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N-terminal diproline and charge group effects on the stabilization of helical conformation in alanine-based short peptides: CD studies with water and methanol as solvent

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Protein folding problem remains a formidable challenge as main chain, side chain and solvent interactions remain entangled and have been difficult to resolve. Alanine-based short peptides are promising models to dissect protein folding initiation and propagation structurally as well as energetically. The effect of N-terminal diproline and charged side chains is assessed on the stabilization of helical conformation in alanine-based short peptides using circular dichroism (CD) with water and methanol as solvent. A1 (Ac–Pro–Pro–Ala–Lys–Ala–Lys–Ala–Lys–Ala–NH_2) is designed to assess the effect of N-terminal homochiral diproline and lysine side chains to induce helical conformation. A2 (Ac–Pro–Pro–Glu–Glu–Ala–Ala–Lys–Lys–Ala–NH_2) and A3 (Ac–DPro–Pro–Glu–Glu–Ala–Ala–Lys–Lys–Ala–NH_2) with N-terminal homochiral and heterochiral diproline, respectively, are designed to assess the effect of Glu...Lys (*i*, *i* + 4) salt bridge interactions on the stabilization of helical conformation. The CD spectra of A1, A2 and A3 in water manifest different amplitudes of the observed polyproline II (PPII) signals, which indicate different conformational distributions of the polypeptide structure. The strong effect of solvent substitution from water to methanol is observed for the peptides, and CD spectra in methanol evidence A2 and A3 as helical folds. Temperature-dependent CD spectra of A1 and A2 in water depict an isodichroic point reflecting coexistence of two conformations, PPII and β-strand conformation, which is consistent with the previous studies. The results illuminate the effect of N-terminal diproline and charged side chains in dictating the preferences for extended-β, semi-extended PPII and helical conformation in alanine-based short peptides. The results of the present study will enhance our understanding on stabilization of helical conformation in short peptides and hence aid in the design of novel peptides with helical structures. Copyright © 2017 European Peptide Society and John Wiley & Sons, Ltd.

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Introduction

Proteins are characterized by the ability to adopt unique folds specific for their sequences [1]. The folds are specified sequentially, and characterizing the basis remains a grand challenge given the size of a typical protein and the complexity of its interactions [2–4]. The problem has been addressed using bottom up approach of simple to incrementally complex models. The models are designed to observe the conformational preferences of the polypeptide chain and addressing the basis with theory using computational modeling [5–7]. The empirical force fields have been applied for simulation of equilibria to address the thermodynamics with rigor [8–10]. Much research has been reported in recent years with oligoalanine as the protein main chain models [11–15]. The models have established that unfolded proteins tend to adopt appreciable order as semi-extended structures in correspondence of polyproline II (PPII) conformation [16–18].

In the interest of protein folding problem, it is useful to perturb oligoalanine model with effects such as to induce their ordering as specific folds to characterize the effect with both experiment as well as theory. Specifically, dissection of effects that concern the interactions of the main chain against interactions of side chains evokes interest. The stereochemical effect of N-terminal modification that is capable of influencing conformation at the level of main chain structure [19–21] and the role of residue stereochemistry in the delineation of protein folding mechanism [22], to increase the stability of proteins [23], to redesign an active

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and specific ion channel [24] and in the design of novel folds are highlighted in the literature [25–29]. The electrostatic interactions among main chain as well as side chains are well recognized for their role in promoting conformational folding in the polypeptide structure [30,31].

The helical fold is of paramount importance in protein folding research as it constitutes a major secondary structure found in natural proteins and controls numerous biological activities and functions [32,33]. The helical fold plays a key role in the vast majority of proteins that are found in the cell and is often found at the interface between proteins mediating protein–protein interactions [34]. The misregulation of helical promoted interactions leads to the disease state. Thus, a better understanding of the helical structure and the elucidation of the factors that dictate its structure and stabilization are of key importance.

The folding simulations of all-alanine peptides and a number of short alanine-based helical peptides with positively or negatively charged residues have highlighted the role of hydrophobic interaction and charged side chains in the folding of α-helical peptides [35]. The diproline segments, covalently constrained diproline surrogate, have been reported as potential nuclei for initiating helical folding in peptides [36–38]. The present study addresses nonapeptide composed of L-amino acids for the effect in N-terminal diproline and charge-group effect over side chains that are capable of ordering the peptide as a helical fold. Due to charged side chains, nonapeptides are water soluble and hence amenable to experiment. The peptides were designed, synthesized and examined with circular dichroism (CD) to assess the possible contribution of extended-β, semi-extended PPII and helical conformation in the equilibrium ensemble. Subtle variations of these conformational possibilities are indicated in the designed peptides with CD. Circular dichroism spectral studies have been employed to identify stable and nascent secondary structure in short peptide fragments which lack tertiary interactions in a number of studies [39–41]. The implications for the understanding of nucleation and stabilization of helical conformation are discussed. The results of the present study will aid in the design of novel peptides with helical structures. The role of helical structures in the rational design of biocompatible hydrogels and inhibition of disease-relevant intracellular or extracellular protein–protein interactions has been reported in the literature [42,43].

Results and Discussion

Design, Synthesis and Characterization of Peptides

The study is implemented with nine-residue peptides (Table 1). The models are primarily oligoalanine sequences having intrinsically helix favoring residue [44]. The model oligoalanine is substituted with internal Lys and Glu residues for imparting solubility. The N-terminal dialanine is substituted with a diproline segment of homochiral and heterochiral structure for possible inducement of helical conformation. The diproline segment, covalently constrained diproline surrogate, has been employed as potential nuclei for inducing helical conformations in peptides [36–38]. Thus, a combination of charge-group effect over side chains and stereochemical effect of the N-terminal structure is examined for possible inducement of helical folds.

A1 is designed to assess the effect of N-terminal homochiral diproline segment and lysine side chains for the inducement of helical conformation. **A2** and **A3**, with N-terminal homochiral and heterochiral diproline segment, respectively, are designed to assess the effect of Glu...Lys (*i*, *i* + 4) salt bridge interactions on the nucleation of helical conformation. The designed peptides were synthesized by manual solid-phase synthesis using standard Fmoc-chemistry. The peptides displayed expected MS peaks in QTOF-ESI-MS, m/z 922 for **A1**, and m/z 981 for **A2** and **A3** (Figure S1, see Supporting Information). Proton nuclear magnetic resonance (^{1}H NMR) spectra for **A1**, **A2** and **A3** recorded at 2.5 mM concentration in 90% H_2O/10% D_2O at 298 K are presented in Figure S2. ^{1}H NMR spectra were recorded at tenfold dilution for each peptide. No noticeable dilution effect on chemical shifts or line widths was observed for the synthesized peptides. This indicates that the peptides do not aggregate in the concentration regime of NMR experiment. **A2** and **A3** manifest similar ^{1}H NMR spectra except in their NH regions in accordance with stereochemically differentiated structure of the peptides (Figure S2). Nuclear magnetic resonance spectra in methanol were characterized by apparent rapid solvent exchange resulting in non-observation of amide-NH resonances. Therefore, no further NMR studies were pursued in methanol.

Table 1. Sequence of the designed nine-residue alanine-based peptides **A1**, **A2** and **A3**. The N-terminal homochiral and heterochiral diproline segment, and amino acids with charged side chains are shown in bold

Model	Alanine-based peptides
A1	Ac–**Pro**–**Pro**–Ala–**Lys**–Ala–**Lys**–Ala–**Lys**–Ala–NH_2
A2	Ac–**Pro**–**Pro**–**Glu**–**Glu**–Ala–Ala–**Lys**–**Lys**–Ala–NH_2
A3	Ac–**D-Pro**–**Pro**–**Glu**–**Glu**–Ala–Ala–**Lys**–**Lys**–Ala–NH_2

Characterization of Conformation of Peptides in Water

The CD spectra of peptides **A1**, **A2** and **A3** in water are displayed in Figure 1. The spectrum of **A1** is characterized by a weak positive peak at ~220 nm and a sharp negative peak below 200 nm. In the absence of interfering signals from aromatic side chains, such a couplet is characteristic for the PPII conformation [45–47]. The

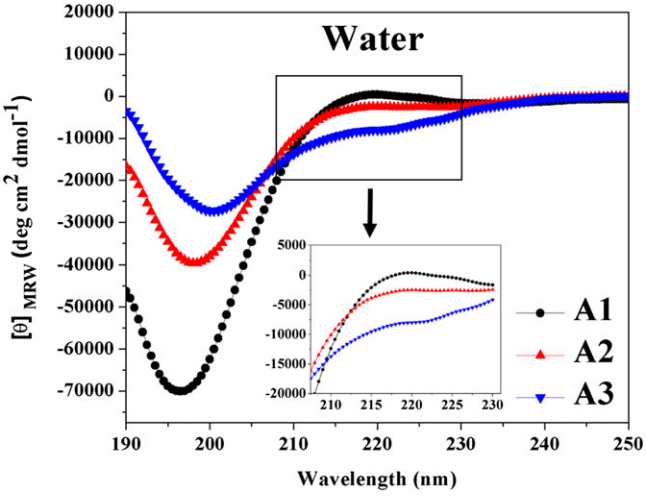

Figure 1. Circular dichroism (CD) spectra of **A1**, **A2** and **A3** in water at 298 K. Y-axis represents mean residue molar ellipticity, and X-axis represents wavelength in nanometer. The peptide concentration is 40 μM. Inset shows the wavelength region (207–230 nm) that includes the characteristic maxima (217–220 nm) for the polyproline II (PPII) conformation in higher magnification.

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CD spectrum of **A1** in water resembles the pattern that Kallenbach and co-workers characterized as a signature for PPII helix [18]. Banerji, *et al.*, reported that tetrapeptide constellated with alternative D- and L-proline displayed a negative CD band at ~199 nm, which highlights that peptide exists in the PPII conformation [48]. The weak positive maximum at 217–220 nm is the most pronounced for **A1**, which suggest that **A1** has, relatively, the highest contribution of the PPII-like conformer.

As shown in Figure 1, the small positive maximum at ~220 disappears, and the absolute value of negative band at ~197 nm decreases along with ~1-nm redshift for **A2** and **A3**. In addition to the negative band at ~197 nm for **A3**, we observe a broad negative shoulder at ~224 nm for **A3** which highlight existence of other conformational folds in the equilibrium ensemble. Thus, observation of variation in the level of ordering of PPII conformation implies the equilibrium that involves the participation of other conformational folds, which highlight the role of N-terminal diproline and Glu...Lys salt bridge interactions in the conformational landscape of the designed peptides. The participation of other conformational folds is evident from the percentage helical content calculated from the mean residue molar ellipticity at 222 nm, which highlights that **A2** and **A3** sample helical conformation, i.e. 9 and 26%, respectively (Table 2). The ellipticity at 222 nm has been employed to estimate the α-helix content from CD spectra in a number of studies [49,50].

Saha, *et al.*, have investigated conformational states for the diproline segment (^{L}Pro–^{L}Pro) found in 606 protein structures in the non-redundant data set [51]. The study highlighted that PPII–PPII and PPII–α are the most favorable conformational states for the diproline segment, which is consistent with the results of the present study that highlights higher PPII content for **A2** as compared to **A3**, which in turn implies lower helical content for **A2** than **A3**.

Raghavender investigated linear hydrophobic sequences containing centrally positioned diproline motifs, heterochiral (DL/LD) and homochiral (LL/DD), for their ability to form β-hairpins [52]. The author reported that the propensity of homochiral diproline segments to adopt PPII conformations is higher than heterochiral segments, which is consistent with the results of the present study. Density Functional Theory (DFT) calculations highlight that the energetics of folding critically depend on the geometrical relationship between backbone peptide units of the polypeptide structure [21]. The effect of the orientation of backbone peptide units of polypeptide main-chain on the energetics of folding has been examined with density functional theory by utilizing end-protected model peptides of LLLL- and DLLL-stereochemical structure. DFT calculations highlighted that enthalpy change and free energy change from an extended state to the folded state is more favorable for Ac–DPro–LPro–LAla_2–NHMe as compared to Ac–Pro_2–Ala_2–NHMe, which is consistent with the present results that highlight more helical structures for **A3** than **A2**.

Table 2. Mean residue molar ellipticity at 222 nm, i.e. $[\theta]_{222}$ and estimated helix content from CD spectra of **A1**, **A2** and **A3** in water and methanol

Model	Water		Methanol	
	$[\theta]_{222}$	Estimated helix content[a] (%)	$[\theta]_{222}$	Estimated helix content[a] (%)
A1	57.38	0	−5730.46	20
A2	−2603.26	9	−22214.72	76
A3	−7767.65	26	−15232.78	52

[a]Helix content was calculated from the mean residue molar ellipticity at 222 nm, i.e. $[\theta]_{222}$ using the following equation, % helix =100 $[[\theta]_{222}$ / {−39500(1−2.57/*n*)}], where *n* is the number of peptide bonds [20].

Effect of Solvent on the Conformation of Peptides

The alcohols, methanol and trifluoroethanol (TFE), have been extensively used in the protein folding and structure examinations by experimental approaches such as NMR, CD, Fourier transform infrared spectroscopy (FTIR), light scattering and fluorescence [53,54]. Methanol plays an important role as a raw material or solvent in numerous enzymatic syntheses due to its low cost and easily supplied industrial organic solvent [55]. Thus, characterization of the conformation of peptides in methanol is of key importance.

The strong effect of solvent substitution from water to methanol is observed on the conformation of **A1**, **A2** and **A3** as shown in Figure 2. With reduced polarity and dielectric strength of methanol as solvent, mutual interactions of suitably placed charged side chains could affect conformation.

As depicted in Figure 2, CD spectra in methanol evidence **A2** and **A3** as helical folds specifically on the basis of observation of two strong negative bands at ~205 and ~224 nm and a positive band at ~193 nm [56]. Thus, there is a combined effect of N-terminal diproline and Glu...Lys salt bridge in the ordering of **A2** to helical conformation in methanol. The negative bands at ~205 and ~224 nm for **A2** are much more pronounced, which suggests that the content of the helical structure is highest for **A2** (Figure 2, Table 2). Hwang, *et al.*, highlighted that local sequence and environment play a significant role in determining whether methanol tightens or loosens the local protein structure [49]. The observed variation in the helical content of **A2** as compared to **A3** in methanol can be explained on the basis of change in the N-terminal diproline segment. The ratio (*R*) of the two negative maxima of the ellipticity, $R = [\theta]_{222}/[\theta]_{207}$, has been employed to estimate the helical type (whether 3_{10}-helix or α-helix) [57–59]. For **A2** and **A3**, the ratio (*R*) is 0.57 and 0.56, respectively, which highlights that **A2** and **A3** adopt 3_{10} helical conformations in methanol.

Lack of Concentration Dependence Observed by Circular Dichroism Spectroscopy

For each peptide **A1**, **A2** and **A3**, CD signature was examined at four different concentrations of the peptide: 40, 60, 80 and

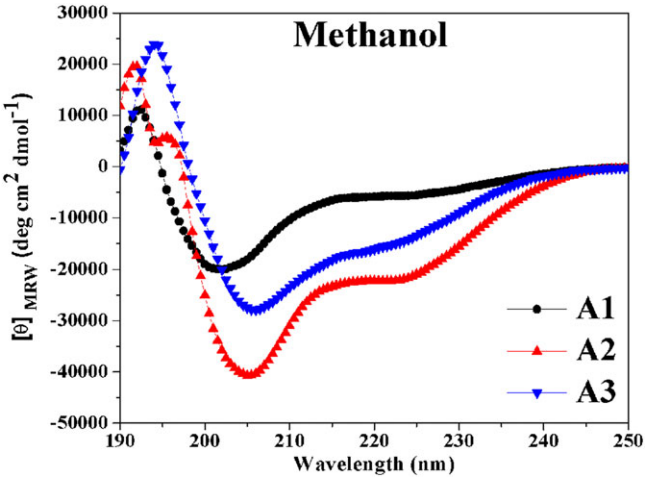

Figure 2. CD spectra of **A1**, **A2** and **A3** in methanol at 298 K. The peptide concentration is 40 μM.

100 μM in water as well as in methanol. For each peptide, no remarkable change was observed, and CD signature was identical at every concentration in water (Figure 3, left panel). These CD spectral results of **A1**, **A2** and **A3** indicate that, for this concentration range, the peptides display near identical signature, signifying a lack of concentration dependence (Figure 3, left panel). Banerji, *et al.*, observed a red shift in the CD signature of a tetrapeptide constellated with alternative D- and L-proline upon an increase in the peptide concentration in water, which highlights the formation of the oligomeric structure of the peptide [48]. The absence of a red shift in the CD signature of **A1**, **A2** and **A3** on concentration variation in water indicates that each peptide exists as a monomeric fold. Guarracino, *et al.*, designed and synthesized short six to eight residue α- and β-peptides by using primary sequence design features that influence helical control and directly compared the helicity across peptides with the most minimal epitopes [60]. The CD signature was examined for three different concentrations of synthesized peptides, i.e. 25, 50 and 100 μM. No remarkable change was seen in the CD signature of the synthesized peptides, and helical signature was identical at every concentration. The peptides display near identical CD signature signifying a lack of concentration dependence for this concentration range.

With the increase in peptide concentration, no marked change of peak position was observed; however, variation in the intensity of the positive peak at 193 nm was observed for **A1**, **A2** and **A3** (Figure 3, right panel). The minimum at ~205 and ~224 nm and a maximum at ~193 nm represent helical structures, and the absolute values at these points indicate the percentage of helical residues in the peptide. The variation in the intensity of the positive peak at 193 nm thus highlights minor changes in the percentage of helical residues in the designed peptides. Any change in the helical signature as a result of concentration variation designates potential helical oligomerization [61]. Thus, based on these results, no oligomerization appeared to occur in the synthesized peptides in methanol, and hence each peptide fold is monomeric in concentration regime of CD experiment in both water and methanol, which is consistent with the results from the NMR spectra as well.

Effect of Temperature and Solvent Variation on the Conformation of Peptides

The main objective of the present study was to address a combined effect of N-terminal diproline and charge groups for the possible inducement of helical folds in the *de novo* designed nonapeptides using CD studies. Thus, a cationic peptide (**A1**) and an ion-pair peptide (**A2**) were selected for the temperature-dependent and solvent variation experiments to assess the effect of positively charged residues and Glu...Lys (*i*, *i* + 4) salt bridges on the nucleation of helical conformation. The CD spectrum of **A1** and **A2** in water measured at different temperatures between 20 and 75 °C at an interval of 5 °C is shown in Figure 4.

On raising the temperature, the weak positive maximum at 217–220 nm observed for **A1** disappears; meanwhile, the absolute value of negative band at ~197 nm decreases. The results indicate that a

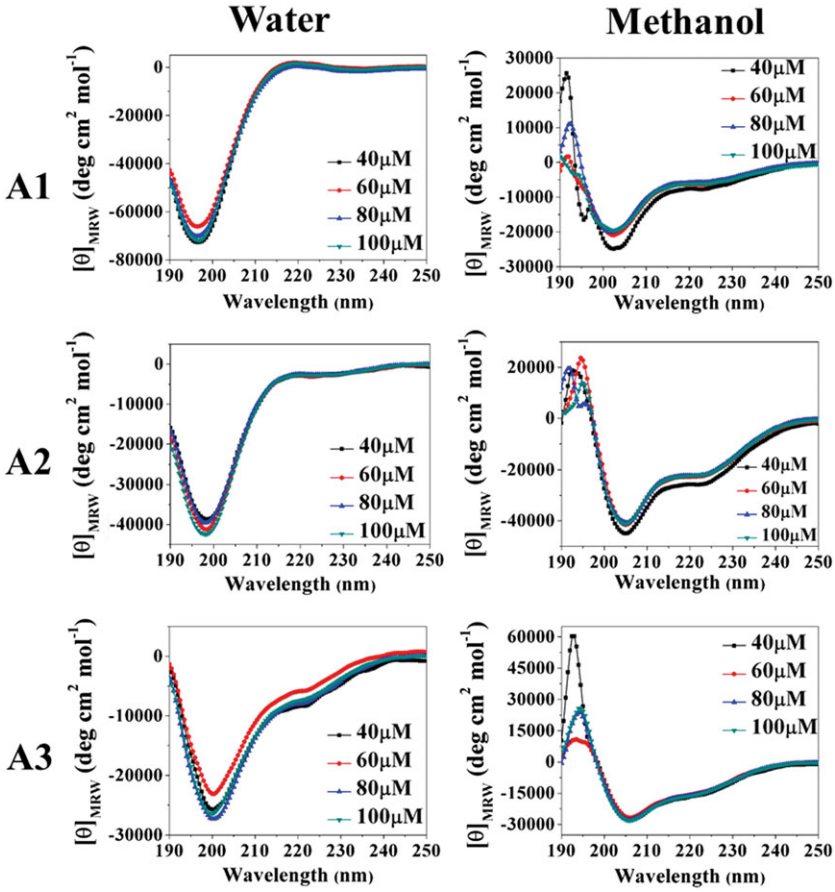

Figure 3. CD spectra of peptides **A1**, **A2** and **A3** in water (left panel) and methanol (right panel) at 298 K in 40–100 μM concentration range. The peptides' structures are concentration independent in their molar ellipticities in both water and methanol, thus apparently, free of aggregation.

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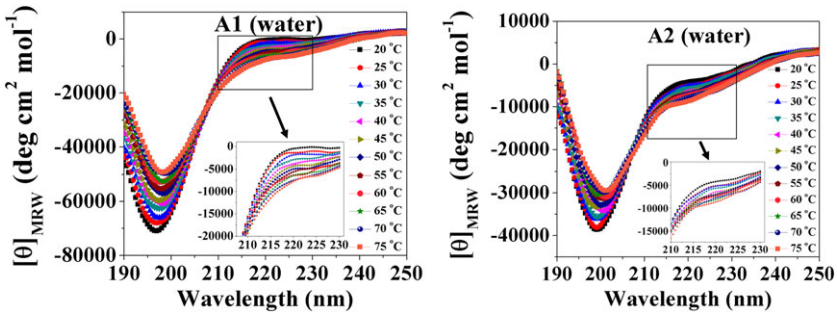

Figure 4. CD spectra of **A1** (left panel) and **A2** (right panel) in water displaying temperature-dependent equilibrium between different conformations. The peptide concentration is 40 μM.

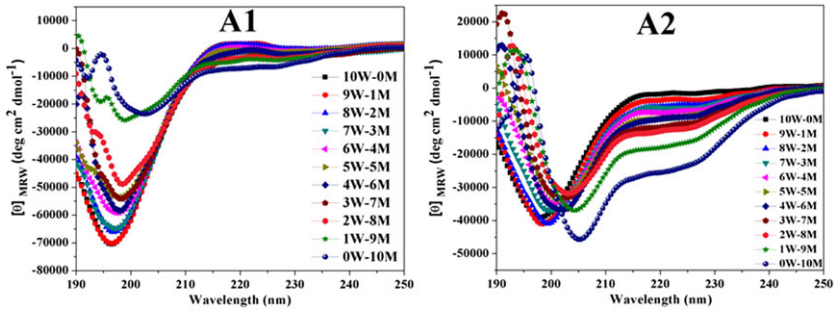

Figure 5. CD spectra of **A1** (left panel) and **A2** (right panel) displaying solvent-dependent equilibrium between different conformations. The peptide concentration is 40 μM. The solvent composition is shown as water (W) to methanol (M) ratio.

β-strand conformer becomes more populated at the expense of PPII conformer at a higher temperature. In addition, a clear isodichroic point at ~207 nm was observed. Accordingly, temperature effect in the CD spectrum can be interpreted in terms of a two-state transition between a PPII and a β-strand conformation in agreement with the result from a CD spectrum of cationic trialanine [62]. Srivastava, *et al.*, highlighted that serine nonapeptide, Ac–(Ser–Ala)$_4$–Ser–NH$_2$, display a PPII-helix conformation in water, which unfolds to extended β-conformation with increase in temperature, apparently in a two-state equilibrium [63]. Mu, *et al.*, performed classical molecular dynamics (MD) studies of trialanine in aqueous solution and highlighted that two extended conformers, β and PPII, of AAA coexist in aqueous solution [64]. Temperature-dependent CD measurement of poly-L-lysine by Tiffany and Krimm also clearly depicted an isodichroic point reflecting the coexistence of two conformations [65]. Shi, *et al.*, analyzed the seven-residue alanine peptide (XAO peptide) by NMR and CD spectroscopy and found a transition between PPII (stabilized at low temperature) and β-strand structure (stabilized at high temperature) [18]. The CD spectrum of **A2** measured at different temperatures between 20 and 75 °C is shown in Figure 4 (right panel). The spectra display strong negative bands at ~197 nm; however, the ellipticity values are smaller as compared to **A1**. The absolute value of negative band at ~197 nm decreases with an increase in temperature, and an isodichroic point at ~207 nm was observed. The results highlight that a β-strand conformer becomes more populated at the expense of PPII conformer at a higher temperature as observed with **A1**.

On increase of methanol concentration, **A1** manifests a shift from the weak positive peak at ~220 nm and a large negative peak around 197 nm, diagnostic for the PPII structure, to a weak negative shoulder at around ~225 nm and negative peak at near ~204 nm, which highlight the existence of helical folds in the equilibrium ensemble (Figure 5). **A2** manifests a clear transition from the PPII conformer to the helical conformation on increase of methanol concentration on account of the observation of strong negative bands at ~205 and ~224 nm, which highlight the role of N-terminal diproline segment as well as Glu...Lys (*i*, *i* + 4) salt bridge interaction on the nucleation of helical conformation.

Conclusions

In the present study, the effect of N-terminal diproline and charged side chains in the alanine-based short peptides has been investigated as an approach to scrutinize the specific role of interactions within the main chain and between side chains in inducement and stabilization of helical conformation. The subtle variations in the intensity of CD bands characterizing PPII signature were observed for the model peptides in water, which highlight contribution of other conformational states of the polypeptide structure in the equilibrium ensemble. Temperature-dependent CD studies in water provided clear evidence that PPII ensemble will melt to extended β-conformation with an increase in temperature, which is consistent with previous studies. Solvent-dependent CD spectra indicate a transition from PPII conformer to helical conformation for **A2**, which illustrates the role of N-terminal diproline segment as well as Glu...Lys (*i*, *i* + 4) salt bridge interactions on the nucleation of helical conformation in alanine-based short peptides. The present study will enhance our understanding on the stabilization of helical conformation in short peptides and hence aid in the design of novel peptides with helical structures.

Experimental Section

Materials

Fmoc-protected amino acids, reagents for solid-phase peptide synthesis, Rink-Amide AM resin, dried solvents–dimethylformamide (DMF), methanol, diethylether and dichloromethane were purchased either from Sigma-Aldrich or Novabiochem-Merck.

Peptide Synthesis

The peptides were synthesized manually on Rink-Amide AM resin as solid support using standard Fmoc chemistry and HOBt/DIC as coupling reagents [66]. The coupling reaction was monitored with standard Kaiser and chloranil tests, each coupling reaction typically required about 6 h. Subsequent deprotection step was carried out by 30% (*v/v*) piperidine–DMF solution. N-terminal was acetylated (—$NHCOCH_3$) with Ac_2O:DIPEA:DMF in 1 : 2 : 20 ratio. The cleavage of the end-protected final polypeptide and simultaneous deprotection of side chains were accomplished with reagent K (82.5% TFA/5% dry-phenol/5% thioanisole/2.5% ethandithiol/5% water). The filtrate from the resin was precipitated with anhydrous diethyl ether and lyophilized in 1 : 4 H_2O:^{t}BuOH solution to obtain peptide in powdered form. The purity of the peptides was assessed by high-performance liquid chromatography over reverse-phase (RP) C18 column (10 μM, 10 mm × 250 mm; Merck) eluting with water/acetonitrile (0.1% TFA) 5–95% gradients and determined to be at least 95% pure by analytical high-performance liquid chromatography.

Mass Spectra

Mass spectra of the synthesized peptides were recorded on QTOF-ESI mass spectrometer. Positive ions were detected in linear/reflectron mode.

Nuclear Magnetic Resonance (NMR)

Proton nuclear magnetic resonance experiments were recorded on Bruker 700-MHz spectrometer at 298 K. Peptide concentration of 2.5 mM was used. The solutions were prepared in 90% H_2O/10% D_2O with 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) as an internal reference. The 1D ^{1}H spectra were recorded for all peptides at tenfold dilution to check the formation of aggregates.

Circular Dichroism (CD)

Circular dichroism measurements were performed on JASCO J-180 CD spectropolarimeter calibrated with D-(+)-10-camphorsulfonic acid (CSA). Data were collected at 298 K in 0.2-cm path length quartz cell with 2-nm bandwidth in far-UV (190–250 nm) range. Scanning was done at 100 nm/min with 1.0-s time constant, in 1-nm steps, and five scans were averaged after background subtraction for solvent water. Because of lack of aromatic residues in the designed peptides, the peptide concentration was determined using the UV absorbance (A_{205}) at 205 nm [67,68]. The observations in millidegree ellipticity were converted to mean residue molar ellipticity $[\theta]_{MRW}$.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article.

Fig. S1. High-resolution QTOF-ESI mass spectra of peptides **A1**, **A2** and **A3**.

Fig. S2. ^{1}H NMR spectra of peptides **A1**, **A2** and **A3** recorded in 90% H_2O/10% D_2O at 298 K on a 700-MHz spectrometer. Insets show the NH region in higher magnification.

