th>
<th>(B)-(A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-G-W-G-NH₂</td>
<td>341.4</td>
<td>342.2</td>
<td>0.8</td>
</tr>
<tr>
<td>Ac-Trpzip-NH₂</td>
<td>332.8</td>
<td>340.5</td>
<td>7.7</td>
</tr>
<tr>
<td>Ac-Trpzipalpha-NH₂</td>
<td>332.6</td>
<td>340.9</td>
<td>8.3</td>
</tr>
</tbody>
</table>

* Excitation and emission wavelength are as in Fig. 3.

Figure 3. Fluorescence characteristics of trpzip, trpzipalpha and reference peptides. The spectra for trpzip (I, thick solid line), Ac-Gly-Trp-Gly-NH₂ (II, dotted thin line) and trpzipalpha (IV, thin solid line) were recorded at a concentration of 8.7 and 2.17 μM, respectively. This was done to ensure equal concentration of tryptophan in the three samples. The mixing experiment (III, thick dotted line), had trpzip (2.17 μM) and the 'KΔF' peptide (3.6 μM). The mixing in this ratio mimics the ratio of W and ΔF in trpzipalpha. All samples were dissolved in 10 mM phosphate buffer, pH 8. Excitation was at 280 nm. The excitation and emission slit widths were set at 10 and 5 nm, respectively. Inset shows the overlap between the absorption spectrum of ΔF [thick line] as in 'KΔF' peptide and emission spectrum of tryptophan [thin line] as in trpzipalpha.
Concentration dependence of CD spectra of trpzipalpha

When trpzipalpha was examined by CD spectroscopy as a function of concentration, the Mean Residue Ellipticity (MRE) was found to rise progressively from 5 to 20 μM in both the far and near UV regions (Fig. 4A,B). This suggested that the peptide was oligomerizing as a function of concentration. It may be noted that the concentration dependent percent rise in MRE was much higher (300%) in the near UV region than was the case in the far UV region (16%). This suggests that the hydrophobic helical segment may constitute the sticky ends for the trpzipalpha monomers to oligomerize. The increment in MRE for both the far and the near UV regions was linear up to a concentration of 20 μM. However, a sudden fall in the two MREs was noticed at a peptide concentration of 40 μM, beyond which MREs began to rise again. Except for a fall in MRE at 40 μM (Fig. 4A,B), a plateau was observed from 20 to 80 μM. It may be noted that the sudden fall in MRE seen at 40 μM was not an experimental error but was seen consistently on repeating the experiment.

CD study of thermal denaturation of trpzipalpha

In order to examine the properties of the monomeric and oligomeric states of trpzipalpha, thermal denaturation was examined by CD at a low concentration of 5 μM as well as at a high concentration of 160 μM. As shown in Fig. 4C, progressive heating of an aqueous solution of trpzipalpha (5 μM) caused a progressive decrease in the intensity of the far UV coulpet until it was significantly reduced at 343 K, suggesting the loss of β-hairpin. However, interestingly, as temperature was increased, the near UV coulpet showed a

Figure 4. Concentration and temperature dependence of the CD spectra of trpzipalpha. Panels A and B show the concentration dependent nonlinear CD patterns of trpzipalpha, in the far UV and the near UV regions, respectively. Panels C and D show CD spectra at a peptide concentration of 5 μM recorded at temperatures 293-343 K at an interval of 10 K. The 293 K and the 343 K curves have been indicated by arrows. The intervening temperature curves showed a regular trend. Increasing temperature affects the near and the far UV spectra differentially: While the β-hairpin represented by the far UV coulpet shows loss in structure (Panel C), the helix represented by the near UV coulpet seems to become stronger (Panel D). The temperature-dependent selective gain in structure for the helical segment is concentration dependent. This gain is seen at 5 μM (Panel E) but no increment in the already high [θ]max (deg cm²/dmol) at 160 μM (Panel F) is seen in the near UV region as temperature is increased from 293 to 343 K. Thin dotted line and thick dashed line in Panels E and F represent spectra at 293 and 343 K, respectively.
progressive rise (Fig. 4D) in intensity. This indicates that as a function of rising temperature, the ΔF containing helix becomes stronger, a likely consequence of temperature-mediated oligomerization of the hydrophobic helical segment in water. Such oligomerization seems to confer thermal stability to the helix but not to the β-hairpin component of trpzipalpha. When the temperature shock experiment was performed at 160 μM, the far UV couplet was found to collapse (Fig. 4F) akin to its behavior at 5 μM [Fig. 4E]. However, at this concentration, the near UV couplet remained invariant in intensity.

Thermal melting comparison of trpzipalpha and trpzip

Trpzip has been described as a peptide with very high-thermal folding energies that folds cooperatively in water. In order to find the effect of the extension of trpzip to trpzipalpha on the thermal melting behavior, we performed a variable temperature CD experiment in aqueous buffer (pH 8) at a peptide concentration of 20 μM. It was interesting to note that the thermal melting curves of the two peptides measured at 228 nm (Fig. 5A) appeared to be nearly the same. The thermal melting temperatures were measured as 62 °C (trpzip) against 57 °C (trpzipalpha). A comparison of the two melting curves indicates a little more flattening (7–20 °C) and a little sharper melting (40–80 °C) of the trpzipalpha curve when compared with trpzip. This suggests the thermal melting of trpzipalpha to be slightly more cooperative than is the case with trpzip. Interestingly, as the CD signal at 228 nm was decreasing with rise of temperature, the CD signal in the near UV (271 nm) was found to increase up to ~50 °C beyond which it too started to fall (Fig. 5B).

Discussion

Even as protein folding is a well-regulated phenomenon, the rules governing it have remained elusive. The first hallmark of water soluble proteins is their natural tendency to bury hydrophobic surfaces and expose the polar sequences. This phenomenon causes proteins to assume globular shapes. The formation of well-defined secondary structures like helices and sheets and their mutual compaction is another hallmark of proteins. The quick digestion of unfolded proteins by proteases vs. the enhanced stability of their folded counterparts is a testimony to the importance of compaction of well-defined secondary structures in protein folding.

A notable success in designing polypeptides that resemble proteins structurally has been the use of a simple binary code of polar and non-polar residues arranged in an appropriate order to drive them into a globular α-helical fold (2). However, the success of binary patterning is not to suggest that nature has always used such a principle for patterning cellular proteins. It is clear that there remain several undiscovered rules and patterns used by nature in designing proteins of the cell. Folding and misfolding of cellular...
proteins are two faces of the same coin. Mutation-induced misfolding of cellular proteins is the cause of several human diseases [13,14]. De novo protein design is a true testing ground for our understanding of protein folding since the designed peptides can be assessed for some of the protein like features indicated above.

In the present study, we have used a kaleidoscopic approach of conjoining two well-defined secondary structures via a flexible linker. The first of these two units of a protein in the making was trpzip [6], a 16 residues long, highly negatively charged, water soluble, Tryptophan rich β-hairpin peptide stabilized by Trp-Trp zipper interactions. This happens to be the shortest peptide to fold cooperatively in water. Our second unit was a didehydrophenylalanine (ΔF) containing apolar octapeptide. This stretch was taken from a helical hairpin eicosapeptide where it assumes a helical fold [8]. Such unsaturation of phenylalanine defines the origin of ΔF. Such unsaturation causes extensive delocalization of the electronic cloud of benzene ring well into the peptide bonds flanking the ΔF residue. The resulting partial double bond character of such bonds causes conformational constraints in the backbone torsion angles causing ΔF peptides to assume helical conformations. The propensity of ΔF residues to induce β turns in short peptides and helical folds in longer ones has been well established [10]. Its ability to engage in weak CH-O or NH-II interactions makes it particularly useful for design of long-range driven super secondary structures [12]. It may be noted that the ΔF containing octapeptide in our design is apolar and highly insoluble in water. Thus in combining trpzip, a β-hairpin with the ΔF containing octapeptide helix, we were combining a highly charged, water-soluble peptide with an apolar water insoluble peptide. In order to facilitate long-range interactions between the helix and the β-hairpin, it was important to choose a permissive linker. Glycine's simplicity and flexibility has rendered it most suitable to occupy the turn regions of natural proteins [2] as well as de novo designed super secondary structures [8]. Hence, we chose a tetracyclic linker to join the β-hairpin with the helical segment. Our surmises were that (a) with the assistance of the water-soluble β-hairpin, the apolar helical octapeptide may be solvated in water, (b) the apolar octapeptide may form a stable helix in water (c) neither of the two structural modules may destabilize each other and (d) in the event that the apolar helix did not gain sufficient stability in a monomeric state, it could dimerize through a hydrophobic effect. The first of these features became immediately apparent when we realized that while the water solubility of trpzipalpha (~0.5 mM) was considerably lower than was the case with trpzip (~5 mM), it was nevertheless a quantum jump for the solubility of the apolar helical segment in water. (The solubility of such apolar peptides in water is <7 μM).

The CD spectra of trpzip vs. trpzipalpha in water showed characteristic signatures of each peptide. Thus, while the CD spectrum of trpzip showed a single excitonic couplet in the far UV, the corresponding spectrum of trpzipalpha showed an additional excitonic couplet in the near UV region (Fig. 2). The former arises from Trp-Trp interactions in a β-hairpin [6] and the latter from the organization of the strongly absorbing ΔF chromophores in a helical backbone [16,17]. The near UV couplet with a positive ellipticity at 271 nm, a negative ellipticity at 302 nm and a cross over at 292 nm was clearly visible in the CD spectrum of trpzipalpha. It may be noted that tryptophans of the β-hairpin give only a very weak, although a characteristic triplet signal in the near UV region [6]. However, this is too weak to cause any interference with the much stronger CD signal due to the ΔF containing helix (Fig. 2). As a result, in the CD spectrum of trpzipalpha, the dominance of the ΔF couplet seems to mask the tryptophan signal completely. One of the first signs of compaction and proximity between the β-hairpin and the helical segment became apparent when we compared the total molar ellipticities of trpzip and trpzipalpha in the far UV couplet. This value [deg cm²/dmol] was reduced from ~700 000 in trpzip to ~400 000 in trpzipalpha. The presence of this couplet in trpzipalpha is a proof of the stability of the β-hairpin in trpzipalpha. The diminished value of molar ellipticity however, may be a sign of perturbation of the β-hairpin by the helical segment.

A second evidence of molecular compaction came by way of fluorescence studies. Inherent in the design of trpzipalpha is the feature that while the tryptophan rich β-hairpin module represented by trpzip is highly fluorescent, the ΔF rich helix has no ability to fluoresce. Further, we hypothesized that in the event that tryptophan and didehydrophenylalanine residues came in close proximity, the latter may quench the fluorescence of the former. This expectation is valid since the fluorescence of tryptophan is well known to be uniquely sensitive to quenching by externally added quenchers or nearby groups in proteins [18]. The blue shifted λmax of emission (at ~332 nm) for both trpzip and trpzipalpha [Fig. 3] indicates that tryptophans in trpzip and trpzipalpha are buried and not exposed to water. However, at identical concentrations (2.175 μM in water), the quantum yield of fluorescence emission due to trpzip appeared to be 2.6-fold higher than the corresponding yield from trpzipalpha. A likely cause of fluorescence quenching in
trpzialpha could be the proximity driven interaction between tryptophans [fluorogens] of the β-hairpin and the ΔFs [quenchers] of the helix. However, the observed quenching could also be caused by the fact that the ε_{280} of ΔF is ~4-fold as high as ε_{280} of tryptophan. Thus the relative absorbances due to trpzip (4 W; ε_{280}~20 000) vs. the helical segment (3ΔFs; ε_{280}~57 000) is ~1:3. Therefore, the observed quenching seen in trpzialpha could be due to non-availability of sufficient number of photons for tryptophans to absorb. To explore this possibility, we mimicked the Trp-ΔF ratio [4:3] of trpzialpha by mixing one part trpzip with 1.5 parts of a water-soluble ΔF containing peptide (Ac-GP (KK..I F) 2 AK-NH2). The quenching caused in this mixture was much less (~19%) in comparison with the dramatic quenching (~62%) seen in trpzialpha (Fig. 3). Since the emission spectrum of tryptophan partially overlaps with the absorption spectrum of ΔF [Fig. 3, inset], the observed quenching may be caused by fluorescence resonance energy transfer [19]. Thus, it is concluded that ΔF quenches the fluorescence of tryptophan and indicates that in trpzialpha, the β-hairpin and the helix are in close proximity [Fig. 6].

Figure 6. Predicted structure of trpzialpha supported by solution studies described in the present paper. The presence of the β-hairpin and the helical segments is based on CD studies. Large blue shifts in fluorescence emission also indicated the presence of a β-hairpin in both trpzip and trpzialpha. The proximity between the two secondary structures is based on fluorescence quenching of tryptophans in the β-hairpin by didehydrophenylalanine in the helix. The model was drawn in INSIGHT II. The coordinates of trpzip and tetraglycyl segment were from Cochran et al. 2001 [6] [PDB number ILE 3] and Ramagopal et al. 2001 [8], respectively. The software was asked to model the didehydrophenylalanyl octapeptide as a helix.

The low water solubility of trpzialpha seemed to suggest that the peptide having an appendage of an apolar helix might have become prone to oligomerization. Further evidence in support of oligomerization came from concentration dependent CD studies [15], which showed a linear rise in MRE selectively in the near UV region [Fig. 4B]. There was a paradoxical decrease in MRE at 40 μM, beyond which MRE continued to rise again, tending to plateau in the higher concentration zone. Any non-linearity in MRE is indicative of a structural change in the peptide monomers or their patterns of intermolecular assembly. However, we do not know the cause for the non-linear pattern of MRE observed for trpzialpha as a function of increasing concentration of the peptide. The plateau seen at 20 and 80 μM[Fig. 4B] suggests the formation of defined oligomers glued together via the ΔF containing helix. This is likely to be the case since ΔF containing peptides have demonstrated the ability to form intermolecularly interacting ΔF zippers [21]. An intermolecular dimerization of trpzialpha through the apolar helix, driven by the hydrophobic effect in water is a distinct possibility. However, we could not confirm this since both trpzip and trpzialpha displayed an anomalous retardation in gel filtration (Sephadex G25 SF, 50 mM phosphate buffer, 100 mM NaCl, pH 8). Thus, the elution volumes of trpzialpha (3158 Da) and trpzip (2054 Da) were 52 and 80 ml, respectively, on a column of total volume 30 ml. It is well known that aromatic substances show greater affinity to Sephadex gels than to aqueous solutions and are thus usually retarded to varying extents compared with non-aromatic substances of similar size [22]. Since a hallmark of both trpzip and trpzialpha is a high content of aromatic residues, the anomalous retardations are indeed likely to be a consequence of interactions between the chromatographic bed and the aromatic residues in the two peptides. Even as trpzialpha has shown a tendency to oligomerize in water, we believe that the units of such oligomers would be the pre-folded ββα monomers with long-range intramolecular interactions between the β-hairpin and the helix. Such a proposition is tenable since, although the CD spectrum of trpzialpha in comparison with that of trpzip shows a characteristically decreased MRE in the far UV couplet, the magnitude of this ellipticity is nearly concentration independent [Fig. 4A]. Therefore, it appears that the trpzip β-hairpin is not involved in oligomerization of trpzialpha. The oligomerization at the helix-helix interface may occur via two modes: (a) the helix of each monomer is in compact intramolecular interaction with its β-hairpin and (b) the helix of each monomer may prefer not to intramolecularly interact with its β-hairpin. It
is well known that the effective concentration of interacting species in the intramolecular situation can vary between \(10^{-10}\) and \(10^{10}\) M \cite{23}. The low values occur when the two groups are held apart by the molecule to which they are attached; extremely high values occur when the two groups are held rigidly in optimal position for interaction even when they are not \cite{23}. The decreased ellipticity of the far UV couplet in trpzipalpha must be because of intramolecular interactions between the hairpin and the helix due to several orders of magnitude higher effective concentrations of the interacting partners in an intramolecular situation facilitated by the flexible tetracyclic linker. Therefore, if the reduction in the far UV ellipticity is a signature of the intramolecularly folded trpzipalpha, the concentration independence of this signal is a proof of the presence of prefolded monomers being the units of the oligomers.

When CD studies were performed as a function of temperature, the pattern of CD changed selectively in the far and near UV regions respectively. Thus at low concentration (5 \(\mu\)M), as the temperature was increased, while the \(\beta\)-hairpin was found to be destabilized, the helical segment showed a rise in the CD signal. On the other hand, at a high concentration of 160 \(\mu\)M, while the \(\beta\)-hairpin continued to show temperature-dependent destabilization, the helical segment showed no change in the CD signal. It therefore appears that at high concentration, the state of aggregation confers sufficient stability to the participating helices. As a result, further increase in temperature causes no further rise in ellipticity. These results suggest that the helical segment is inherently more stable to temperature than is the case with the \(\beta\)-hairpin.

In order to find the effect of linking the apolar helical octapeptide to the \(\beta\)-hairpin via the tetracyclic linker, on the cooperativity of thermal melting of trpzip, a variable temperature CD experiment at 228 nm was conducted for both trpzip and trpzipalpha. In addition, the melting of trpzipalpha was also monitored at 271 nm. It appeared from the melting profiles at 228 nm that trpzipalpha unfolds a little more cooperatively than is the case with trpzip. This appears to be the case from a greater flatness of the melting curve in the temperature range of 7–20 °C for trpzipalpha when compared with the trpzip. In addition, beyond 20 °C, the melting curve of trpzipalpha falls a little more steeply than is the case with trpzip \cite{5A}. This gives a higher \(T_m\) of 62 °C to trpzip in comparison to a \(T_m\) of 57 °C for trpzipalpha. It may be noted that the \(T_m\) observed by Cochran \textit{et al.} for the parent trpzip 4 peptide is 70 °C, and therefore 8 °C higher than the value obtained by us. The only difference between the two peptides is the presence of a free N-terminal amino group in the trpzip 4 studied by Cochran \textit{et al.} and an acetyl capping in our peptide. This may suggest the importance of free N-terminal amino group in the stability of trpzip. Interestingly, the melting profile of trpzipalpha showed a divergence of melting behaviors at 228 nm vs. 271 nm. While the CD signal showed a progressive fall at 228 nm as temperature was increased, at 271 nm it showed a rise up to a temperature of \(\sim\)50 °C, beyond which it decreased \cite{5B}. It therefore appears that temperature influences the two modules of trpzipalpha in two different ways. Such dichotomy between stabilities in different domains of the same protein has been observed in Hyaluronate Lyase \cite{20}.

The Meccano set approach towards protein design used in this study is versatile in terms of the diversity of structures it can improvise giving us an opportunity to fathom the language of tertiary interactions in proteins. A sustained effort along this path promises to provide us proteins of our design that may match or surpass the proteins of nature.

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