Evidence that the β-Peptide *14*-Helix is Stabilized by β^3-Residues with Side-Chain Branching Adjacent to the β-Carbon Atom

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Dedicated to Professor *Dieter Seebach* on the occasion of his 65th birthday

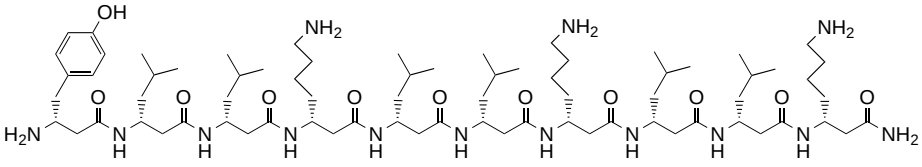
Oligomers of β-substituted β-amino acids ('β^3-peptides') are known to adopt a helical secondary structure defined by 14-membered ring hydrogen bonds ('*14*-helix'). Here, we describe a deca-β^3-peptide, **1**, that does not adopt the *14*-helical conformation and that may prefer an alternative secondary structure. β^3-Peptide **1** is composed exclusively of residues with side chains that are not branched adjacent to the β-C-atom (β^3-hLeu, β^3-hLys, and β^3-hTyr). In contrast, an analogous β-peptide, **2**, containing β^3-hVal residues in place of the β^3-hLeu residues of **1**, adopts a *14*-helical conformation in MeOH, according to CD data. These results illustrate the importance of side-chain branching in determining the conformational preferences of β^3-peptides.

Introduction. – The modern era of β-peptide research began publicly in mid-1996, when *Seebach* and co-workers published their first study on the synthesis and conformational characterization of short β-amino acid oligomers [1]. These workers showed *via* two-dimensional NMR analysis that oligomers comprised of β-substituted β-amino acids ('β^3-amino acids') and containing as few as six residues could adopt a specific helical conformation in organic solvents. This helix has *ca.* three residues per turn and is defined by a series of 14-membered-ring H-bonds between backbone amide groups ($C{=}O(i)\cdots H{-}N(i-2)$); we have designated this secondary structure the '*14*-helix' [2]. The seminal paper [1] showed that short α-amino acid oligomers do not form α-helices in MeOH, in striking contrast to the strong *14*-helical propensity displayed by short β-peptides in the same solvent. Prior to this report, there was a modest literature on secondary-structure formation by β-amino acid polymers, but no high-resolution structural data were available [3].

Independently of *Seebach* and co-workers, we had become interested in the possibility that β-peptides might display discrete folding patterns [4], and, in late 1996, we reported *14*-helix formation by oligomers of *trans*-2-aminocyclohexanecarboxylic acid (ACHC) [2]. Our approach was to use covalent constraints (rings) to pre-organize the backbone for a specific secondary structure. This approach enhances *14*-helical stability relative to pure β^3 sequences [5] and provides access to secondary structures that have not been observed in the absence of ring constraints [6][7]; nevertheless, the observation that short β^3-peptides fold to the *14*-helix is a continuing source of amazement to many chemists. This discovery is a tribute to *Seebach*'s remarkable molecular intuition.

The study of unnatural oligomers with well-defined folding propensities ('foldamers') has become widespread over the past decade [8]. Among the increasingly diverse foldamer backbones under study, β-peptides are perhaps the most thoroughly developed [9]. Numerous β-peptide secondary structures have been documented, including four different helices, two different reverse turns, two different types of sheet, and strand-paired structures reminiscent of nucleic acids [10]. In addition, the first steps have been taken toward β-peptide tertiary structure [11]. Designed β-peptides have displayed a number of different biological activities [12][13]. Increasing the number of β-peptide shapes that can be rationally selected is likely to enhance the utility of these foldamers for biological applications. Here, we report preliminary data suggesting that altering the side-chain branching of β^3-residues can profoundly influence the conformation favored by short β-peptides.

Results. – The studies reported here, involving β-peptides **1**–**3**, arose from an unexpected observation. Molecule **1** was prepared as part of our effort to identify β-peptides that form amphiphilic helices and self-associate in aqueous solution. Protein designers have shown that this type of aggregation can be a harbinger of helical bundle tertiary structure [14]. We observed self-association of β-peptide **4** in aqueous solution



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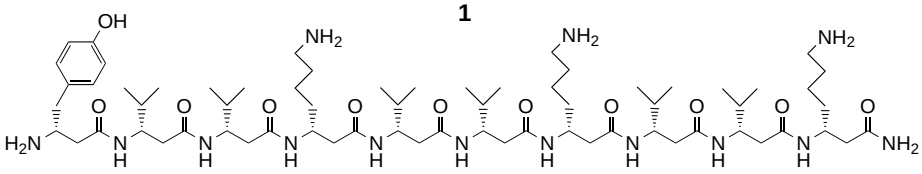

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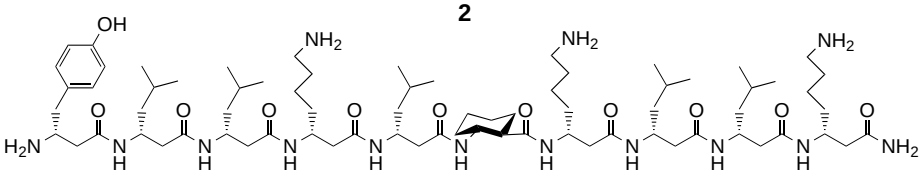

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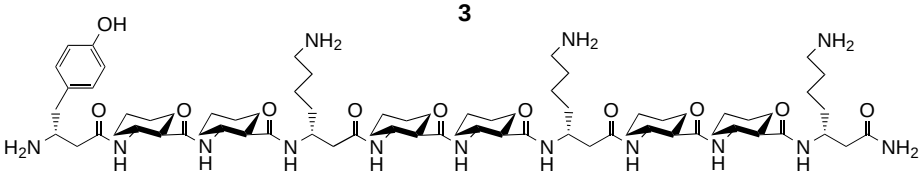

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[11]; in this case, the hydrophobic face of the *14*-helix is formed by the pre-organized *trans*-2-aminocyclohexanecarboxylic acid (ACHC) residues. β-Peptide **1** is an analogue of **4** in which, if a *14*-helix were formed, the hydrophobic face would be comprised of flexible β^3-hLeu residues. We planned to compare the behaviors of **1** and **4** to determine the role played by conformational pre-organization in intermolecular helix-bundle formation. (The N-terminal β^3-hTyr residue was included to provide a UV chromophore.)

Analysis of **1** by circular-dichroism (CD) spectroscopy yielded a surprise. In MeOH, a solvent that strongly promotes *14*-helix formation among β^3-peptides [15][16], **1** displays a CD signature quite different from that of the *14*-helix (*Fig. 1*). The CD spectrum of **1** features a weak minimum at 220 nm and a strong maximum at 203 nm, while the canonical CD spectrum of *14*-helical β^3-peptides in MeOH is a maximum at 215 nm and a minimum at 195 nm. (This paper focuses on β-peptides that contain β^3-residues derived from α-D-amino acids; β-peptides comprised of β^3-amino acids derived from α-L-amino acids show opposite CD extrema, of course.) The CD spectrum displayed by **1** is reminiscent of the CD signature of the β-peptide *12*-helix (defined by a series of twelve-membered-ring H-bonds between backbone amide groups, $C{=}O(i)\cdots H{-}N(i+3)$) [6][17]; however, we cannot assign any specific conformation to **1** in the absence of high-resolution structural data.

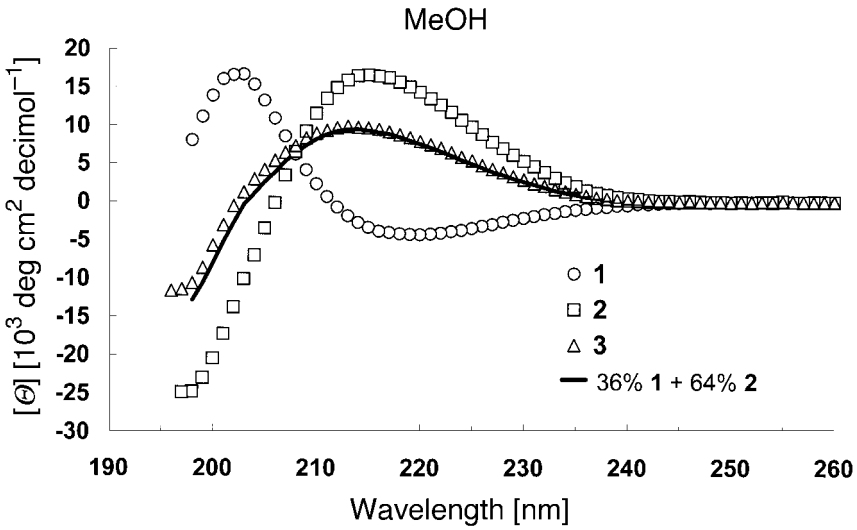

Fig. 1. *CD Spectra of β-peptides in MeOH at 25°.* The concentration of each β-peptide is 0.1 mM. The data are normalized for β-peptide concentration and number of residues.

Observations reported by *Gung et al.* [18] and *Hamuro et al.* [13] suggest that β^3-residues bearing side chains with branch points adjacent to the β-C-atom (*e.g.*, β^3-hVal) may be more prone to *14*-helix formation than are β^3-residues that lack branching at this position (*e.g.*, β^3-hLeu). Therefore, we examined β-peptide **2**, an analogue of **1** in which all six β^3-hLeu residues have been replaced with β^3-hVal residues. Indeed, the CD spectrum of **2** in MeOH (*Fig. 1*) shows a standard *14*-helical signature. The dramatic

difference between the CD spectra of **1** and **2** in MeOH indicates that β^3-hVal and β^3-hLeu have very distinct conformational preferences, which is consistent with the view that β^3-residues, as a class, are quite malleable [19].

To probe further the intrinsic conformational preferences of β^3-hLeu, we examined **3**, an analogue of **1** in which a single β^3-hLeu residue near the middle of the sequence has been replaced by ACHC. ACHC has a very high predisposition for the *14*-helical conformation. *Seebach et al.* observed high *14*-helical propensity in a β-peptide containing mostly β^3-residues and a central ACHC residue [20]. A comparison of the CD spectra of **3** and **1** in MeOH (*Fig. 1*) shows that the single β^3-hLeu → ACHC change exerts a major effect on folding. β-Peptide **3** displays a maximum consistent with *14*-helix formation, at *ca.* 213 nm, but this maximum is slightly blue-shifted and less intense relative to the maximum displayed by **2**.

The CD spectra of **1**–**3** display an isodichroic point, which led us to wonder whether the CD spectrum of **3** could be reproduced by some combination of the CD spectra of **1** and **2**. Indeed, a curve generated with 36% **1** and 64% **2** matches the CD spectrum of **3** almost perfectly (*Fig. 1*). Thus, if the spectra of **1** and **2** represent 'pure' conformational states, then perhaps the folding of **3** may be described as a mixture of those two states. This mixture may be achieved by *1*) equilibration between the two conformational states, each formed along the entire length of **3**; or *2*) by different portions of **3** adopting different conformations; or *3*) by some combination of explanations *1*) and *2*).

The CD spectra of **1**–**3** were examined in aqueous solution (*Fig. 2*). In all three cases, the intensity of the CD extrema are substantially diminished in H_2O relative to MeOH. Diminution in CD intensity upon moving from MeOH to H_2O is universally observed for β-peptides comprised largely or exclusively of β^3-residues [15][16], and

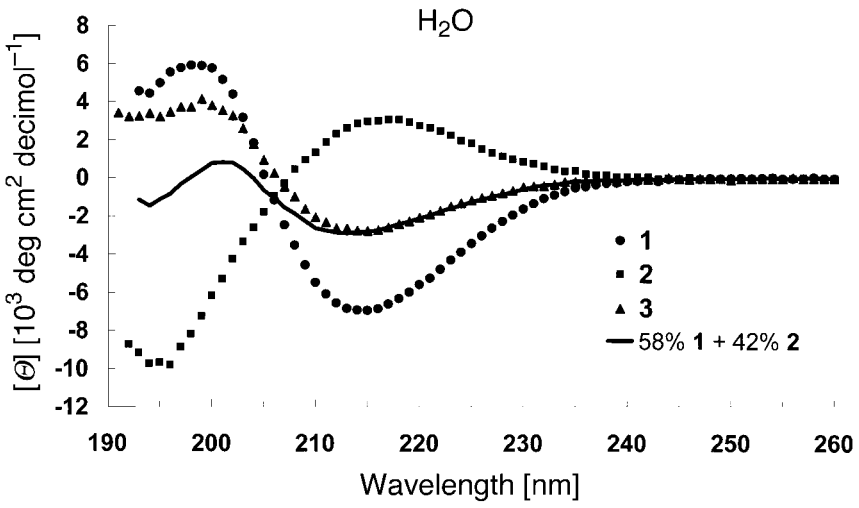

Fig. 2. *CD Spectra of β-peptides in 10 mM aqueous* Tris, *pH 7.2 at 25°*. The concentration of each β-peptide is 0.1 mM. The data are normalized for β-peptide concentration and number of residues. (Note that the vertical scales in *Figs. 1* and *2* are different.)

this effect is attributed to diminution in the extent of folding in H_2O relative to MeOH. MeOH and other alcohol solvents are also known to promote α-helix formation among conventional peptides (α-amino acid residues) [21].

The shapes of the CD spectra for **1** and **2** in H_2O are comparable to the shapes observed in MeOH. These shape similarities suggest that both **1** and **2** adopt the same folding patterns in H_2O and in MeOH, but that the population of the folded conformation is diminished in each case in H_2O relative to MeOH. This interpretation of the CD data carries the assumption that the 'random coil' state for β-peptides shows little or no CD in this spectral region, an observation that has some experimental support [13][22].

The CD spectrum of **3** in H_2O has a qualitatively different shape from the CD spectrum of **3** in MeOH (maximum at 213 nm in MeOH *vs.* minimum at 215 nm in H_2O). This difference can be explained in the context of the hypothesis described above to rationalize the behavior of **3** in MeOH. We postulate that **3** populates two different folded states in MeOH: one state is represented by **1** and the other represented by **2**. The resulting CD signal is comprised of a combination of the CD shapes of these two states. The change in CD signal for **3** in H_2O relative to MeOH may be attributable to variation of the proportion of the two folded states accessed by **3** in the different solvents. We probed this expanded hypothesis by trying to fit the CD spectrum of **3** in H_2O *via* combination of the CD spectra of **1** and **2** in H_2O. As seen in *Fig. 2*, the experimental CD spectrum of **3** in H_2O is reproduced moderately well with a curve generated by 58% **1** and 42% **2**, although the fit is not as good as that in MeOH (*Fig. 1*). Interestingly, the major contributor to the best fit of the CD spectrum of **3** is reversed from **2** to **1**, when the solvent is changed from MeOH to H_2O. Thus, if the two-folded-state hypothesis for **3** is correct, then the conformation represented by **2**, the *14*-helix, is disfavored for **3** in H_2O relative to the other folded conformation, while MeOH exerts the opposite conformational preference on **3**.

The structural hypothesis offered above for the behavior of **3** posits that the solution behavior of this β-peptide arises from a mixture of three conformational states: *14*-helix, another folded state represented by **1**, and random coil (unfolded). If the proportions of these three change as a function of solvent, then we should not see an isodichroic point when CD spectra of **3** obtained in various H_2O/MeOH solvents are overlaid. The data in *Fig. 3* verify this prediction.

In the *14*-helical conformation, β-peptides **1**–**3** would display well-defined hydrophobic surfaces comprised of the β^3-hLeu, β^3-hVal, and/or ACHC residues. This feature could lead to hydrophobically-driven β-peptide self-association in H_2O, as has been observed for **4** [11]. We probed for self-association among **1**–**3** by conducting variable-concentration ^{1}H-NMR studies in H_2O (*Fig. 4*). In all three cases, there is little or no change in terms of line shape or chemical shift of the amide or aromatic H-atoms between *ca.* 0.3 mM and *ca.* 3 mM. In contrast, **4** displays profound broadening and substantial changes in chemical shifts over this concentration range [11]. Self-association of **4** at or above *ca.* 1 mM was confirmed by analytical ultracentrifugation [11]. For **1**, we used analytical ultracentrifugation to confirm that there is no aggregation at 1.4 mM (*Fig. 5*). Thus, the CD data presented in *Fig. 2* represent the behavior of **1**–**3** in the absence of self-association.

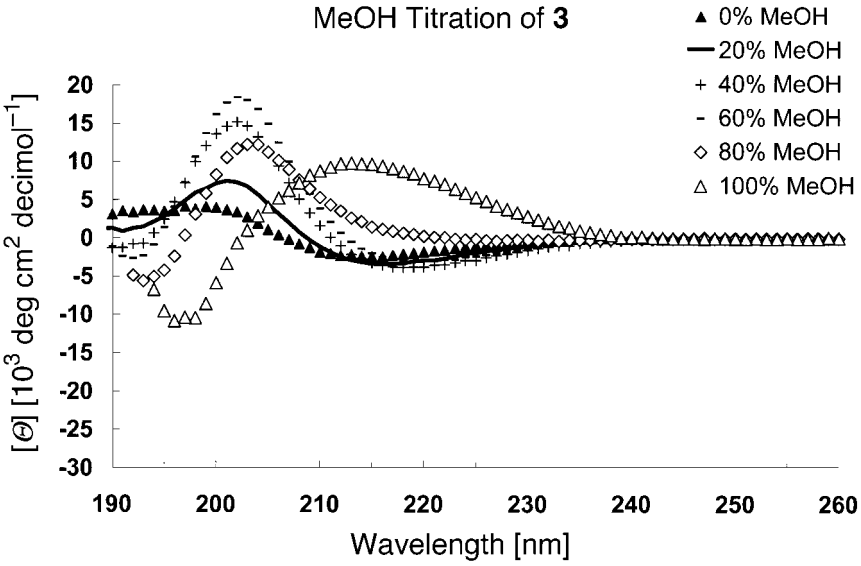

Fig. 3. *CD Spectra of* **3** *in various ratios of MeOH/aqueous* Tris *buffer at 25°*. The concentration of each β-peptide is 0.1 mM. The data are normalized for β-peptide concentration and number of residues.

Discussion. – β^3-Amino acid residues display considerable conformational plasticity. β-Peptides comprised exclusively of these residues can adopt the *14*-helix [1]. Certain combinations of β^3- and β^2-residues can lead to the *10*/*12*-helix, a remarkable conformation in which there are two different types of intramolecular H-bonds [23]. We have recently shown that β^3-residues can be incorporated into the *12*-helix, if they are combined with appropriately constrained residues [19]. In other sequence contexts, β^3-residues can participate in antiparallel-sheet secondary structure [24]. The results reported here suggest that a folded conformation other than the *14*-helix may be accessible to β-peptides constructed exclusively from β^3-residues. This alternative fold is promoted by β^3-residues bearing side chains that are not branched adjacent to the β-C-atom. High-resolution NMR analysis should provide an incisive test of this hypothesis.

Experimental Part

General Procedures. Matrix-assisted laser desorption-ionization time-of-flight mass spectra (MALDI-TOF-MS) were obtained on a *Bruker REFLEX II* spectrometer with a 337-nm laser and the α-cyano-4-hydroxycinnamic acid matrix. The instrument was calibrated to a standard mixture of leu^5-enkephalin ($[M + H]^+$ 556.28), angiotensin I ($[M + H]^+$ 1296.7) and neurotensin ($[M + H]^+$ 1672.9).

Materials. Et_2O was anh. The highest available grade of all other solvents was purchased and used without further purification. Fmoc-Amide resin (4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxyacetamido-ethyl resin; polystyrene resin functionalized with a *Knorr* linker; loading 0.63 mmol/g) was obtained from *Applied Biosystems*. *Tris* was purchased from *Sigma*. All other reagents were purchased from *Aldrich*.

β-Peptides were purified by RP-HPLC on a *Vydac C4* semiprep. column with a flow rate of 3 ml/min. Solvent *A* and solvent *B* for RP-HPLC were 0.045% trifluoroacetic acid (TFA) in *Millipore* water and 0.036% TFA in MeCN, resp. β-Peptide purity was assessed with a linear gradient of 5–95% solvent *B* over 57 min on a

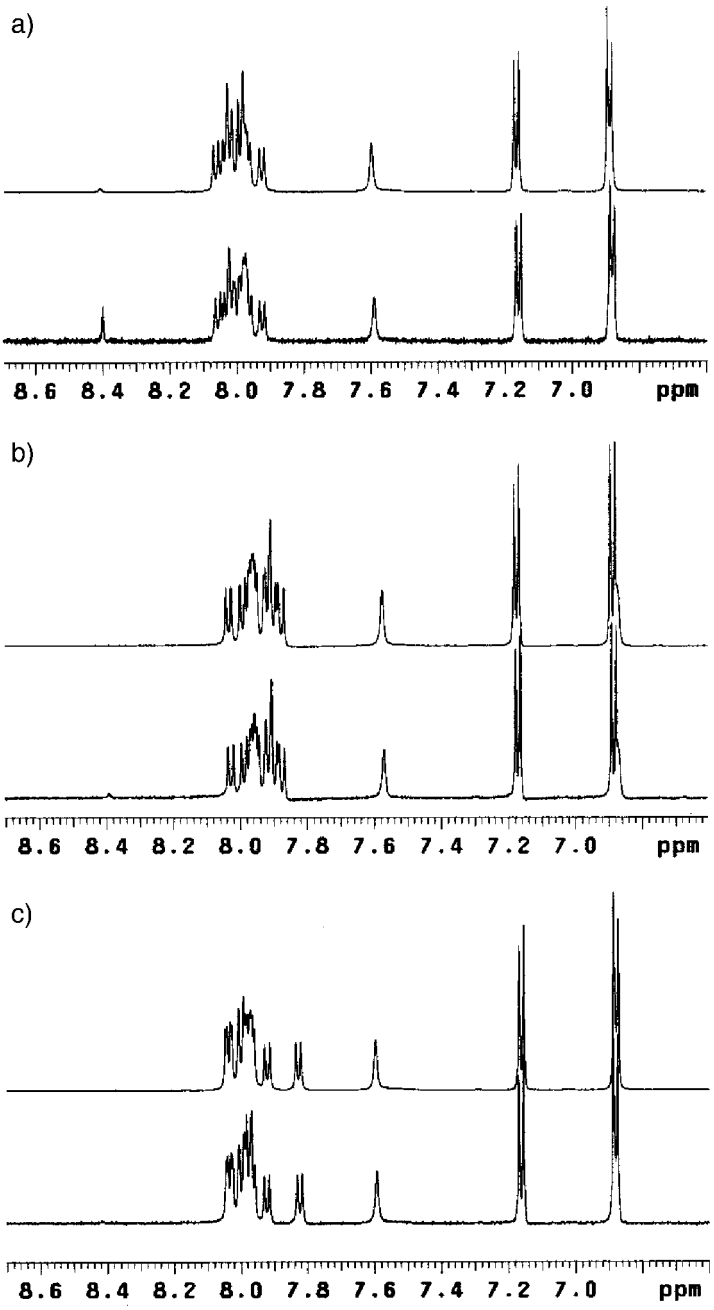

Fig. 4. *Amide/aromatic regions of ^{1}H-NMR spectrum of the β-peptides studied at various concentrations:* a) *β-peptide* **1** *at 3.7 mM* (top) *and 0.4 mM* (bottom); b) *β-peptide* **2** *at 2.6 mM* (top) *and 0.3 mM* (bottom); c) *β-peptide* **3** *at 2.9 mM* (top) *and 0.3 mM* (bottom). Similarity of spectra at high and low concentrations for all three β-peptides suggests that these sequences are not prone to self-aggregation in this concentration range. Data were acquired at 600 MHz in H_2O/D_2O 9:1 containing 100 mM CD_3COOD (pH 3.8). (The CD data displayed in *Figs. 1–3* were obtained in 10 mM *Tris*; CD data for **1**–**3** in 100 mM AcOH (not shown) were within experimental uncertainty of data in 10 mM *Tris*).

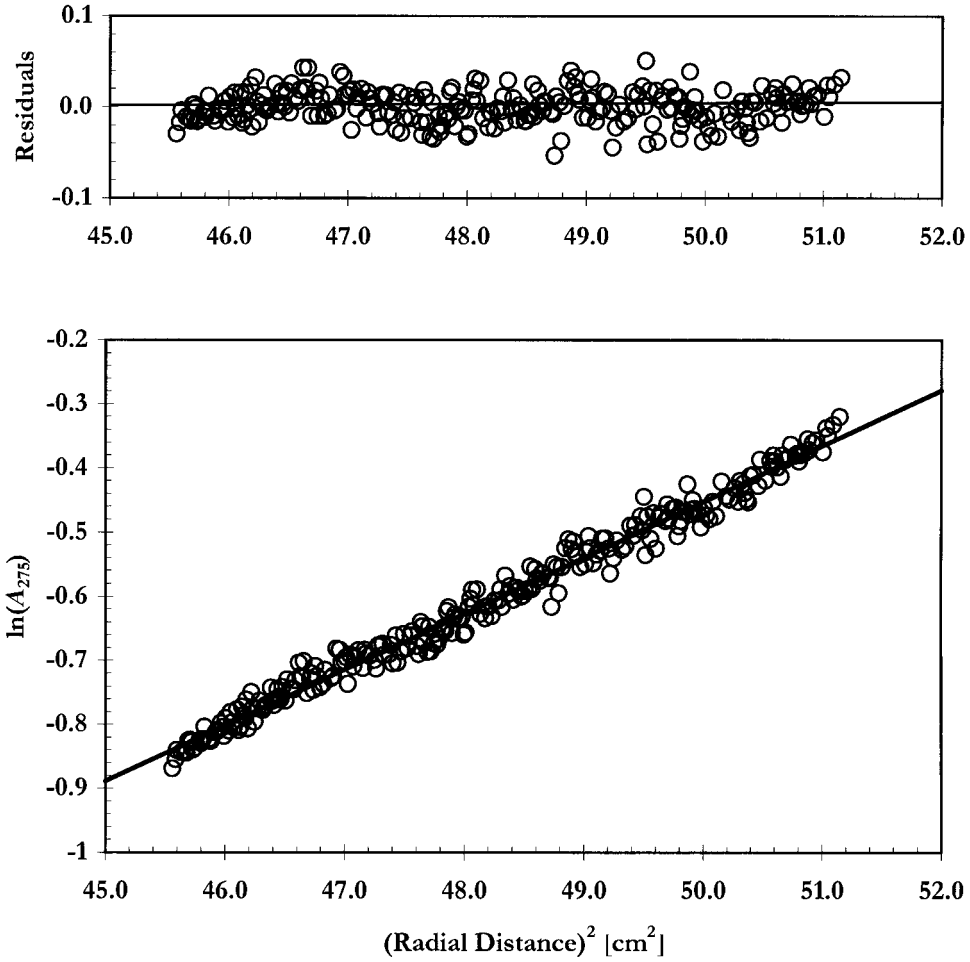

Fig. 5. *Representative data from sedimentation equilibrium analytical ultracentrifugation studies with* **1** *at 1.4 m*M *in aqueous 40 m*M Tris, *pH 7.2, 24°*. Data shown were acquired at 45 krpm with 0.001-cm step size. Linear least-squares fitting of data to a single ideal species model resulted in molecular-weight estimates consistent with **1** monomer (fit shown as solid line). The fits were found to be adequate as judged by random distribution of residuals around zero (shown on top).

Vydac C4 anal. column, monitoring at 220 nm. The purity of each β-peptide after RP-HPLC was greater than 95%.

Synthesis of β-Peptides. General Procedures for Solid-Phase Synthesis. Fmoc-Protected acyclic β-amino acid monomers [15][25] and ACHC [26] were synthesized as described previously. β-Peptides were synthesized on Fmoc-amide resin (25 μmol scale) on an *Applied Biosystems* model *432A* (*Synergy*) automated peptide synthesizer by a standard Fmoc/*t*-Bu strategy with *O*-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxy-1*H*-benzotriazole (HOBt) as coupling reagents. Two-hour coupling times were employed. The program module controlling Fmoc deprotection was modified to extend the deprotection time automatically, if necessary. Cleavage from the resin and simultaneous deprotection of the side-chain protecting groups were accomplished by stirring the resin in a soln. of TFA/ethane-1,2-dithiol/H_2O 95 : 2.5 : 2.5 (8 ml) for 3 h. The resin was removed *via* filtration through glass wool and rinsed with additional

TFA. The combined filtrate was concentrated under a stream of N_2. A minimal amount of MeOH was added to dissolve the crude β-peptide, and Et_2O (10 ml) was added to form a white precipitate. The mixture was cooled in an acetone/dry ice bath for 5 min and then centrifuged, and the Et_2O was decanted from the white pellet. A new portion of Et_2O (10 ml) was added, the mixture was stirred with a spatula, cooled in an acetone/dry ice bath for 5 min and centrifuged, and the Et_2O was decanted from the white pellet. Any Et_2O remaining with the white solid was evaporated under a gentle stream of N_2. After purification *via* RP-HPLC as described below, each β-peptide was lyophilized.

β-Peptide **1**. The crude β-peptide was dissolved in HPLC solvent *A*/MeOH 10:1 with sonication and purified by RP-HPLC with a linear gradient from 30 to 42.5% solvent *B* over 25 min. MALDI-TOF-MS: 1384.2 ($[M+H]^+$), 1406.3 ($[M+Na]^+$), 1422.2 ($[M+K]^+$); calc. for $C_{73}H_{134}N_{14}O_{11}$ (*M*): 1383.0.

β-Peptide **2**. The crude β-peptide was dissolved in the HPLC solvent *A* and purified by RP-HPLC with a linear gradient from 31 to 41% solvent *B* over 20 min. MALDI-TOF-MS: 1300.1 ($[M+H]^+$), 1322.1 ($[M+Na]^+$), 1338.1 ($[M+K]^+$); calc. for $C_{67}H_{122}N_{14}O_{11}$ (*M*): 1298.9.

β-Peptide **3**. The crude β-peptide was dissolved in the HPLC solvent *A* and purified by RP-HPLC with a linear gradient from 38 to 48% solvent *B* over 20 min. MALDI-TOF-MS: 1382.0 ($[M+H]^+$), 1404.0 ($[M+Na]^+$), 1420.0 ($[M+K]^+$); calc. for $C_{73}H_{132}N_{14}O_{11}$ (*M*): 1381.0.

CD Spectroscopy. Each lyophilized β-peptide was dissolved in *Millipore* water to create a 2 mg/ml stock soln. The concentration of β-peptide in each stock soln. was determined by UV absorbance. We assume the extinction coefficient of each β-peptide is 1420 $cm^{-1}\,M^{-1}$ at 275 nm, the extinction coefficient of α-tyrosine [27]. The concentration determined by mass (the β-peptide molecular weight was calculated on the basis of one TFA counterion per amine) was within 80% of the concentration determined by UV absorbance for each β-peptide stock soln. Aliquots of the *ca.* 2 mg/ml β-peptide stock solns. were lyophilized, and MeOH or 10 mM Tris (pH 7.2) were added to create 0.1 mM β-peptide solns. CD Spectra were obtained on an *Aviv 202SF* spectrometer at 25° with 1-mm path length cells and 10-s averaging times. The CD signal of the corresponding buffer soln. was subtracted from the CD spectrum of each β-peptide soln. Data were converted to ellipticity (deg cm^2 $dmol^{-1}$) by means of the equation:

$$[\Theta] = \psi M_r / 100lc$$

where ψ is the CD signal in degrees, M_r is the molecular weight divided by the number of residues, l is the path length in decimeters, and c is the concentration in g/ml.

MeOH Titration. Aliquots of a *ca.* 2 mg/ml stock soln. of β-peptide **3** were lyophilized to dryness and combined with *Tris* buffer (10 mM *Tris*, pH 7.2) and/or MeOH to create 0.1 mM solns. of **3** in varying proportions of MeOH and *Tris* buffer. The CD spectra of the solns. of **3** were obtained as described above.

^{1}H-NMR Spectroscopy. Samples were prepared by dissolving the lyophilized β-peptides in H_2O/D_2O 9:1 containing 100 mM CD_3COOH (pH 3.8, uncorrected). Concentrations of the resulting solns. were determined by UV absorbance [27][28]. All spectra were recorded at 600 MHz on a *Varian INOVA* spectrometer at 24°, and H_2O signal suppression was achieved by selective presaturation.

Equilibrium-Sedimentation Anal. Ultracentrifugation (AU). Purified β-peptide **1** was dissolved in aq. 40 mM *Tris*, pH 7.2, to the appropriate concentration. Equilibrium-sedimentation studies were conducted on a *Beckman* model *XL-A* analytical ultracentrifuge at 24°. At each rotor speed, data were acquired every 2–4 h, until three successive spectra were identical (typically, equilibrium was reached after 24 h at a particular rotor speed). Analysis was performed on data with 0.001-cm step size, recorded at several rotor speeds ranging from 25 to 52 krpm. Data for **1** could be fit to a single ideal species model [29]; the quality of the fits were adequate as judged by the randomness of the residuals. The molecular weight of **1** was calculated to be 1387 g mol^{-1}. A partial specific volume of 0.848 ml g^{-1} was calculated based on molecular composition by the method of *Durchschlag* and *Zipper* [30], and the solvent density was determined experimentally to be 0.999338 g L^{-1} (measurement performed on an *Anton Parr DMA 5000* densitometer).

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