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Comparison of Design Strategies for Promotion of β-Peptide 14-Helix Stability in Water

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Many short β-peptides adopt well-defined conformations in organic solvents, but specialized stabilizing elements are required for folding to occur in aqueous solution. Several different strategies to stabilize the 14-helical secondary structure in water have been developed, and here we provide a direct comparison of three such strategies. We have synthesized and characterized β-peptide heptamers in which variously a salt bridge between side chains, a covalent link between side chains, or two cyclically constrained residues have been incorporated to promote 14-helicity. The incorporation of a salt bridge does not generate significant 14-helicity in water, according to CD and 2D NMR data. In contrast, incorporation either of a lactam bridge between side chains or of cyclic residues results in stable 14-helices in water. The β-peptides featuring trans*-2-aminocyclohexanecarboxylic acid (ACHC) residues show the highest 14-helical backbone stability, with hardly any sensitivity to pH or ionic strength. The β-peptides featuring side-chain-to-side-chain cyclization show lower 14-helical backbone stability and higher sensitivity to pH and ionic strength, but increased order between the side chains because of the cyclization.*

Introduction

Foldamers are oligomers that can adopt predictable secondary structures,[1] and foldamers that display stable conformations in aqueous solution provide attractive scaffolds for development of biologically active molecules.[2] β-Peptide foldamers have received considerable attention in this respect.[3] These β-amino acid oligomers are typically straightforward to prepare, and they provide access to a variety of ordered backbone conformations. Among the regular β-peptide conformations, the 14-helix has been most intensively studied, in terms of sequence/stability relationships and biological applications.[2,4] The 14-helix is defined by the formation of $i, i-2$ C=O···H–N backbone hydrogen bonds that encompasses a 14-atom ring. 14-Helical β-peptides have been shown to bind to specific proteins[5] and to display antibacterial[6] and antifungal[7] activities.

Most β-peptides studied to date have been composed partially or entirely of β^3-amino acid residues, because protected β^3-amino acids are readily available in enantiopure form from the analogous α-amino acids by the Arndt–Eistert process.[4] β-Peptides containing exclusively β^3-residues generally fold to the 14-helix in organic solvents, but special design features are required to enable 14-helical folding in water.[2,4]

The first clear evidence for 14-helix formation in water was obtained for β-peptides containing residues incorporating a six-membered ring constraint, such as *trans*-2-aminocyclohexanecarboxylic acid (ACHC).[8] These constrained residues show 14-helix propensities much larger than those of β^3-residues. However, it was subsequently shown that alternative design strategies, based entirely on β^3-residues, can also generate 14-helicity in water. Both Seebach et al.[9] and Cheng and DeGrado[10] showed that 14-helicity is promoted in aqueous solutions by $i, i+3$ spacing of β^3-residues bearing oppositely charged side chains. The 14-helix has approximately three residues per turn; therefore, this $i, i+3$ spacing allows intrahelical salt bridge formation. Initial all-β^3 designs featured charge–charge interactions at two of the three helical faces of the 14-helix; however, subsequent studies showed that residues with side chain branching adjacent to the backbone—such as β^3-homovaline (β^3-hVal)—can replace some of the charged side chains.[11] Covalent linkage of two side chains at β-amino acid positions i and $i+3$ through a disulfide bridge was shown to lead to the stabilization of the 14-helix in methanol.[12] Covalent linkage of two side chains at β-amino acid positions i and $i+3$ through amide bonds, as an alternative to ion pairing, resulted in the promotion of 14-helicity in water.[13]

It would be valuable to know the relative helix-stabilizing efficacies of the different strategies outlined above. However, published examples do not allow the necessary comparisons, because of differences in β-amino acid composition and β-peptide length. We therefore decided to prepare and analyze a homologous set of β-peptide heptamers (Scheme 1) that would enable us to assess the relative extents of 14-helix promotion provided by salt bridging, covalent side chain linkage, and ACHC incorporation in a consistent β^3-amino acid context. Although it is not yet possible to quantify the population of 14-

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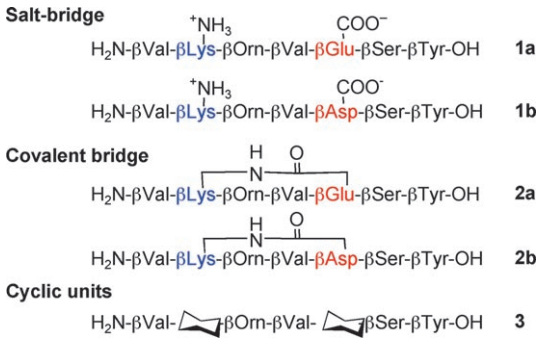

Scheme 1. β-Peptides illustrating the three different strategies for 14-helix stabilization in water: salt bridge formation between side chains (**1a** and **1b**), covalent bridge formation between side chains (**2a** and **2b**), and cyclic residues (**3**), with the cyclohexyl symbol representing *trans*-2-aminocyclohexanecarboxylic acid.

helix (or of any other β-peptide secondary structure) in a given β-peptide, we find that a combination of 2D NMR and circular dichroism (CD) measurements allows us to draw clear qualitative distinctions among the 14-helix-stabilizing strategies.

Results and Discussion

Design and synthesis of the β-peptides

A β-peptide composed of seven residues can form two full turns of the 14-helix containing up to five intramolecular backbone hydrogen bonds. We chose positions two and five to incorporate the different helix-stabilizing elements, and kept the other five positions constant in order to isolate the impact of the variable elements on 14-helicity. At positions one and four, β^3-hVal residues were incorporated, because a side chain branch point adjacent to the backbone appears to promote 14-helicity.[11a,14] Position seven is β^3-hTyr; the UV absorbance of the aromatic side chain is useful for HPLC purification and concentration determination. Hydrophilic residues were placed at positions three (β^3-hOrn) and six (β^3-hSer) to promote aqueous solubility (Scheme 1).

β-Peptides **1a** and **1b** contain complementary basic and acidic side chains at positions two and five, respectively, which allows formation of a salt bridge in the 14-helical conformation. Placing of the basic residue near the N terminus and the acidic residue near the C terminus leads to a more favorable interaction of the charged side chains with the 14-helix dipole than would the alternative arrangement.[10] The β^3-hOrn was placed next to the β^3-hLys in order to generate the most favorable charge–charge interactions in β-peptides **1a** and **1b**;[10,15a] placement of the ornithine at position six could possibly have resulted in significant interference with the acidic residue at position five. The acidic residue at position five was varied between β^3-hAsp and β^3-hGlu in order to determine whether the length of the tether between the carboxylate and the backbone influences the contribution of ion pair formation to helix stability. The basic lysine residue at position two had been replaced previously by an ornithine, without significant changes in the stability of the helix.[13]

β-Peptides **2a** and **2b** were formed from precursors with the sequences of **1a** and **1b**, respectively, but in **2a** and **2b** the side chains from the residues at positions two and five had been linked covalently through amide formation. In β-peptide **3**, pre-organized ACHC residues occupy positions two and five. ACHC has a stronger 14-helix propensity than any β^3-residue.[8,15]

All β-peptides were synthesized by previously described protocols on solid-phase, with the specific differences of the β-amino acids and the on-bead side chain cyclization being taken into consideration.[13,16] After purification by preparative RP-HPLC, the expected products, identified by mass spectrometry, were isolated in 9–35% yields, with >99% purity (see the Supporting Information).

Comparisons by 2D NMR spectroscopy

2D NMR provides the strongest spectroscopic evidence for helical folding in solution, through the observation of NOEs between protons from residues that are not sequentially adjacent. Because 2D NMR analysis is labor-intensive, we analyzed only a subset of our β-peptides—**1b**, **2b**, and **3**—in this way. Each was examined at a concentration of 1–2 mM in a phosphate-buffered H_2O/D_2O (9:1, 10 mM) mixture at pH 7.4 and 10 °C. TOCSY data (Supporting Information) were used for a complete assignment of proton chemical shifts. ROESY data (Supporting Information) were evaluated to identify medium-range NOEs that indicate β-peptide folding. Three NOE patterns involving backbone protons are diagnostic for 14-helix formation: 1) between an amide proton and the proton at the β-position two residues toward the C terminus [NH(*i*)–C_βH(*i*+2)], 2) between an amide proton and the proton at the β-position three residues toward the C terminus [NH(*i*)–C_βH(*i*+3)], and 3) between the axial proton at the α-position and the proton at the β-position three residues toward the C terminus [C_αH(*i*)–C_βH(*i*+3)] (Figure 1, top).

β-Peptide **1b**, stabilized by a salt bridge, showed mainly sequential and intraresidue NOEs; few of the characteristic medium-range NOEs were detected (Figure 1). The resonances for **1b**, especially those corresponding to the amide protons of β^3-hLys2 and β^3-hAsp5, displayed considerable overlap (Supporting Information). Poor proton resonance dispersion is commonly observed when the extent of folding is low. The NH(β^3-hLys2)–C_βH(β^3-hVal4) NOE might have been present but could not be assigned unambiguously because of resonance overlap.

Side-chain-cyclized β-peptide **2b** showed a larger dispersion of the amide signals than **1b**, indicating a higher degree of folding of the β-peptide. Even though **2b** displayed some resonance overlap, we were nevertheless able to assign three important medium-range NOEs involving amide protons and all four of the possible C_αH(*i*)–C_βH(*i*+3) NOEs, for residue pairs 1/4, 2/5, 3/6, and 4/7 (Figure 1 and Supporting Information). Among these last four NOEs, the strongest was the correlation between β^3-hOrn3 and β^3-hSer6, which suggests that the bridged residues are particularly well ordered. Many other medium-range NOEs involving side chain protons of **2b** were observed. These medium-range side-chain-to-side-chain and

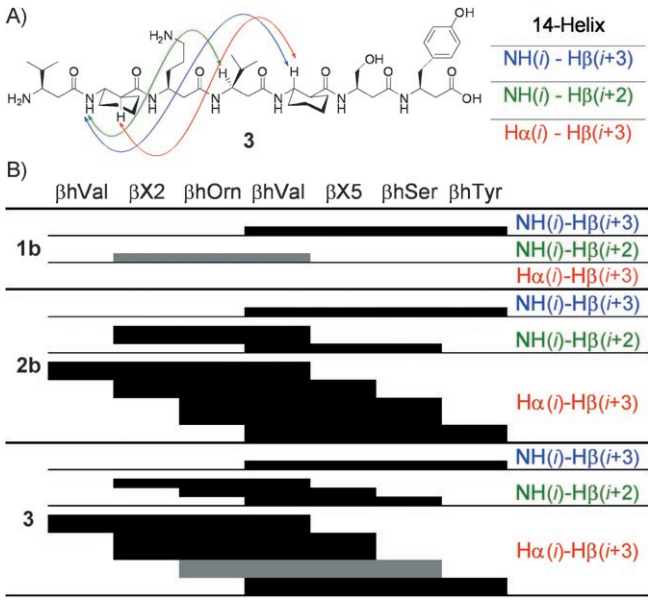

Figure 1. A) NOE interactions characteristic of the 14-helix, illustrated with β-peptide **3**, and B) summary of observed NOE correlations characteristic of a 14-helix (thickness of the bars reflects intensities of the connectivities; gray represents ambiguously assigned NOE).

side-chain-to-backbone NOEs were typically weaker than the inter-backbone medium-range NOEs, but could be unambiguously assigned and provide important information concerning the conformational order of the side chains. Medium-range NOEs involving the side chains were observed for the residue couples 2/5, 3/6, and 4/7 (Table 1). The two protons at the δ-position of β^3-hLys2 could be differentiated in the spectra, suggesting a high degree of order in this lactam-bridged side chain. The $C_\delta H$ and $C_\varepsilon H$ protons of β^3-hLys2 were found to correlate with the backbone of β^3-hAsp5. Comparable side-chain-to-backbone medium-range NOE correlations were also observed for the β^3-hOrn3/β^3-hSer6 couple, which is not connected through a covalent bridge. The β^3-hVal4/β^3-hTyr7 couple featured, in addition to a side-chain-to-backbone medium-range NOE, side-chain-to-side-chain medium-range NOEs. The aliphatic protons of β^3-hVal4 featured medium-range correlations with the aromatic protons of β^3-hTyr7. The combination of both the typical backbone medium-range NOE pattern for a 14-helix with the additionally observed medium-range correlations involving the side chains provides strong evidence for a well ordered 14-helix structure for **2b**.

The six amide protons of β-peptide **3**, featuring the cyclic residues, showed excellent dispersion between 8.1 and 7.6 ppm, which led to well-resolved TOCSY and ROESY data and indicates a high degree of folding. A large number of NOEs diagnostic for the 14-helix, including four correlations in the amide region, were observed for **3** (Figure 1). Three of the four possible $C_\alpha H(i)$–$C_\beta H(i+3)$ NOEs were observed; the NOE between C_αH(Orn3)–C_βH(Ser6) could not be unambiguously assigned because of overlap between the $C_\beta H$ units of β^3-hOrn3 and β^3-hSer6. The highest intensity was found for a connectivity at an internal position: C_αH(ACHC2)–C_βH(ACHC5). Fewer medium-range NOEs involving protons at side-chains were observed than in the case of **2b**. The observed medium-range side chain NOEs were limited to the residue pairs 1/4 and 4/7 (Table 1). The absence of NOEs involving the side chains of residue couple 2/5 might result from the restrained nature of the cyclic β-amino acid residue at these positions. However, the absence of side-chain-to-backbone medium-range NOE correlations for the β^3-hOrn3–β^3-hSer6 pair is striking, as this pair is the same in **2b** and **3**. The lower number of medium-range NOEs involving side-chains in **3** relative to **2b** suggests a more dynamic character of the side chains of **3**, despite the more rigid backbone.

No NOE inconsistent with the 14-helix conformation was detected in any of the three β-peptides examined by 2D NMR. β-Peptides **2b** and **3** featured many NOEs characteristic of the 14-helix, with additional medium-range NOEs involving the side chains. Overall, the NMR results indicate that β-peptides **2b** and **3** have well populated 14-helical conformations in water. β-Peptide **1b**, which could form a single intrahelical salt bridge, does not seem to display significant 14-helicity, in contrast with, for example, long β-peptides that feature two sequential salt bridges.[17] The use of cyclic residues as biasing elements (**3**) might preorganize the backbone to promote a 14-helix in which the side chains have a certain degree of freedom. The use of a covalent bridge appears to have an additional stabilizing effect on the conformations of the side chains, as suggested by the higher number of observed medium-range NOEs involving side chain protons for **2b** relative to **3**.

Table 1. Medium-range side chain NOEs observed among the four different *i*, *i*+3 residue pairs of **2b** and **3** in water.

	β^3-hVal1/β^3-hVal4	β^3-hLys2/β^3-hAsp5	β^3-hOrn3/β^3-hSer6	β^3-hVal4/β^3-hTyr7
2b		$C_\delta H(2)$–$C_\beta H(5)$ $C_\delta H(2)$–$C_\beta H(5)$ $C_\varepsilon H(2)$–$C_\beta H(5)$ $C_\varepsilon H(2)$–NH(5)	$C_\gamma H(3)$–$C_\beta H(6)$ $C_\gamma H(3)$–$C_\beta H(6)$ $C_\delta H(3)$–$C_\beta H(6)$	$C_\delta H(4)$–$C_\beta H(7)$ $C_\delta H(4)$–$C_\varepsilon H(7)$ $C_\delta H(4)$–$C_\zeta H(7)$ $C_\gamma H(4)$–$C_\varepsilon H(7)$
3	$C_\delta H(1)$–$C_\beta H(4)$			$C_\delta H(4)$–$C_\beta H(7)$ $C_\delta H(4)$–$C_\varepsilon H(7)$ $C_\gamma H(4)$–$C_\beta H(7)$ $C_\gamma H(4)$–$C_\varepsilon H(7)$

Comparisons by circular dichroism spectroscopy

Circular dichroism (CD) measurements were performed in order to compare the extents of 14-helical folding among the five β-peptides discussed here. Figure 2 shows the CD signatures of each β-peptide in methanol and in aqueous sodium phosphate buffer (10 mM, pH 7.4; all samples were evaluated at 20 °C). In methanol each heptamer displays the characteristic CD pattern of a 14-helix, with a minimum around 215 nm. The mean residue ellipticity (MRE) at 215 nm can be used as a very approximate basis for comparing the extent of 14-helical folding among different β-peptides.[2]

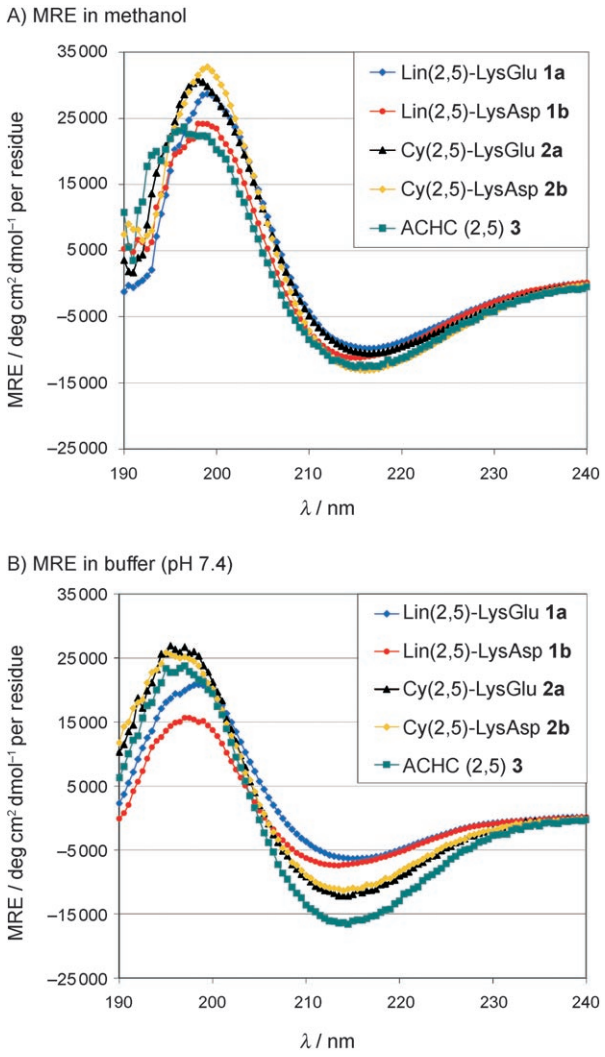

Figure 2. CD spectra of β-peptides **1**–**3** in A) methanol and B) phosphate buffer (pH 7.4, 20 °C) normalized to mean residue ellipticity (MRE).

The CD spectra of β-peptides **1**–**3** in methanol display similar MRE values around 215 nm, which suggests that these five β-peptides display similar extents of 14-helicity in this helix-stabilizing solvent. Changing from methanol to aqueous buffer, however, results in significant differences among the β-peptides in terms of MRE at 215 nm; this suggests differences in the favorability of 14-helical folding among these molecules. β-Peptides **1a** and **1b** feature MRE (215 nm) values of around -7000 deg cm^2 dmol^{-1}, with the minimum shifted to higher wavelength for **1a**. β-Peptides **2a** and **2b** feature MRE (215 nm) values of around $-11\,000$ deg cm^2 dmol^{-1}, which is similar to the MRE values displayed by these β-peptides in methanol. β-Peptide **3** features an MRE (215 nm) value of around $-16\,000$ deg cm^2 dmol^{-1}; this minimum is the most intense among all of the β-peptides analyzed in aqueous buffer. The CD minimum at 215 nm for **3** in water is modestly but significantly more intense than that of **3** in methanol. Overall, the data agree well with previous observations,[15a] and they suggest that only **2a**, **2b**, and **3** display significant 14-helicity in water.

Analysis of the CD data suggests that β-peptides **1a** and **1b**, which can form an intra-14-helical salt bridge, show the least 14-helicity in aqueous solution among the five heptamers we examined. The small MRE (215 nm) in water for a heptameric β-peptide stabilized by one salt bridge is in line with previous results.[18] The lengths of the side-chains—that is, β-peptides **1a** vs. **1b**—do not produce drastic changes for these two β-peptides, but has a more fine-tuning effect. The more intense minima near 215 nm for β-peptides **2a**, **2b**, and **3** relative to **1a** and **1b** allow us to conclude that either *i*, *i*+3 side chain linkage or the use of preorganized ACHC residues significantly promotes 14-helical folding relative to simple all-β^3 sequences that can form a potentially stabilizing internal salt bridge between side chain groups. The lengths of the lactam bridges differ by one CH_2 group between β-peptides **2a** and **2b**, and the effect on helix stability is minimal. Larger changes within this bridging element, however, do result in significant changes in the helix stability.[13] β-Peptide **3** features the most intense CD signal at 215 nm, which might indicate that the ACHC-based strategy is most effective among those examined here for 14-helix stabilization.

In order to evaluate the stabilities of the 14-helices of β-peptides **1**–**3** under different environmental conditions, CD spectroscopy measurements were performed at different pH values (Figure 3). In addition to the data displayed in Figure 2 at pH 7.4, data were acquired at pH 1.8, 3.6, and 9.6.

Significant pH-dependent differences in 14-helical folding were observed among the five heptamers. β-Peptides **1a** and

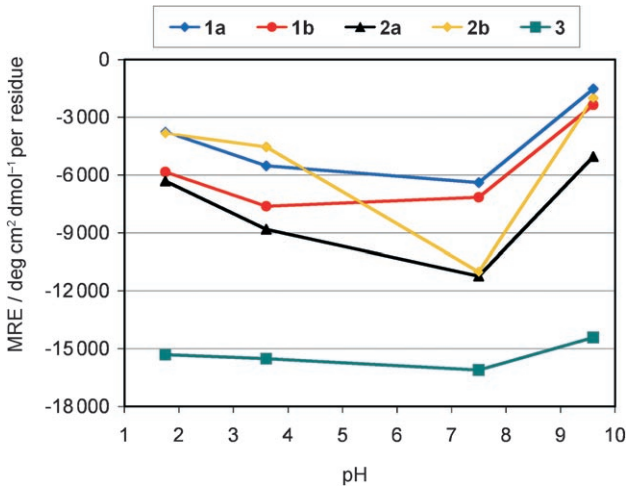

Figure 3. Effect of pH on the stabilities of the 14-helices, expressed in MRE at 215 nm at four different pH values.

1b each show a substantial decrease in 14-helicity with increasing pH from 7.4 to 9.6, suggesting that little or no 14-helix population remains at high pH. Decreasing the pH from 7.4 to 1.8 has a smaller effect on 14-helix population. β-Peptides **2a** and **2b** feature a relatively larger pH dependence of the 14-helicity than **1a** and **1b**, with higher apparent populations of the helical conformations at neutral pH for series **2** than for series **1**. For both series the destabilizing effect at

high and low pH could result from interactions between the helix macrodipole and the charged states at the N and/or C termini. An increase in the pH results in deprotonation of the backbone ammonium at the N terminus and loss of the stabilizing interaction between the positive terminal charge and the helix macrodipole; comparable effects result from protonation of the C-terminal carboxylate at low pH. 14-Helical folding of β-peptide **3**, however, shows very little susceptibility to changes in pH. This behavior is surprising, since terminal charge–helix macrodipole interactions should be comparable for **3** and for the other β-peptides in this series. This insensitivity to pH suggests that the ACHC residues in **3** are so highly preorganized for the 14-helical conformation that the presence or absence of terminal charge–helix macrodipole interactions has no effect on the extent of helix formation. This interpretation, in turn, raises the possibility that **3** approaches complete population of the 14-helical conformation in aqueous solution, a conclusion that is consistent with the results obtained for other short ACHC-containing β-peptides, featuring similar MRE values.[15a]

We used CD spectroscopy to examine the effect of changing ionic strength (analyzed by varying NaCl concentration between 0 and 1.6 M) on the extent of 14-helicity in pH 7.4 aqueous buffer (Figure 4). β-Peptide **1b** shows a strong and **2b** a mild decrease in the CD intensity at 215 nm with increasing NaCl concentrations, which suggests that salt destabilizes the

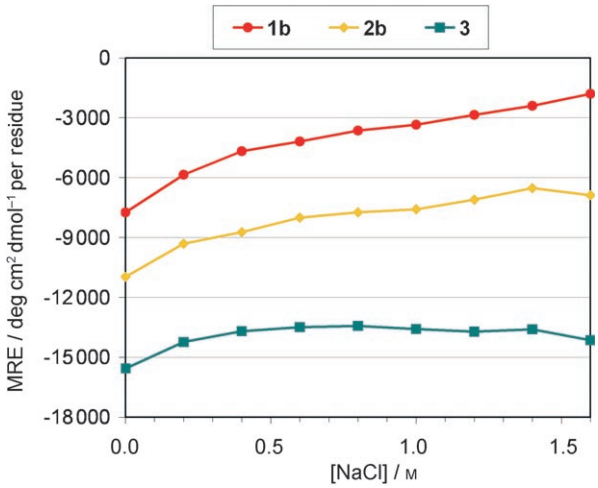

Figure 4. Effect of [NaCl] on the 14-helix, expressed in MRE at 215 nm.

14-helical conformation. The effect of NaCl is particularly pronounced for **1b**, with a decrease of 77% in the intensity at 215 nm. This large effect presumably arises because the intrahelical salt bridge available to **1b** promotes the formation of the small population of 14-helix detected in the absence of NaCl, and the intramolecular electrostatic attraction is screened as ions are added to the solution. β-Peptide **2b** apparently retains significant 14-helicity even at 1.6 M NaCl, since the intensity at 215 nm has declined by only 37% relative to aqueous buffer without any salt. In this case the decrease might arise from ionic screening of the charge–macrodipole interactions. The CD spectra of β-peptide **3** show an overall loss of only 9% of the intensity at 215 nm. This trend supports the view that a small number of ACHC residues strongly promote 14-helix formation.

Conclusions

We have conducted the first direct comparison of three distinct strategies developed to stabilize 14-helix secondary structures in β-peptides. Our results suggest that incorporation of a small proportion of ACHC residues (two out of seven in our system) represents the most effective way to promote 14-helicity in water. In addition, a covalent side-chain linkage of appropriate length is effective at supporting the 14-helix conformation in aqueous solution, but is more susceptible to changes in the environment. On the other hand, noncovalent side-chain linkage, through formation of a single ion pair, exerts only a weak helix-promoting effect in aqueous solution. This last observation presumably explains the use of multiple ion pairing interactions that have been necessary to stabilize 14-helical conformations in other systems.[9–11,17] The necessity for multiple ion pairs limits the number of residue positions that can be used to achieve desired functions, such as binding to a target biomolecule. Moreover, the use of ion pairing for 14-helix stabilization creates a high level of dependence on environmental conditions (such as pH and ionic strength). The findings presented here show that incorporation either of a lactam bridge or of a small proportion of preorganized ACHC residues results in short, helix-forming β-peptide sequences that offer great flexibility for incorporation of functionally important side chains on a rigid backbone structure.

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Keywords: 14-helices · beta-peptides · conformation analysis · cyclic peptides · foldamers

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