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N and N+1 cap positional effects of (D) alanine in the 310 type helical model peptide Boc-(D)Glu-Ala-Ala-Lys-NHMe

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The N (Ala_2) and N+1(Ala_3) cap positional alanine in the 3_{10} helical model peptide Boc-(D)Glu_1-Ala_2-Ala_3-Lys_4-NHMe, **2** is substituted with a (D)alanine. An NMR enquiry using solvents that promote appreciable ordering of peptide **2**, against random conformation of the parent (L)Glu_1 peptide **1**, establishes that D alanine partially disorders the type II' turn templated 3_{10} type helical fold and distorts it in a position dependent manner, more strongly from N cap position than from N+1 cap position.

Keywords: 3_{10} Helix, II' turn, reverse turn, D amino acids, NMR studies

The translational incorporation of non-coded α-amino acids into native protein structures arouses an interest in better characterizing the effects of specific types of noncoded amino acids on specific kinds of protein secondary structural elements[1,2]. Among noncoded α-amino acids that require particular attention for potential use in protein incorporation studies are the amino acids of D chirality. The stereochemical features of D amino acids make them compatible with different kinds of folded elements of the protein tertiary structure, such as β-turn and helical type elements[3,4]. The right handed protein helical element has backbone torsional angles that are also accessible to a D amino acid. However, these are energetically less favoured than for an L amino acid[5]. Consequently, a helical type folds weakens following the L to D amino acid substitution[6], but whether the effect may be position dependent, dependent on the helix geometry, or accompanied by any specific consequence on the helix geometry, are issues that need also to be addressed.

A stereochemically directed type II' turn element, serving as helix template, ordered modeled peptides of the general structure Boc-(D)Glu_1-Xxx_2-Yyy_3-Lys_4-Aaa_5-Bbb_6-OMe into 3_{10} type helices end capped by Boc-(D)Glu_1[7-9]. The geometry of the helical type fold seems to be amino acid dependent. It was of interest, therefore, to exploit the model to generally assess the D amino acid effect in a helical type fold and to particularly assess if the effect might be position dependent.

Herein is analysed the tetrapeptide Boc-(D)Glu_1-Ala_2-Ala_3-Lys_4-NHMe **2** for the effect of (L)Ala to (D)Ala substitution in its two internal positions. The diastereomeric peptides **3** and **4,** with (D)Ala as the Xxx_2 or Yyy_3 positional residue, are thus compared with parent peptide **2** and with its (L)Glu_1 diastereomer **1**. The solvent dependent NMR inquiry, undertaken specifically in solvents which promote the ordering of peptide **2** as against the unordered nature of peptide **1**, establishes that the D amino acid weakens the model and distorts its helical type element in a strongly position dependent manner. The tetrapeptides **1-4** are shown thus,

1 Boc-(L)Glu-(L)Ala-(L)Ala-(L)Lys-NHMe,
2 Boc-(D)Glu-(L)Ala-(L)Ala-(L)Lys-NHMe,
3 Boc-(D)Glu-(D)Ala-(L)Ala-(L)Lys-NHMe and
4 Boc-(D)Glu-(L)Ala-(D)Ala-(L)Lys-NHMe.

Experimental Section

Synthesis

The standard amino acid protections were achieved using the reported procedures and the coupling of the protected amino acids and peptides were by solution phase methodology using isobutyl chloroformate/N-methyl morpholine as the coupling reagents and trifluoroacetic acid for N-terminal deprotection[10]. Progress of all coupling reactions was monitored by thin layer chromatography on manually prepared silica gel plates, with (i) $CHCl_3$:MeOH, 9:1 and

(ii) *n*-BuOH:AcOH:H_2O, 4:1:1 as the solvent systems. After every coupling step the product was purified over a silica gel (100-200 mesh) column eluting with a chloroform-methanol gradient, and was checked for identity as well as purity. The side chain deprotections of the final peptides were by hydrogenolysis in methanol, and the products were purified on a (Hitachi) HPLC system over a Lichrosorb RP 18 (15 μm, 250×10 mm) reverse phase column eluting with 15% water in methanol and with UV detection at 220 nm. The requisite HPLC elutes on lyophilization gave the final peptides with purity higher than 95%.

NMR studies

NMR experiments were carried out on a Varian VXR 300 MHz spectrometer. The 1D and 2D ^{1}H NMR spectra were obtained from peptides in $CDCl_3$-DMSO-d_6 3:1 and in neat DMSO-d_6, using tetramethylsilane (δ 0.00 ppm) and DMSO (δ 2.5 ppm) as the internal standards. The observed chemical shifts and line widths were essentially invariant in the concentration range 10-40 mM, suggesting the absence of any perceptible intermolecular association in any peptide. All results in this report are with 10 mM peptide solutions. Temperature coefficients of amide resonances in DMSO-d_6 were extracted from spectra at six different temperatures over the range 298-323 K. $^3J_{NH\alpha}$ coupling constants were extracted directly from 1D spectra. The ^{1}H,^{1}H-COSY spectra were a total of 256 experiments, 16 scans each or more with relaxation delay of 1.5 s, size 1K, and width shifted sine bell window multiplication for spectral processing. The ROESY spectra were a total of 512 experiments, 32 scans each or more with relaxation delay of 1.5 s, 300 ms mixing time, size 2K, and the spectral processing were with shifted sine bell window multiplication in both the dimensions.

Results

Chemical shift assignments in ^{1}H NMR spectra are achieved with 2D COSY spectra, to identify the spin systems, and with 2D ROESY spectra, to assign the specific Ala spin systems based on $d_{\alpha N}$(i, i+1) type NOEs. The assigned chemical shifts of all peptides in the two solvent systems, $CDCl_3$-DMSO-d_6 mixture (3:1) and neat DMSO-d_6, are shown in **Table I**.

Table I — ^{1}H NMR chemical shifts (δ, ppm) in $CDCl_3$:DMSO-d_6 (3:1) mixture

Residue	NH	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	$C^{\delta}H$	$C^{\varepsilon}H$
1						
(L)Glu	6.96	3.95	1.90	2.25	—	—
(L)Ala	8.26	4.18	1.32	—	—	—
(L)Ala	7.99	4.18	1.34	—	—	—
(L)Lys	7.62	4.18	1.90	1.62	1.62	2.78
NHMe	7.41	2.65	—	—	—	—
2						
(D)Glu	8.95	4.01	2.05/1.82	2.39/2.22	—	—
(L)Ala	8.82	4.01	1.41	—	—	—
(L)Ala	7.56	4.18	1.46	—	—	—
(L)Lys	7.54	3.99	1.77	1.54/1.17	1.77/1.52	2.95/2.73
NHMe	7.01	2.67	—	—	—	—
3						
(D)Glu	7.55	3.58	1.91	2.19	—	—
(D)Ala	8.54	4.09	1.35	—	—	—
(L)Ala	8.30	4.20	1.38	—	—	—
(L)Lys	7.79	4.09	1.65	1.34	1.65	2.82
NHMe	7.49	2.61	—	—	—	—
4						
(D)Glu	8.66	4.01	1.92	2.42/2.25	—	—
(L)Ala	8.79	4.01	1.40	—	—	—
(D)Ala	8.16	3.58	1.50	—	—	—
(L)Lys	4.01	1.84	1.84	1.71	1.71	2.91/2.86
NHMe	7.30	2.72	—	—	—	—

The backbone fold and degree of its ordering

NOEs in $CDCl_3$-DMSO-d_6 mixture: The ROESY spectra of all the peptides were analyzed and all important NOEs are summarized in **Table II**. The $d_{\alpha N}(i, i+1)$ type NOEs appear in all peptides. However, their relative intensities were widely variable. The extended chain conformers are thus sampled by all peptides but to a variable extent. No additional NOEs appear in peptide **1**, and hence the peptide is generally unordered. All the $d_{NN}(i, i+1)$, $d_{\alpha N}(i, i+2)$ and $d_{\alpha N}(i, i+3)$ type NOEs appear in peptide **2**, and characterize it as an appreciably ordered helical type fold according to $d_{\alpha N}(i, i+3)$ NOEs, and as a 3_{10} type fold according to $d_{\alpha N}(i, i+2)$ NOEs[16]. No d_{NN} type NOE appears between Glu_1 and Ala_2 in the peptide, no $d_{\alpha N}(i, i+2)$ and $d_{\alpha N}(i, i+3)$ type NOEs are observed to its Glu_1 αH and an especially strong NOE appears between Glu_1 αH and Ala_2 NH. The pattern is consistent with the suggested ordering of Boc-(D)Glu-Ala-Ala as a type II' β turn element[11]. The $d_{NN}(i, i+1)$ type NOEs also appear from Ala_2 onwards in peptide **3** and **4**, but no medium range NOE is observed in either one. Thus, compared with peptide **2**, peptide **3** and **4** feature a backbone fold which is either partially disordered or specifically distorted.

Solvent shielded amide NHs in DMSO-d_6: The amide chemical shifts in all peptides in DMSO-d_6 are noted to be linearly dependent on temperature (**Figure 1**). The derived coefficients of NH resonances are shown in **Table III**. All the NH coefficients in peptide **1** are > 3ppb/K, the characteristic value for a disordered peptide owing to the solvent exposed nature of the NHs.

The temperature coefficients of three C-terminal NHs in rest of the peptides are < 3ppb/K. The NHs are thus solvent shielded and are possibly intramolecularly H-bonded[12]. A type II' turn, with Ala_3 NH H-bonded to Boc C=O, followed by consecutive 3_{10} turns, with Lys_4NH H-bonded to Ala_2 C=O, are indicated as modeled in **Figure 2**. As discussed earlier, the proposed model places (D)Glu_1 as the stereochemically favored first corner residue in the type II' turn element, and Ala_2 as the second corner residue in the turn as well as the N-cap residue in 3_{10} type helical segment in the model.

The temperature coefficients of apparently H-bonded amide NHs are variable. Possibly the NH differ in their relative solvent accessibilities. The apparent NH accessibilities are comparable in peptide **2** and **4**, but are somewhat larger in peptide **3**, possibly due to relatively more disordered or distorted backbone fold.

Table II — Observed NOE connectivities in peptides **1** to **4** in $CDCl_3$:DMSO-d_6 (3:1) mixture

	NOE connectivities	Observed NOEs
1	(L)E_1-A_2-A_3-K_4-NHMe	
	$d_{NN}(i, i+1)$	—
	$d_{\alpha N}(i, i+1)$	E_1-A_2, A_2-A_3, A_3-K_4, K_4-NHMe
	$d_{\alpha N}(i, i+2)$	—
	$d_{\alpha N}(i, i+3)$	—
2	(D)E_1-A_2-A_3-K_4-NHMe	
	$d_{NN}(i, i+1)$	A_2-A_3, A_3-K_4*, K_4-NHMe
	$d_{\alpha N}(i, i+1)$	E_1-A_2, A_2-A_3, A_3-K_4, K_4-NHMe
	$d_{\alpha N}(i, i+2)$	E_1-A_3, A_2-K_4, A_3-NHMe
	$d_{\alpha N}(i, i+3)$	A_2-NHMe
3	(D)E_1-(D)A_2-A_3-K_4-NHMe	
	$d_{NN}(i, i+1)$	A_2-A_3, A_3-K_4, K_4-NHMe
	$d_{\alpha N}(i, i+1)$	E_1-A_2, A_2-A_3, A_3-K_4, K_4-NHMe
	$d_{\alpha N}(i, i+2)$	—
	$d_{\alpha N}(i, i+3)$	—
4	(D)E_1-A_2-(D)A_3-K_4-NHMe	
	$d_{NN}(i, i+1)$	A_2-A_3, A_3-K_4, K_4-NHMe
	$d_{\alpha N}(i, i+1)$	E_1-A_2, A_2-A_3, A_3-K_4, K_4-NHMe
	$d_{\alpha N}(i, i+2)$	—
	$d_{\alpha N}(i, i+3)$	—

*Not observed due to resonance overlap.

The stability of the backbone fold

Evidence for salt bridging: The formation of the salt bridge in peptide **2** consequent to its helical type ordering was evidenced in the dispersal of its Glu^{γ} and Lys^{ε} methylene proton chemical shifts. As a matter of fact, many of the diastereotopic methylene protons in Glu_1 and Lys_4 side chains in peptide **2** appear as well resolved resonances in both $CDCl_3$-DMSO-d_6 mixture and neat DMSO-d_6 (see **Table I**), but are ill resolved multiplets in peptide **1**. A dispersal of diastereotopic proton resonances is also noted in peptide **4** but only in $CDCl_3$-DMSO-d_6 mixture and not at all in peptide **3** in either solvent. Thus, Lys_4-Glu_1 salt bridge is indicated in case of peptide **2** irrespective of the solvent, only in $CDCl_3$-DMSO-d_6 mixture in case of peptide **4**, and not at all in case of peptide **1** and **3**. Apparently, the salt bridge strength and, thus, the degree of helical type ordering of the peptides is **2** > **4** > **3**.

Glu_1NH deshielding in $CDCl_3$-DMSO-d_6 mixture: The formation of the salt bridge in peptide **2** was also evidenced in the deshielding of its Glu_1NH, which provided the principal means to assess the degree of its helical type ordering. The basis for such a conformation dependent deshielding of Glu_1NH in the peptide is evident from **Figure 2**. The NH is found to occupy a region which roughly corresponds to the deshielding zone of Glu^{γ} carboxylate. From the

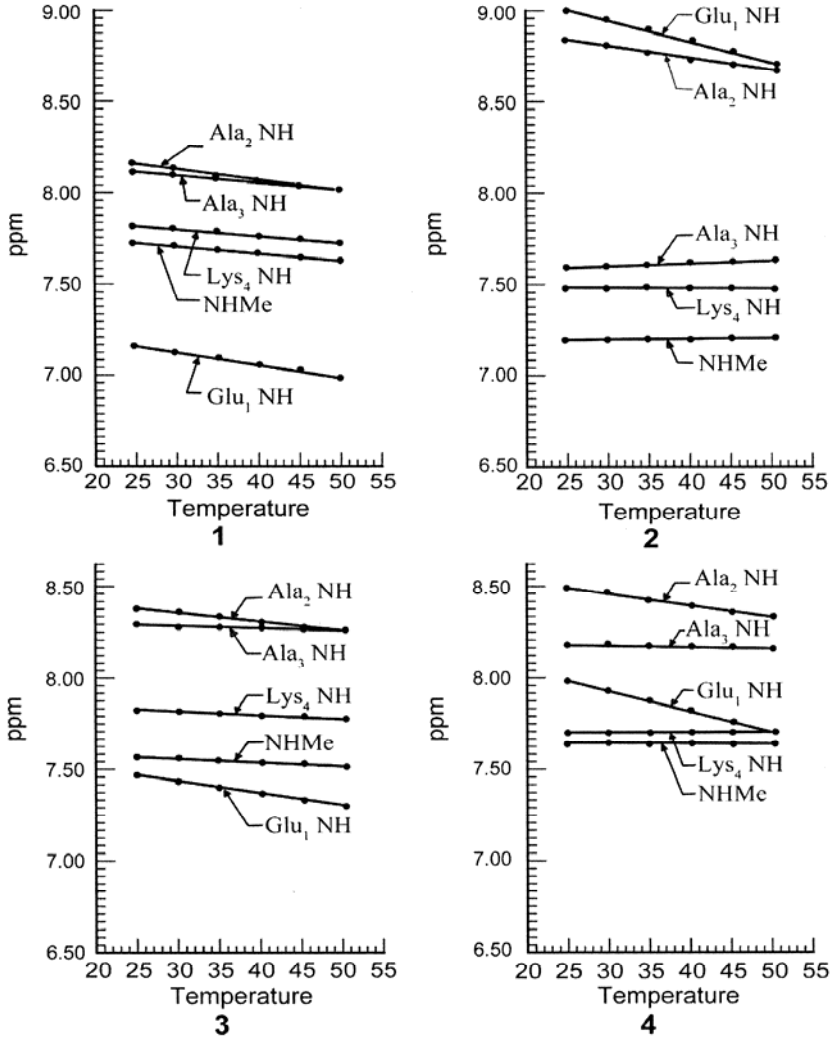

Figure 1 — The NH chemical shifts in peptides **1** to **4** in DMSO-d_6 as a function of temperature

summary of the data in **Table IV**, Glu_1NH in the case of peptide **1** is seen to resonate at δ 6.96 in $CDCl_3$-DMSO-d_6 mixture, while in the case of peptide **2**, **3** and **4**, the NH resonates 1.99, 1.59 and 1.70 ppm downfield, respectively. The extent of Glu_1 NH deshielding implies that the contribution of the helical component to the conformation of the peptides is **2** > **4** > **3**.

Solvation effect in Glu_1NH shift: The difference shift of Glu_1NH in $CDCl_3$-DMSO-d_6 mixture and in neat DMSO-d_6 is given in **Table IV**, as the dδ (solv) value. In a solvent of higher polarity and higher dielectric strength, the backbone fold in a peptide may weaken or its salt bridge may be partially disrupted, and the effect is expected to manifest in an upfield shift of Glu_1NH, towards a positive dδ (solv). In the disordered peptide **1**, which lacks salt bridge, Glu_1 NH is at a lower field position in DMSO-d_6 than in $CDCl_3$-DMSO-d_6 mixture, and hence the dδ (solv) is negative. The dδ (solv) is also negative in peptide **2**, and the implication is that the backbone fold/or the salt bridge in the peptide is nearly as well ordered in DMSO-d_6 as in $CDCl_3$-DMSO-d_6 mixture. The dδ (solv) values in peptide **3** and **4** are positive. Hence the backbone fold or the salt bridges in these peptides are relatively more disordered in DMSO-d_6. From the magnitude of the dδ (solv), the solvation induced disordering is quite pronounced in peptide **4** while in the already partially disordered peptide **3** it is relatively weaker. The strength of the backbone fold and/or of the salt bridge in the peptides is thus again **2** > **4** > **3**.

Temperature coefficient of Glu_1NH in DMSO-d_6: The Glu_1 NH temperature coefficient in the peptides is found to vary widely (**Table III**). The coefficient in the prototype peptide **1** is 6.43 ppb/K

Table III — Coupling constants and the calculated φ torsional angles in specified solvents and the amide temperature coefficients in DMSO-d_6

Residues	$^3J_{NH\alpha}$ ($CDCl_3$-DMSO-d_6) (Hz)	φ	$^3J_{NH\alpha}$ (DMSO-d_6) (Hz)	φ	dδ/dT (DMSO-d_6) (ppb/k)
1					
(L)Glu	5.1	—	7.2	—	6.43
(L)Ala	4.8	—	6.3	—	5.37
(L)Ala	6.9	—	7.5	—	3.30
(L)Lys	7.5	—	7.5	—	3.39
NHMe	—	—	—	—	3.20
2					
(D)Glu	—	—	3.3	+55°	12.5
(L)Ala	4.3	–63°	4.5	–65°	6.58
(L)Ala	7.2	–86°	7.5	–89°	1.71
(L)Lys	6.6	-82^0	6.6	–82°	0.16
NHMe	—	—	—	—	0.03
3					
(D)Glu	—	—	6.9	+ 84°	7.12
(D)Ala	6.0	–83°	6.3	–78°	5.34
(L)Ala	7.2	–86°	7.5	–89°	1.51
(L)Lys	7.8	–92 °	8.1	–91°	2.38
NHMe	—	—	—	—	2.44
4					
(D)Glu	—	—	5.1	+ 70°	11.5
(L)Ala	5.6	–74°	5.7	+ 75°	6.89
(D)Ala	6.6	–72°	6.9	–65°	1.42
(L)Lys	6.9	–84°	7.2	–86°	0.37
NHMe	—	—	—	—	0.17

and it is progressively larger in the peptides **3**, **4** and **2**. A reasonable interpretation is that the more ordered a peptide, the larger will be the upfield shift of Glu_1 NH due to thermal disordering of its backbone fold or its salt bridge. Thus, the Glu_1 NH coefficients imply an appreciable degree of ordering of peptide **2**, an intermediate degree of ordering of peptide **4** and a relatively weak degree of ordering of peptide **3**. Thus, even this line of reasoning indicates that the ordering of the peptides is **2** > **4** > **3**.

The geometry of the backbone fold

Coupling constants: $^3J_{NH\alpha}$ coupling constants, extracted directly from 1D spectra in specified solvents are shown in **Table III**. The calculated φ torsional angles[13] for peptides **2**, **3** and **4**, which most closely match the backbone folds evidenced in other data, are listed alongside. It may be noted that the φ torsional angles calculated for D chiral residues from their observed coupling constants can be related to a suitably modified Karplus type relationship for L amino acids[14].

Glu_1 NH signal is a doublet in peptide **1** irrespective of the solvent, but is a singlet in every other peptide in $CDCl_3$-DMSO-d_6 mixture and doublet in DMSO-d_6. The coupling constant in this solvent is particularly small for peptide **2** and is progressively larger for peptides **4** and **3**. The calculated φ torsional angle for peptide **2** is in close agreement with the first corner φ torsional angle +60°

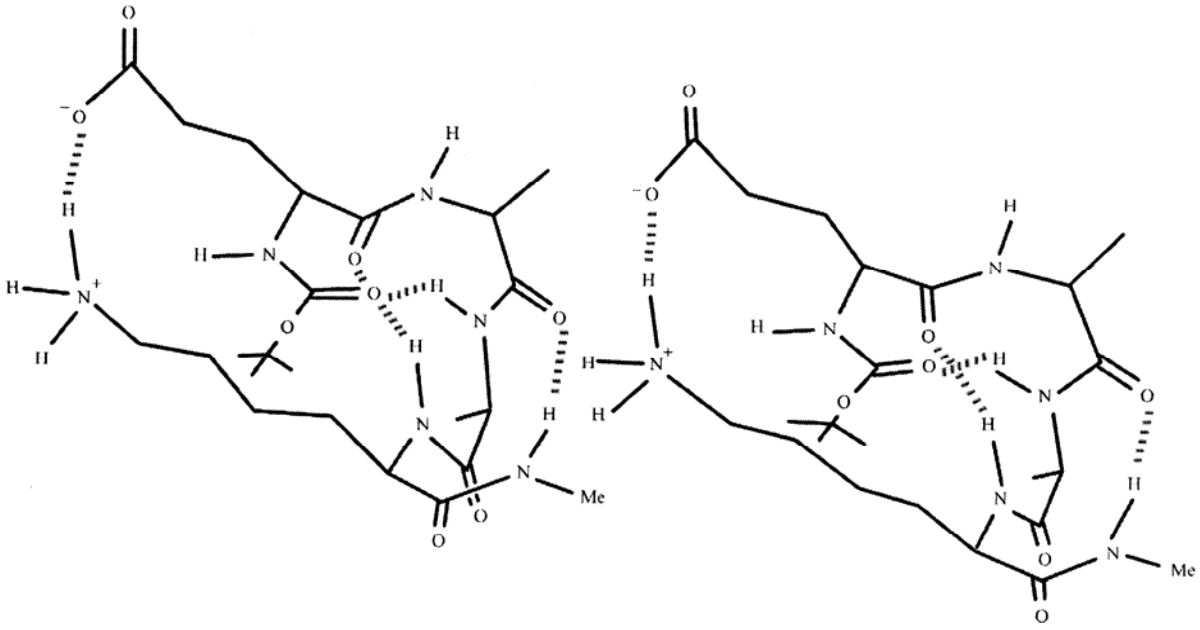

Figure 2 — Boc-(D)Glu-(L)Ala-(L)Ala-(L)Lys-NHMe **2** modelled as consecutive II′-3_{10}-3_{10} type turn. The φ, ψ torsional angles for Glu_1 are 60°, –120° and all other residues are –74°, –4°

Table IV — Glu_1 NH chemical shifts (δ, ppm) in specified solvents and the difference shift in the two solvents [d δ(solv)]

Peptide	$CDCl_3$-DMSO-d_6	DMSO-d_6	dδ (solv)
1	6.96	7.14	–0.18
2	8.95	8.98	–0.03
3	7.55	7.45	+ 0.10
4	8.66	7.98	+ 0.68

in a standard type II' turn, but is nearly 30° larger for peptide **3** and 15° larger for peptide **4**. Assuming that Glu_1 φ torsional angle in $CDCl_3$-DMSO-d_6 mixture is at the most +55° in every peptide, the type II' turn element in peptide **2** is well ordered in DMSO-d_6, and is progressively more disordered in peptides **4** and **3**.

Since no peptide appears to be fully ordered even in $CDCl_3$-DMSO-d_6 mixture, all the calculated φ torsional angles are an ensemble average overall of conformers populated. However, peptide **2**, the most well ordered, possibly most closely reflects the geometry of type II' turn initiated 3_{10} fold in its coupling constants. Indeed it's Ala_2-Ala_3-Lys_4- NHMe segment is a helical type fold but with a non ideal geometry. The Xxx_2 positional φ torsional angle in the peptide is close to an ideal helical torsional angle[15] but is smaller than the second corner torsional angle-80° in a standard type II' turn[16]. The Ala_3 and Lys_4 torsional angles in the peptide are, on the other hand, larger than even the standard 3_{10} angle –74° (Ref 17). A comparison between peptides in $CDCl_3$-DMSO-d_6 mixture indicates that D alanine in Xxx_2 position increases the Xxx_2 positional torsional angle in the model by 20°, its Lys_4 torsional angle by 10° but has no effect on its Yyy_3 positional torsional angle. D Alanine in Yyy_3 position on the other hand, increases the Xxx_2 positional φ torsional angle in the model by 11°, has almost no effect on its Lys_4 torsional angle, but diminishes its Yyy_3 positional torsional angle by 14°. These torsional perturbations surely are contributed by the induced disordering in both peptides **3** and **4**, but appear to include geometrical effects, as is particularly evident in the relatively diminished Yyy_3 positional torsional angle in peptide **4** despite it relatively disordered nature as evidenced by the non appearance of the medium range NOEs.

In contrast to the large increase in Glu_1 φ torsional angle in peptide **3** and **4** in DMSO-d_6, the torsional angles in their helical segments are only marginally larger in this solvent, and in fact are smaller by at least 5° in Ala_2 in peptide **3** and in Ala_3 in peptide **4**. Thus, a solvation change which has no perceptible effect on the salt bridge integrity in peptide **2** but partly disrupts the salt bridge in peptide **3** and **4**, appears to have a complex effect on the backbone fold in partly randomizing it and partly distorting it.

Discussion

The 3_{10} type helical model peptide Boc-(D)Glu_1-Xxx_2-Yyy_3-Lys_4-NHMe was examined for effects of D Ala and L Ala in its Xxx_2 and Yyy_3 positions. The helix destabilizing stereochemical effect of a D amino acid[6,18] was expected to be mirrored in the present model and it was of particular interest to assess whether or not the effect might be position dependent.

The 3_{10} type helical model peptide is indeed destabilized by D alalnine and in a strongly dependent manner. The adverse stereochemical effect of L to D amino acid substitution in a right handed helical type fold[6] is thus mirrored in this model. The energetic cost of the chiral inversion is quite substantial in the N cap position than in the N+1 cap position. Specific geometrical consequences are noted to also accompany the site specific chiral inversions in the model. In the relatively H-bond reinforcing solvent $CDCl_3$-DMSO-d_6 mixture, the consecutive φ torsional angles in peptide **2** are +55°, -63°, –86°, –82°, in peptide **3** are +55°, –83°, –86°, –92° and in peptide **4** are +55°, –74°, –72°, –84°. The observed torsional angle perturbations include effects from the relatively disordered nature of both peptides **3** and **4**, and yet a torsional angle compression at (D)Ala_3 is quite clearly manifest in peptide **4**, and an appreciable torsional angle expansion at (D)Ala_2 is manifest in peptide **3**. The appreciable torsional angle perturbation in the residue which has its R group stereochemically inverted implies that, besides its energetic cost, the L and D amino acid substitution in a helix may also be accompanied by a localized geometrical distortion.

The observation of D amino acid effects in an unusually simple helical model may facilitate a theoretical analysis of the basis for D amino acid effects in the stability and geometry of an ordered helical structure. The particularly significant issues raised in the course of a theoretical scrutiny of the results are those of the substantial ordering of peptide **2** against the random conformation of (L)Glu_1 diastereomer **1**, and the specific perturbations in the stability and geometry of peptide **2** on its position specific (L)Ala to (D)Ala substitutions.

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