The Helix-Destabilizing Propensity Scale of D-Amino Acids: The Influence of Side Chain Steric Effects

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Abstract: Although D-amino acids are found in various naturally occurring peptides and frequently used for structure−activity studies, not much is known about their impact on the helical secondary structures formed by L-amino acids. Although several previous accounts reported on the α-helical propensity of L-amino acids, the present contribution addresses this subject for the first time for the corresponding D-enantiomers of all of the proteinogenic amino acids. Thus, the helix-destabilizing abilities of the 19 D-amino acids in the host sequence acetyl-KLALKLALxxLKLALKLA-amide ($x^{9,10}$-KLA) were evaluated by means of circular dichroism (CD) spectroscopy, nuclear magnetic resonance, and reversed-phase HPLC. CD and HPLC data enabled calculation of differences in the free energy of helix formation for D-amino acid x relative to glycine ($\Delta\Delta G_t$) or to the corresponding L-amino acid ($\Delta\Delta G_{D\text{-}L}$). The data show that the helix-destabilizing propensity is highly dependent on the amino acid side chain and not related to the structure propensity of the corresponding L-amino acid. In consequence, the D-amino acids can be grouped into (i) weak helix destabilizers (D-His, D-Asp, D-Glu, D-Cys, D-Gln, D-Asn, D-Ser), (ii) medium helix destabilizers (D-Leu, D-Arg, D-Met, D-Lys, D-Trp, D-Ala), and (iii) strong helix destabilizers (D-Thr, D-Phe, D-Val, D-Ile, D-Tyr, D-Pro). Accordingly, the D-isomers of bulky and *β*-branched amino acids are the most effective in destabilizing the amphipathic KLA-helix by induction of turn-like structures. Such D-isomers disrupt the secondary structure in a manner similar to that of L-proline.

Introduction

The functionality of many biological molecules is determined by chirality. Although among amino acids the L-form is by far the predominant enantiomer found in nature, examples of the occurrence of D-amino acids are also known. Thus, some bacterial peptides[1,2] and the amphibian skin peptides dermorphin[3] and deltorphin,[4] which are related to the mammalian hormones and neurotransmitters, contain D-amino acids. Free D-amino acids are present in mammalian tissues and body fluids.[5−7] It has been shown recently that a normal codon for the corresponding L-amino acid is present in the cDNA at positions where D-amino acids were found and that an enzyme-catalyzed reaction enables the conversion of L- to D-amino acids by a posttranslational reaction.[8,9] In addition, the occurrence of D-aspartate and D-serine in amyloid protein related to Alzheimer's disease[10] may indicate a relationship between epimerization and neurodegenerative disorders. The presence of D-amino acids in peptides or proteins may serve several purposes, such as (a) increasing their proteolytic stability,[11,12] which is due to the stereospecificity of most exo- or endoproteases, or (b) changing the conformation of a peptide by enhancing the formation of turn structures.[13]

When L-amino acid residues in a helical peptide sequence are replaced by the corresponding D-amino acids, destabilization of the α-helices occurs,[14] thus allowing the use of D-amino acid analogues of peptides in structure−activity studies[15] to probe the relationship between conformational domains and bioactivity.[16,17] By conformational analysis using a helical coiled-coil peptide, the contribution of individual amino acids to the stability of α-helices has shown that an L-Ala → D-Ala substitution destabilizes the secondary structure by 4 kJ/mol.[14] The incorporation of an adjacent pair of D-amino acids into an α-helical

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model peptide enhances the effect of helix disturbance.[18] ^{1}H NMR and guanidine hydrochloride denaturation experiments confirm that D-amino acids cause only a local change in structure and flexibility at the position of substitution[19] with a free energy contribution to helix stability of +4.5 kJ/mol.[20] On the basis of this effect double D-amino acid replacement sets have been used to localize helical domains in neuropeptides.[18,21] Moreover, the method has been employed to study the role of peptide helicity on the interaction of antibacterial peptides with lipid bilayers and biological membranes.[22,23]

In contrast to the numerous studies which have been carried out concerning the α-helical propensity of L-amino acids using statistical analyses of proteins of known structure,[24] site-directed mutagenesis of proteins,[25−28] and host−guest analyses of helical peptides,[29−32] little is known about the intrinsic structure propensities of D-amino acids incorporated into peptides consisting of L-amino acids. Other than the studies using particular D-amino acid substitutions mentioned above, there has been no systematic evaluation of the impact of specific D-amino acids on α-helix propensity. In the present work an amphipathic model peptide was used as host sequence to determine the relative helix-destabilizing abilities of the 19 common D-amino acids by conformational analysis.

Experimental Section

Peptide Synthesis and Purification. The 18-residue peptides acetyl-KLALKLALxxLKLALKLA-amide (x^{9,10}-KLA) with substitutions of all 20 natural amino acids and the corresponding D-amino acid analogues at positions 9 and 10 were synthesized on a 433A peptide synthesizer (PE Biosystems, Weiterstadt, Germany) by solid-phase methods using standard Fmoc (N_α-9-fluorenylmethoxycarbonyl) chemistry on TentaGel S RAM resin (Rapp Polymere, Tübingen, Germany) as described previously.[18] The N-terminus was acetylated using a mixture of acetic anhydride/diisopropylethylamine (DIEA)/dimethylformamide (DMF) (1:2:7) for 30 min. The peptides were cleaved from the resin support with a solution of 2% triisopropylsilane, 5% phenol, and 5% water in trifluoroacetic acid for 3 h. The crude products were precipitated with diethyl ether and purified by preparative reversed-phase chromatography on PolyEncap A300 (10 μm, 250 × 20 mm i.d., Bischoff Analysentechnik GmbH, Leonberg, Germany), using an acetonitrile−0.1% trifluoroacetic acid solvent system. The purified peptides (>95% according to HPLC analysis) were characterized by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (Voyager-DE STR, PerSeptive Biosystems, Framingham, MA) using an α-cyano-4-hydroxycinnamic acid matrix, which gave the expected $[M+H]^+$ mass peaks for each peptide. The peptide content of lyophilized samples was determined by quantitative amino acid analysis (LC 3000, Biotronik-Eppendorf, Maintal, Germany).

Circular Dichroism (CD) Measurements. CD spectra were measured at 25 °C on a J-720 spectropolarimeter (Jasco, Tokyo, Japan), in a quartz cell of 0.1 cm path length, over the range 190−260 nm. The instrument was calibrated with an aqueous solution of (+)-10-camphorsulfonic acid. Peptide concentrations were 5×10^{-5} M in 30% trifluoroethanol (TFE)/70% 0.05 M KH_2PO_4 (v/v), pH 2.4. Spectra were the average of a series of five scans made at 0.1 nm intervals. The CD results are reported as mean residue molar ellipticity [Θ] in deg cm^2/dmol. The fraction of helix (f) which is assumed to be linearly related to the ellipticity at 222 nm was calculated using $f = [\Theta]_{222} - [\Theta]_{coil}/[\Theta]_{helix} - [\Theta]_{coil}$. $[\Theta]_{helix} = (-44000 + 250T)/(1-3/N)$ and $[\Theta]_{coil} = 2220 - 53T$ represent the mean residue ellipticity of a complete helix and complete random coil, respectively, where T is the temperature in °C and N is the chain length in amino acid residues.[33] To determine the helix propensity the difference in the free energy of helix formation for the D-amino acid X relative to Gly ($\Delta\Delta G_t$) was calculated by assuming that the helix−coil transition is a cooparative two-state transition according to the equation: $\Delta\Delta G_t = \Delta G_{t,X} - \Delta G_{t,Gly}$, where $\Delta G_{t,X} = -RT \ln[f/(1-f)]$.[34]

NMR Spectroscopy. One- and two-dimensional ^{1}H NMR spectra were recorded at 295 K on Bruker DRX600 and DMX750 spectrometers in standard configuration using an inverse triple-resonance probe equipped with three-axis self-shielded gradient coils. The peptides (L-Phe^{9,10}-KLA; D-Phe^{9,10}-KLA; L-Trp^{9,10}-KLA; D-Trp^{9,10}-KLA; L-Leu^{9,10}-KLA; D-Leu^{9,10}-KLA) were dissolved in 30% TFE-d3/70% water (v/v) at a peptide concentration of 1 mM. The pH was adjusted to 2.4 with 0.1 M DCl. The set of spectra obtained for every peptide consisted of a P.COSY,[35] a TOCSY[36] using a DIPSI-2rc mixing sequence,[37] and nuclear Overhauser enhancement spectroscopy (NOESY).[38] Water suppression was achieved by presaturation for the P.COSY and using a 3-9-19 WATERGATE sequence[39] for the other spectra. The mixing time was 100 and 150 ms for TOCSY and NOESY, respectively. All two-dimensional spectra were recorded with 2048 complex points in the acquisition dimension and 512 complex points in the indirect dimension, using 16 transients for P.COSY and TOCSY and 32 transients for the NOESY. ^{1}H 90° pulses were performed using a 25 kHz field and for the DIPSI sequence a field strength of 10 kHz was used. Gradients for the WATERGATE had a strength of 20 G/cm, whereas a gradient of 30 G/cm was used during the mixing time of TOCSY and NOESY experiments.

All spectra were processed using XWINNMR (Bruker AG). The final data matrix contained 4096 real points in the acquisition dimension and 2048 real points in the indirect dimension. The spectra were referenced relative to tetramethylsilane (TMS) by using a value of 3.88 ppm for the trifluoroethane (TFE) resonance. Sequential assignment was obtained using the sequence-specific assignment approach.[40]

Reversed-Phase HPLC and Free Energy Changes. Chromatographic measurements were performed on a Jasco HPLC system (Jasco GmbH, Germany), consisting of two PU-980 pumps, an AS-950 autoinjector, and a UV-975 detector operating at 220 nm. The sample concentration was 1 mg/mL of peptide in the eluent. The injection volume was 20 μL. Runs were performed at 25 °C (thermostated

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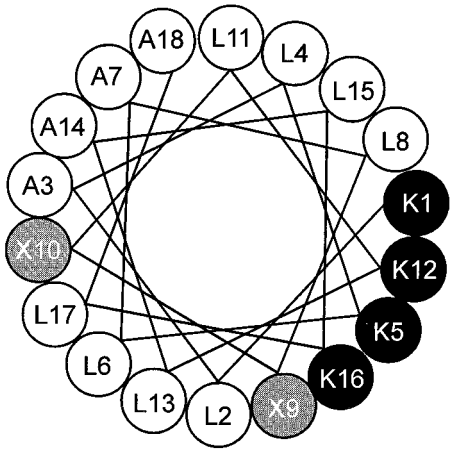

Figure 1. Helical wheel projection of the amphipathic α-helix acetyl-KLALKLALxxLKLALKLA-amide ($x^{9,10}$-KLA).

system), and at an eluent flow rate of 1 mL/min. Mobile phase A was 0.1% trifluoroacetic acid in water and B was 0.1% trifluoroacetic acid in 80% acetonitrile/20% water (v/v). Gradient retention times were determined using a linear gradient 5−95% B in 40 min. Isocratic retention times were determined using eluents consisting of 0.1% of trifluoroacetic acid in water containing different concentrations of acetonitrile (25−45%).

The isocratic retention times (t_R) are used to calculate the capacity factors k' using the following equation: $k' = (t_R - t_0)/t_0$, where t_R is the retention time of the peptide, and t_0 is the column void time. Data for t_0 were obtained by injecting a liquid mixture with a volume composition different from that of the eluent. A linear relationship exists between ln k' of peptides and the organic mole fraction ϕ, according to $\ln k' = \ln k_{0'} - S\phi$, where S is a constant, and $k_{0'}$ is the capacity factor in the absence of the organic solvent.[41] The capacity factor k' is linearly related to the equilibrium partition coefficient between the stationary and mobile phase according to $k' = \varphi K$, where φ is the phase ratio of the column, which is a constant for a given column and was estimated to be 0.1, as previously described.[42] Accordingly, free energy changes (ΔG_{assoc}) which are associated with transfer of the peptide from the aqueous mobile phase to the hydrophobic stationary phase could be calculated from the equation: $\Delta G_{assoc} = -RT\,(\ln k' - \ln \varphi)$.

Results and Discussion

Rationale for Host Model Peptide. The 18-residue peptides ($x^{9,10}$-KLA) used in this study are based on the well-characterized sequence acetyl-KLALKLALKALKLALKLA-amide (KLA) which exhibits a high intrinsic α-helical propensity according to Chou and Fasman analysis.[24] The distribution of hydrophilic lysines and hydrophobic leucines and alanines is characteristic of an amphipathic helix[43] as illustrated in Figure 1. To suppress unfavorable helix dipole interactions all peptides were N-terminally acetylated and C-terminally amidated.[44] In contrast to numerous peptide models used to study the helical propensities of L-amino acids which form unusually stable helices in water,[34,45] the KLA-peptide undergoes a monomeric helix to coil transition. Previous CD studies of KLA and KLA analogues at different pH, peptide concentrations, and structure-inducing conditions have shown that small differences in helicity could be measured in TFE/water solvent systems.[18,23,46] KLA is nearly unstructured in aqueous solution and adopts an amphipathic α-helical structure with a helix fraction of >80% in the presence of 30−50% of the α-helix-inducing solvent TFE. A change in KLA concentration from 50 μM to 1 mM has no influence on CD.[20,46] NMR, 3D structure calculation, and guanidine hydrochloride titration experiments in the presence of 30% TFE demonstrated that the helix-destabilizing effect of D-amino acids can be sensitively determined based on a helix−coil two-state transition.[19,20]

Chromatographic studies showed that KLA has the ability to form an amphipathic helix during reversed-phase HPLC.[18,46,47] In contrast to the random coil conformation in aqueous mobile phase (fraction of helix, $f < 0.15$), the stationary phase-bound KLA adopts a helical conformation.[21,46] Because the helix formation provides a contribution to free energy changes, the retention behavior of KLA is related to the stability of the helix. This enables determination of free energy changes which are associated with the coil → helix transition during transfer of the peptide from the aqueous mobile phase to the hydrophobic stationary phase. Recent experiments using D-amino acid replacement sets of KLA demonstrated that D-amino acids destabilize the amphipathic α-helix, leading to a decrease of helicity as determined by CD and NMR studies and a decrease of hydrophobic interaction during reversed-phase HPLC.[18,19,21] It was shown that the reduction of chromatographic retention times correlates linearly with the decrease of helicity, whereas the destabilizing effect of D-amino acids is enhanced by replacement of two adjacent amino acids with their D-enantiomers. Because replacements at positions 9 and 10 of KLA exhibit the largest effect on helicity and retention behavior as previously shown, $x^{9,10}$-KLA was selected for this study.

CD Studies. To determine the helix-destabilizing effect of D-amino acids in the host sequence $x^{9,10}$-KLA, CD spectra of two sets of KLA with positions 9 and 10 substituted by L-amino acids and D-amino acids were measured and compared to the $Gly^{9,10}$-KLA analogue. CD spectra were obtained for all peptides after they were dissolved in aqueous solutions containing 30% of the helix-inducing solvent TFE at pH 2.4 (Figure 2).

All $x^{9,10}$-KLA-peptides with L-amino acids in the x positions adopt helical structures with fractions of helix between 0.8 and 1.0 (except for proline, $f = 0.51$). The molar ellipticity at 222 nm of peptides with x = L-Ala, L-Arg, L-Lys, L-Leu, L-Met, L-Trp, L-Phe, L-Ser, L-Gln, L-Glu, L-Cys, L-Thr, and L-Asn was determined to be in the range from −30 000 to −34 000 deg cm^2/dmol corresponding to a complete helix formation. The molar ellipticity at 222 nm of KLA with x = L-Val, L-Ile, L-Trp, L-Tyr, L-Asp, and L-His was determined to be −28 722, −28 333, −28 722, −25 444, −28 611, and −29 778 deg cm^2/dmol, respectively. It was found that measured CD spectra and the negative ellipticity at 222 nm, characteristic of helical conformations, were strongly influenced by the incorporated D-amino acids. The results of $[\Theta]_{222}$, the calculated values of fraction of helix (f), and the difference in the free energy of helix formation for D-amino acid x relative to Gly ($\Delta\Delta G_t$) are shown in Table 1. The molar ellipticity at 222 nm was determined to be in the range from −11 385 to −28 222 deg cm^2/dmol. Differences in the free energy of helix formation for D-amino analogues relative to the corresponding L-amino acid analogue ($\Delta\Delta G_{D\text{-}L} = \Delta G_{t,D} - \Delta G_{t,L}$) were calculated (Table 2). The inversion of amino acid configuration results in free energy

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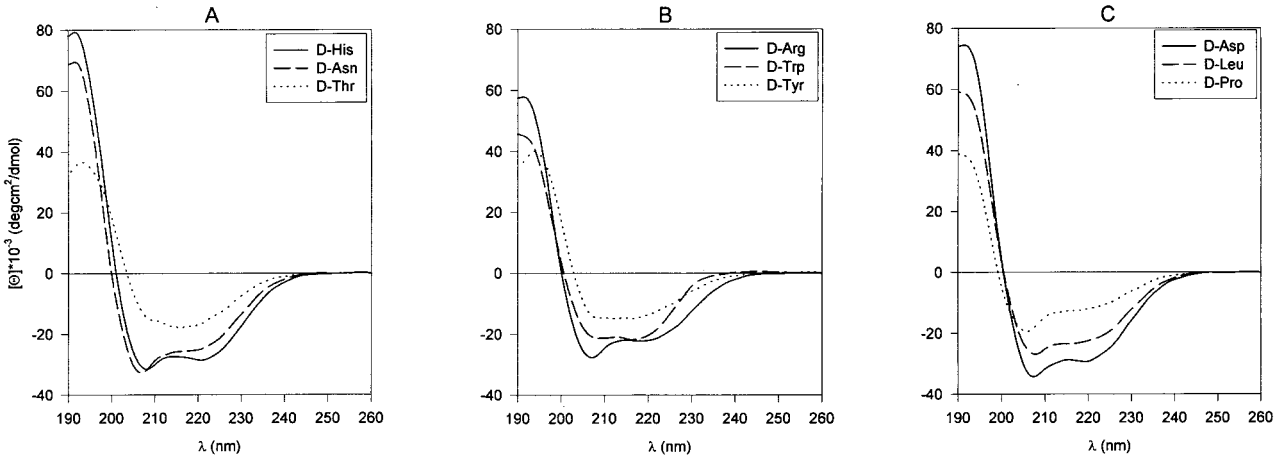

Figure 2. CD spectra of $x^{9,10}$-KLA peptides in 30% TFE/70% 0.05 M KH_2PO_4 (v/v), pH 2.4. Peptide concentration is 5×10^{-5} M.

Table 1. Mean Residue Ellipticity at 222 nm, Fraction of Helix, and Difference in Free Energy of Helix Formation Relative to Glycine of $x^{9,10}$-KLA Peptides Measured by CD; Conditions: 25 mM Phosphate Buffer, 30% TFE, pH 2.4; Peptide Concentration is 5×10^{-5} M

amino acid x	$[\Theta]_{222}$ (deg cm^2/dmol)	fraction of helix, f	$\Delta\Delta G_t$[a] (kJ/mol)
D-His	−28 222	0.900	−0.63
D-Asp	−27 833	0.888	−0.32
Gly	−27 389	0.875	0
D-Glu	−24 778	0.794	+1.48
D-Cys	−24 556	0.787	+1.58
D-Gln	−24 500	0.785	+1.61
D-Asn	−24 167	0.775	+1.76
D-Ser	−23 667	0.759	+1.97
D-Leu	−21 611	0.696	+2.77
D-Arg	−21 333	0.687	+2.87
D-Met	−21 111	0.680	+2.95
D-Lys	−19 667	0.636	+3.44
D-Trp	−18 444	0.598	+3.84
D-Ala	−16 722	0.545	+4.38
D-Thr	−15 556	0.509	+4.74
D-Phe	−15 278	0.500	+4.82
D-Val	−15 167	0.497	+4.86
D-Ile	−14 333	0.471	+5.11
D-Tyr	−12 500	0.414	+5.68
D-Pro	−11 389	0.380	+6.04

[a] $\Delta\Delta G_t = \Delta G_{t,X} - \Delta G_{t,Gly}$, where $\Delta G_{t,X} = -RT \ln[f/(1-f)]$.

Table 2. Influence of Inversion of Amino Acid Configuration on Free Energy Changes of Helix Formation Measured by CD; Conditions: 25 mM Phosphate Buffer, 30% TFE, pH 2.4; Peptide Concentration is 5×10^{-5} M

amino acid x	$[\Theta]_{222}$ (deg cm^2/dmol)	$\Delta\Delta G_t$[a] (kJ/mol)
L-Asp	−28 611	
D-Asp	−27 833	+0.66
L-His	−29 778	
D-His	−28 222	+1.76
L-Tyr	−25 444	
D-Tyr	−12 500	+4.52
L-Trp	−28 467	
D-Trp	−18 444	+4.69
L-Ile	−28 333	
D-Ile	−14 333	+5.84
L-Val	−28 722	
D-Val	−15 167	+5.95

[a] $\Delta\Delta G_t = \Delta G_{t,D} - \Delta G_{t,L}$, where $\Delta G_{t,X} = -RT \ln[f/(1-f)]$.

changes in the range from 0.6 to 6.0 kJ/mol depending on the side-chain of the D-amino acid. The peptides with x = D-His and D-Asp have the highest negative values of $[\Theta]_{222}$ and negative $\Delta\Delta G$-values, indicating that the D-isomers of these two amino acids have a lower α-helix disturbing propensity than glycine, which is the weakest helix former of the commonly occurring amino acids with the exception of proline.[32] D-Glu, D-Cys, D-Gln, D-Asn, and D-Ser in positions 9 and 10 of KLA destabilize the helix compared to glycine by 1.48−1.97 kJ/mol. In contrast, amino acids with bulky and β-branched side-chains such as D-Tyr, D-Phe, D-Ile, D-Val, and D-Thr as well as D-proline are most effective in destabilization of the amphipathic α-helical peptide KLA ($\Delta\Delta G_t = 4.74-6.04$ kJ/mol).

NMR Spectroscopy. To gain more insight into the conformational effects of D-amino acids, the secondary structure of three pairs of KLA peptides which exhibit different helicities (L-Phe^{9,10}-KLA; D-Phe^{9,10}-KLA; L-Trp^{9,10}-KLA; D-Trp^{9,10}-KLA; L-Leu^{9,10}-KLA; D-Leu^{9,10}-KLA) was investigated using ^{1}H NMR spectroscopy. The peptides were dissolved in aqueous solution containing 30% of the structure-inducing solvent TFE-d_3 and the measurements were performed at 295 K. After sequential assignments were obtained using standard sequence-specific assignment strategy,[40] the chemical shifts of the H^α protons and the pattern of nuclear Overhauser effects (NOEs) in the NOESY spectra were analyzed. Regions from the NOESY spectrum from the two tryptophan peptides (L-Trp^{9,10}-KLA; D-Trp^{9,10}-KLA) that contain most of the information regarding the secondary structure are shown in Figure 3.

All peptides investigated having L-amino acids in positions 9 and 10 of KLA clearly exhibit the NOE pattern of an α-helix in the NOESY spectrum. In the region of the signals between amide and H^α protons (Figure 3a for L-Trp^{9,10}-KLA) the intensities of the αN(i,i+1) cross-peaks are weaker than those of the αN cross-peaks. A complete set of αN(i,i+3) and αN-(i,i+4) cross-peaks can be identified. In the region of the amide−amide cross-peaks (Figure 3b for L-Trp^{9,10}-KLA) not only intense NN(i,i+1) cross-peaks are present, but also a full set of NN(i,i+2) peaks. In addition, almost all of the crucial $\alpha\beta$(i,i+3) peaks can be found (not shown), even though the unambiguous identification of such peaks is somewhat difficult because of heavy overlap of signals from identical side-chains. The peaks ($\alpha\beta$(6,9) and $\alpha\beta$(7,10)) are clearly visible for the positions 9 and 10. The chemical shifts of the H^α protons are in agreement with the NOE-pattern; all are in the range that can be expected for a helical structure, i.e., shifted upfield relative to values for random-coil peptides.[48]

The situation changes for the three peptides investigated in which amino acids 9 and 10 have been replaced by their D-analogues. Fewer and weaker NOEs can be observed, indicat-

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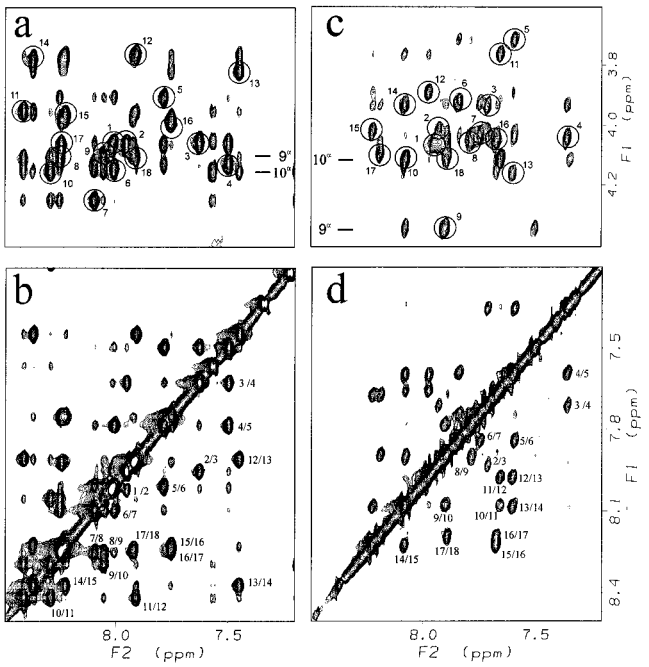

Figure 3. Regions from the NOESY spectrum of L-Trp^{9,10}-KLA and D-Trp^{9,10}-KLA where most of the signals used for the analysis of the secondary structure can be found. (a) αN-region of the NOESY of L-Trp^{9,10}-KLA. The αN(i,i) peaks are marked with a circle and the residue number is given. The positions of the H$^{\alpha}$ of Trp^9 and Trp^{10} are indicated at the side of the spectrum. (b) NN-region of L-Trp^{9,10}-KLA. The NN cross-peaks are labeled with the number of the amino acids involved. (c) αN-region of the NOESY of D-Trp^{9,10}-KLA. The αN(i,i) peaks are marked with a circle and the residue number is given. The positions of the H$^{\alpha}$ of Trp^9 and Trp^{10} are indicated at the side of the spectrum. (d) NN-region of D-Trp^{9,10}-KLA, the NN cross-peaks are labeled with the number of the amino acids involved.

ing an overall loosening of the structure although the main features are still consistent with a helical structure. Most of the αN(i,i+1) cross-peaks are still weaker than the αN(i,i) cross-peaks (Figure 3c for D-Trp^{9,10}-KLA). The αN(9,10) and αN-(10,11) cross-peaks are almost as intense as the corresponding intraresidue peaks, but that can be attributed to chirality at the α-position. The αN(i,i+4) peaks are missing, but weak αN-(i,i+3) cross-peaks can be observed except for the area around the D-amino acids. This can be expected for D-amino acids but the αN(8,11) peak, where no D-amino acid would be involved, is absent as well. The NN(i,i+2) cross-peaks have disappeared (Figure 3d for D-Trp^{9,10}-KLA). Although many $\alpha\beta$(i,i+3) cross-peaks are still detectable (not shown), the $\alpha\beta$(6,9) and $\alpha\beta$(7,10) peaks are not observed. Again this can be attributed to the opposite chirality at the α-carbon of amino acid 9 and 10. If the peptide would be still helical, however, intense cross-peaks from the β-protons of D-Trp^9 and D-Trp^{10} to side chains of the amino acids in positions 12 and 13 should be present. These peaks are extremely weak. Although the chemical shifts of the H$^{\alpha}$ protons are still largely in the range of helical values, the signal of D-Trp^9 has moved downfield as shown in Figure 3c. Taken together this indicates a kink in the helical structure in the region of amino acids 9 and 10.

In summary, the analysis of the proton NMR spectra of various KLA peptides consisting exclusively of L-amino acids reveals a stable helix that extends along the full sequence of the 18 amino acids. After incorporation of two adjacent D-amino acids in positions 9 and 10, the peptides appear to be less structured even though the less sensitive indicators of a helical structure are still present. The helix appears to be kinked in the region around the D-amino acids, indicated by an altered pattern of NOEs. These results are in agreement with previous NMR studies which described structural effects of double D-amino acid replacement in helical model peptides consisting exclusively of leucine, alanine, and lysine.[19]

Table 3. RP-HPLC Retention Times[a] of x^{9,10}-KLA Peptides

amino acid x	retention time (min)	amino acid x	retention time (min)	Δ
L-Val	28.1	D-Val	22.7	−5.4
L-Ile	28.7	D-Ile	23.5	−5.2
L-Thr	25.7	D-Thr	20.9	−4.8
L-Ala	26.6	D-Ala	23.1	−3.5
L-Tyr	27.8	D-Tyr	24.6	−3.2
L-Trp	28.5	D-Trp	25.3	−3.2
L-Ser	24.2	D-Ser	21.2	−3.0
L-Arg	21.6	D-Arg	18.8	−2.8
L-Lys	21.1	D-Lys	18.4	−2.7
L-Leu	28.5	D-Leu	25.9	−2.6
L-Asn	23.3	D-Asn	20.8	−2.5
L-Met	27.8	D-Met	25.5	−2.3
L-Phe	28.5	D-Phe	26.3	−2.2
L-Gln	23.5	D-Gln	21.5	−2.0
L-Glu	24.6	D-Glu	23.0	−1.6
L-Asp	24.4	D-Asp	23.1	−1.3
L-His	20.8	D-His	19.7	−1.1
L-Cys	27.3	D-Cys	26.3	−1.0
L-Pro	21.2	D-Pro	21.6	+0.4
Gly	23.3			

[a] The retention times were determined on PolyEncap A300; mobile phase A was 0.1% trifluoroacetic acid in water, and B was 0.1% trifluoroacetic acid in 80% acetonitrile/20% water (v/v), linear gradient 5−95% B in 40 min.

Free Energy Changes in Reversed-Phase Chromatography. The formation of an amphipathic α-helical structure in peptides induces a preferred hydrophobic binding domain and provides at least in part a contribution to retention on reversed-phase columns.[18,42,49] Thus, free energy changes measured by HPLC retention times are sensitive to conformational changes (random coil in the aqueous phase → α-helix of the bound peptide). The pairwise substitution of two adjacent amino acids by their D-enantiomers is advantageous because it influences secondary structure without changing other properties of the peptide such as side-chain hydrophobicity, functionality, or charge distribution. Therefore, differences in the free energy of helix formation for each D-amino acid analogue relative to the corresponding L-amino acid analogue ($\Delta\Delta G_{D\text{-}L}=\Delta G_{assoc,D} - \Delta G_{assoc,L}$) can be calculated by comparison of the retention behavior using the chromatographic capacity factor (k') which is linearly related to the equilibrium partition coefficient (K) between stationary phase and mobile phase.

The reversed-phase HPLC retention times of two sets of KLA with positions 9 and 10 substituted by all naturally occurring L-amino acids and their corresponding D-enantiomers were measured. The retention times determined by applying a commonly used acetonitrile gradient in aqueous 0.1% trifluoroacetic acid are listed in Table 3. Comparing the retention times of L-amino acid peptides with those of the related D-amino acid analogues, we find that the D-amino acid peptides (with the exception of proline) were eluted at shorter times. This change in retention time depends on the amino acid and is attributed to the effect of L- to D-amino acid replacement on hydrophobicity of the amphipathic KLA helix, and thus is related to helix formation. To calculate free energy data, chromatographic capacity factors (k') were determined from isocratic elutions.

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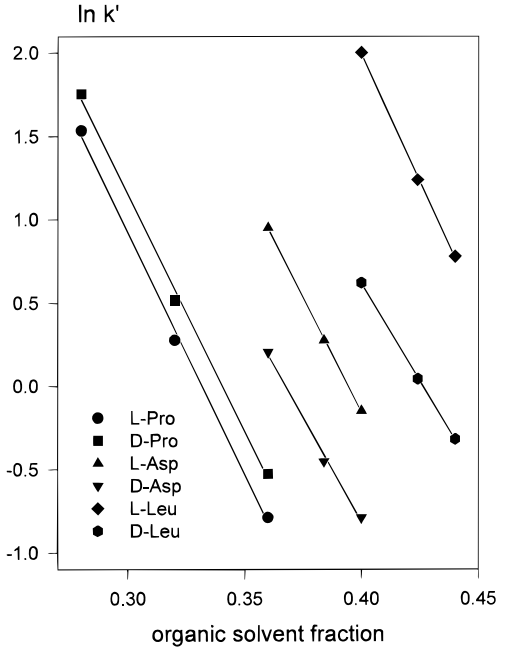

Figure 4. Change in chromatographic capacity factors (ln k') for L-Pro^{9,10}-KLA, D-Pro^{9,10}-KLA, L-Asp^{9,10}-KLA, D-Asp^{9,10}-KLA, L-Leu^{9,10}-KLA, and D-Leu^{9,10}-KLA with increasing content of acetonitrile in water (0.1% trifluoroacetic acid).

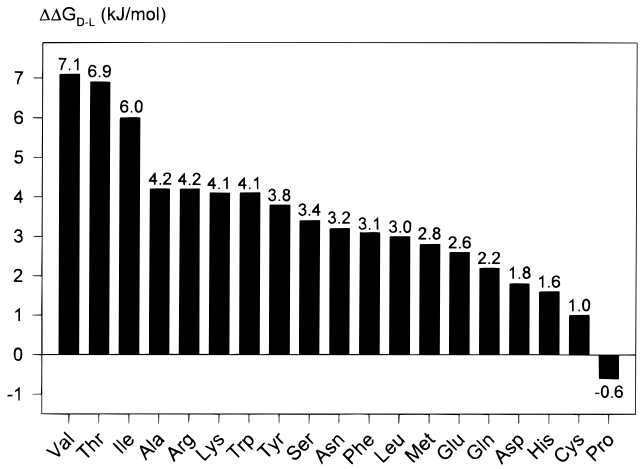

Figure 5. Influence of inversion of amino acid configuration on free energy changes in reversed-phase HPLC. The difference in the free energy changes for each D- amino acid analogue relative to the corresponding L-amino acid analogue was determined from capacity factors (ln k') according to $\Delta\Delta G_{D\text{-}L} = \Delta G_{assoc,D} - \Delta G_{assoc,L}$, where $\Delta G_{assoc} = -RT\ (\ln k' - \ln \varphi)$.

The capacity factors (ln k') were plotted as a function of the acetonitrile concentration in the eluent. As shown in Figure 4, the increase in ln k' with decreasing acetonitrile concentration was very close to linear with all peptides. The difference in the free energy changes for each D-amino acid analogue relative to the corresponding L-amino acid isomer was determined according to $\Delta\Delta G_{D\text{-}L} = \Delta G_{assoc,D} - \Delta G_{assoc,L}$. Figure 5 shows the influence of inversion of amino acid configuration on the calculated free energy changes.

The HPLC data confirm the results of CD measurements. When we compare the HPLC data with the free energy changes measured by CD, the results provide consistent findings. Despite different structure induction (TFE vs hydrophobic surface) the free energy changes are in qualitative agreement. The large variation in retention times and free energy changes indicate that destabilization of an amphipathic α-helix by D-amino acids is highly dependent on the amino acid side chain. In comparison with the corresponding L-amino acid, the incorporation of D-isomers of valine, threonine, and isoleucine strongly destabilize the helical structure by 6.0−7.1 kJ/mol. The increased destabilization of the helical conformation by side chains which are branched at the β-position might be caused by a potential steric clash between the side-chain and the preceding turn of the helix. In marked contrast, the change of configuration for amino acids having more flexible side chains has a considerably smaller influence on the stability of the amphipathic α-helix ($\Delta\Delta G_{D\text{-}L} = +4.2$ to $+1.0$ kJ/mol). The destabilizing propensity differs among charged and polar D-amino acids. These differences cannot be explained on the basis of the experiments performed. Note that inverting the configuration of proline does not significantly influence the helicity of the model peptide. Thus, D-proline possesses no additional helix-disturbing effect compared to the L-form, which has been shown to be the most effective helix-destabilizing L-amino acid.[32,34]

Conclusions

Host−guest analysis using an amphipathic helical model peptide resulted in helix-destabilizing propensity scales of D-amino acids in an L-amino acid environment. Conformational analyses and studies of hydrophobic binding provided free energy changes of helix formation caused by incorporation of two adjacent D-amino acids relative to glycine and to the corresponding L-amino acid, respectively. In comparison with the corresponding L-amino acids, all D-amino acids were shown to be helix-destabilizing by induction of turn-like structures. The helix-destabilizing propensity of the D-amino acids is highly dependent on the amino acid side chain and is not related to the structure propensity of the corresponding L-amino acid. Whereas the destabilizing effect of histidine and aspartic acid is similar to that of glycine, showing that these D-isomers have the highest propensity to form an α-helical structure in this system, D-amino acids with bulky and β-branched side chains strongly destabilize α-helical structures. D-proline disrupts the α-helical structure to roughly the same extent as L-proline. The results demonstrate that the α-helix propensity of D-amino acids incorporated into a peptide consisting of L-amino acids is mainly determined by steric effects of the amino acid side chains. Knowledge of the different effects of D-amino acids on secondary structure formation could be important in structure−activity studies of peptide ligands and in the de novo design of peptides and proteins with specific structural features.

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