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Relationship between side-chain branching and stoichiometry in β^3 -peptide bundles

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Abstract

The stability and stoichiometry of β^3 -peptide bundles is influenced by side-chain identity. β^3 -peptides containing β^3 -homoleucine on one helical face assemble into octamers, whereas those containing β^3 -homovaline form tetramers. From a structural perspective, the side chains of β^3 -homoleucine and β^3 -homovaline differ in terms of both side-chain length and γ -carbon branching. To evaluate the extent to which these two parameters control β^3 -peptide bundle stoichiometry, we synthesized the β^3 -peptide Acid-3Y, which contains β^3 -homoisoleucine in place of β^3 -homoleucine or β^3 -homovaline. Acid-3Y assembles into a stable tetramer whose stability resembles that of the previously characterized Acid-VY tetramer. These results suggest that β^3 -peptide bundle stoichiometry is dominated by the presence or absence of γ -carbon branching on core side chains.

Keywords

Biomaterial; Protein design; Protein folding; Self-assembly

1. Introduction

We previously reported that certain β^3 -peptides self-assemble spontaneously in aqueous solution into discrete, octameric bundles whose kinetic and thermodynamic metrics resemble those of natural proteins.^{1–5} The β^3 -peptide bundles we described undergo cooperative, two-state folding transitions, exchange core amide protons slowly, and exclude hydrophobic dyes; the structures of five such bundles are known in atomic detail.^{1,2,5,6} These initially reported octameric assemblies were designed without the benefit of high-resolution structural data, and were expected to assemble as dimers or tetramers. The observation of a discrete octameric array, with a hydrophobic core composed entirely of solvent-inaccessible β^3 -homoleucine residues, was unanticipated. The structures now available provide the opportunity to study and evaluate the relationship between core residue identity and bundle stoichiometry, kinetic stability, thermodynamic stability, and the cooperativity of unfolding.

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It is well known that the stability and stoichiometry of helical bundle proteins found in nature is influenced by the identities of residues found at the **a** and **d** positions of the heptad repeat.⁷ In complexes characterized by parallel, dimeric coiled coils (such as those in bZIP proteins), these positions are often occupied by leucine residues.^{8,9} Substitution of core leucines in the bZIP protein GCN4 for valine or isoleucine results in three- and four-helix bundles that differ from the dimeric wildtype.⁸ In addition to the effect on bundle stoichiometry, the identities of residues at core positions can also affect the relative orientation of helices; both parallel and antiparallel coiled coils are known.¹⁰ For example, certain GCN4 variants form a tetrameric bundle consisting of parallel helices, in contrast to the antiparallel conformation in most well-characterized four-helix bundles.⁸

The stability and stoichiometry of β^3 -peptide bundles is likewise influenced by side-chain identity. Although β^3 -peptides containing β^3 -homoleucine at the four core positions (indicated by X in Fig. 1a) assemble into octameric bundles, those containing β^3 -homovaline at these positions assemble into tetramers (Fig. 1a).⁴ At the constitutional level, β^3 -homovaline and β^3 -homoleucine differ not only by the presence or absence of a methylene group but also by the extent of branching at the γ -carbon (Fig. 1b). To evaluate the relative contribution of these two parameters to β^3 -peptide bundle stoichiometry, we synthesized the β^3 -peptide Acid-3Y, which is analogous to Acid-1Y and Acid-VY in every respect except for the inclusion of β^3 -homoisoleucine—which is γ -branched but isomeric with β^3 -homoleucine—at all four core positions. Here we report that Acid-3Y assembles into a stable tetramer in solution whose thermodynamic metrics are virtually identical to that of the previously reported Acid-VY tetramer.⁴ These results suggest that β^3 -peptide bundle stoichiometry is controlled in full or in part by the presence or absence of branching at the γ -carbon.

2. Results and discussion

To determine whether Acid-3Y assembled into a bundle with discrete stoichiometry, we first performed sedimentation equilibrium analytical ultracentrifugation (SE-AU) experiments at concentrations of 27.5 μ M, 137.5 μ M and 275 μ M (Fig. 2).

The AU data fit best to a two-state monomer- n -mer equilibrium model. Minimizing the RMSD of the fit as a function of all n returned $n = 4.37$ with an RMSD of 0.00761. Setting $n = 4$ resulted in a comparable fit with an RMSD of 0.00968 (Fig. 2a). Holding the value of n constant to model other potential integer stoichiometries between 2 and 8 resulted in poorer fits with higher RMSD values (0.02675, 0.01115, 0.03172 for $n = 3, 5$ and 8 respectively) and residuals with systematic errors (Fig. 2b–d). These results are consistent with a tetrameric stoichiometry for the Acid-3Y bundle. The $\ln K_a$ value for Acid-3Y derived from the tetramer fit is 40.9 ± 1.5 ($K_a = 5.79 \times 10^{17} \text{ M}^{-3}$), a value close to the $\ln K_a$ values of 38.2 ± 0.8 and 35.1 ± 0.8 ($K_a = 3.89 \times 10^{16} \text{ M}^{-3}$ and $1.75 \times 10^{15} \text{ M}^{-3}$) reported for the tetrameric bundles formed from Zwit-VY and Acid-VY, respectively, which each possess β^3 -homovaline at all four core positions.⁴ Although the data acquired at 137.5 μ M Acid-3Y fit best to a model in which $n = 4.93$, this deviation from a tetramer model is most likely a numerical bias at that particular concentration; the data acquired at 27.5 μ M and 275 μ M Acid-3Y fit best to models in which $n = 4.35$ and 4.40 respectively, corresponding closely to the $n = 4.37$ obtained from the global fit. Overall, the AU data suggest that Acid-3Y exists as a predominantly tetrameric species in solution, although we cannot rule out the contribution of higher order species.

We next used wavelength-dependent circular dichroism (CD) spectroscopy to characterize concentration and temperature-dependent changes in the Acid-3Y secondary structure and provide further evidence for the tetrameric stoichiometry suggested by the SE-AU data (Fig.

3a–c). Acid-3Y underwent a concentration-dependent increase in 3_{14} -helical structure (as judged by the molar residue ellipticity at 212 nm, MRE_{212}) between 20 and 100 μ M (Fig. 3a). This behavior is characteristic of a concentration-dependent equilibrium between a partially structured monomer and a folded oligomer, and mimics the behavior of previously characterized β^3 -peptide bundles, whether octameric (Zwit-1F, Zwit-8L*, Acid-1Y, Zwit-EYYK, Acid-1Y^{LL}, Acid-1Y^{LL}*)^{1,2,5,6} or tetrameric (Zwit-VY and Acid-VY).⁴ A plot of MRE_{212} as a function of Acid-3Y concentration fit to a monomer-tetramer equilibrium model with $\ln K_a = 35.7 \pm 0.8$ (Fig. 3b), in reasonable agreement with the value calculated from the AU data ($\ln K_a = 40.9 \pm 1.5$).¹¹

In addition, the temperature dependent CD spectra of Acid-3Y showed a concentration-dependent increase in T_m , which further suggests self-association (Fig. 3c). The T_m (defined as the maximum in a plot of $\delta MRE_{212} \cdot \delta T^{-1}$) of Acid-3Y at concentrations of 6.25 μ M (63% folded), 12.5 μ M (69% folded) and 25 μ M (80% folded) peptide are 73°C, 82°C and 85°C respectively. These values reflect a thermal stability similar to that of Acid-VY, which at 80 μ M concentration (88% folded) has a T_m of 85°C.⁴ Both Acid-3Y and Acid-VY appear more stable than Acid-1Y, which at 100 μ M concentration (92% folded) exhibits a T_m of 79°C.² Taken together, the CD and SE-AU data indicate that Acid-3Y assembles into a thermally stable tetrameric β^3 -peptide bundle.

We carried out 1-anilino-8-naphthalenesulfonate (ANS) binding experiments to further characterize the hydrophobic packing in Acid-3Y bundles. The fluorescence of ANS increases in a hydrophobic environment, making it a useful probe of access to a protein core. Most well-folded proteins possess a solvent-sequestered core; they bind ANS poorly and do not cause a significant increase in fluorescence.¹² Molten globules, such as α -lactalbumin, bind ANS well, resulting in changes in fluorescence between 60- and 100-fold.¹³ The fluorescence increase of 10 μ M ANS in the presence of 25 μ M Acid-3Y (80% tetramer) relative to ANS alone was 1.3-fold; this value increased only to 2.8-fold in the presence of 400 μ M Acid-3Y (100% tetramer, Figure 3D). The increased fluorescence of ANS upon addition of Acid-VY ranged similarly from 1.1-fold at 25 μ M (91% tetramer) to 1.2-fold at 300 μ M (98% tetramer). The analogous values for the octameric Acid-1Y bundle were 1.1-fold (25 μ M peptide, 74% octamer) and 1.6-fold (400 μ M peptide, 98% octamer). Although these changes in ANS fluorescence are measurable, the values are small, suggesting low accessibility of the tetramer hydrophobic surface. This minimal concentration-dependent increase in ANS fluorescence upon addition of Acid-3Y suggests that it, like all previously reported bundles, possesses a minimally exposed hydrophobic core.

3. Conclusion

In this work we have shown that the β^3 -peptide Acid-3Y, which contains four β^3 -homoisoleucine residues on one 3_{14} -helical face, assembles in aqueous solution into a well-folded, tetrameric bundle. The stability of this bundle, as judged by SE-AU, CD and ANS binding, is virtually identical to that formed by analogous β^3 -peptides containing β^3 -homovaline in place of β^3 -homoisoleucine.⁴ By comparison, analogous peptides containing a β^3 -homoleucine face assemble into octameric bundles, irrespective of side chain identity at most other positions. The difference in oligomeric states between peptides containing β^3 -homoleucine and β^3 -homoisoleucine might initially be surprising, as leucine and isoleucine possess the same molecular weight, and similar van der Waals surface areas and hydrophobic indices at neutral pH.¹⁴ β^3 -homoisoleucine, however, is predicted by molecular mechanics calculations to possess only one favorable rotamer (g^-) in the context of a 3_{14} -helix, due to torsional restraints imposed by branching at the γ -carbon.¹⁵ β^3 -homovaline is also branched at the γ -carbon, and this conformational restraint may guide packing in the

hydrophobic core of β^3 -peptide bundles, leading to a preference for tetrameric stoichiometry.

In the absence of structural information, it is not possible to directly compare the packing arrangements and preferences in the hydrophobic cores of α -peptide and β -peptide bundles. Nevertheless, the observation that replacing leucine with isoleucine in both cases leads to a change in association state highlights the role of side-chain connectivity in dictating bundle stoichiometry. Understanding the effects of side-chain regio- and stereochemistry in both natural and unnatural self-associating systems is a significant challenge for the field of foldamer design.

In summary, the experiments presented herein compelling evidence that substitution at the γ -carbon of a hydrophobic β^3 -amino acid residue plays an important role in defining bundle stoichiometry. Future work will include efforts towards obtaining a high-resolution structure of Acid-3Y and Acid-VY, which will allow us to explore the influence of core residue identity on strand orientation preferences (parallel vs. antiparallel) in tetrameric bundles and help illuminate how the growing collection of protein-like β -peptide bundles might be exploited for higher order functionality.

4. Experimental section

4.1 General methods

Fmoc-protected α -amino acids and Wang resin were purchased from Novabiochem (San Diego, CA). (7-azabenzotriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate (PyAOP) and 1-hydroxy-7-azabenzotriazole (HOAt) were purchased from ChemPep (Wellington, FL) and Oakwood Products (West Columbia, SC). Dimethylformamide (DMF), *N*-methyl morpholine (NMM), trifluoroacetic acid (TFA) and piperidine were purchased from American Bioanalytical (Natick, MA). All other reagents were purchased from Sigma-Aldrich. β^3 -peptides were synthesized with a MARS Microwave Assisted Reaction System (CEM corp., Matthews, NC). Mass spectra were acquired with an Applied Biosystems Voyager-DE Pro MALDI-TOF mass spectrometer (Foster City, CA). Reversed-phase HPLC was performed using a Varian Prostar HPLC and Vydac semi-preparative (C8, 300 Å, 5 μ m, 25 mm $\times$ 250 mm) or analytical (C8, 300 Å, 5 μ m, 4.6 mm $\times$ 150 mm) columns, using water/acetonitrile gradients containing 0.1% TFA. Circular dichroism (CD) spectra were acquired with a Jasco J-810 Spectropolarimeter (Tokyo, Japan) equipped with a Peltier temperature control. Analytical ultracentrifugation (AU) was performed using a Beckman XL-I instrument (Fullerton, CA). ^1H NMR experiments were performed on a Bruker 500 MHz instrument. All CD, AU and ANS binding experiments were performed in buffer A (10 mM NaH_2PO_4 , 200 mM NaCl, pH 7.1).

4.2 β^3 -peptide preparation

Fmoc-protected β^3 -amino acids were synthesized according to methods described by D. Seebach.¹⁶ Acid-3Y was synthesized on a 25 μ mole scale using standard Fmoc solid-phase chemistry and Wang resin loaded with β^3 -homoaspartic acid as previously described.¹⁷ One cycle of peptide elongation consisted of two deprotection steps and one coupling step. Fmoc removal was performed first with 20% piperidine in DMF (50% power at 400 W maximum, 70°C, ramp 2 min, hold 4 min) followed by 2% 1, 8-diazabicyclo[5.4.0]undec-7-ene (DBU) in DMF (50% power at 400 W maximum, 40°C, ramp 2 min, hold 2 min). Coupling reactions were performed using 3 equiv. Fmoc- β^3 -amino acid, PyAOP and HOAt and 8 equiv. diisopropylethylamine (DIEA) in DMF (50% power at 400 W maximum, 60°C, ramp 2 min, hold 6 min). The resin was washed extensively with DMF in between each step. Once the β^3 -peptide sequence was complete, the resin was subjected to a final round of

deprotection, washed extensively with DMF followed by methanol, then dried overnight under nitrogen. Cleavage of the peptide from resin was performed with a cocktail containing 2.5% each of 3,6-Dioxa-1,8-octanedithiol, triisopropylsilane and water in TFA (50% power at 400 W maximum, 38°C, ramp 2 min, hold 30 min). The cleavage cocktail was removed by rotary evaporation and the peptide was dissolved in 20% acetonitrile in water, and lyophilized before purification by HPLC. Semi-preparative HPLC was performed using a linear gradient of 20% to 80% acetonitrile in water containing 0.1% TFA.

Purity and identity of the peptide were assessed using both analytical HPLC and MALDI-TOF. Mass spectra were obtained using peptide samples in a α -cyano-4-hydroxycinnamic acid (CHCA) matrix. The purified peptide was then lyophilized and reconstituted in buffer A for subsequent characterization.

4.3 Sedimentation equilibrium analytical ultracentrifugation

Pure lyophilized Acid-3Y was resuspended in buffer A at three different concentrations (275 μ M, 137.5 μ M and 27.5 μ M) and allowed to reach sedimentation equilibrium in an analytical ultracentrifuge at 4 different speeds (60, 50, 42, and 36 krpm). Centrifugation was performed in an AN 60-Ti 4-hole rotor equipped with six-channel, carbon-epoxy composite centerpieces (Beckman, Fullerton, CA). Absorbance was monitored at 280 nm for samples at 275 μ M and 137.5 μ M concentration, and at 230 nm for samples at 27.5 μ M. Data were collected with a step size of 0.001 cm with scans occurring at 3 hour intervals. Samples were determined to be at equilibrium when no significant changes in radial concentration were observed in 3 successive scans as determined by the program Match within the HeteroAnalysis software suite (available from <http://vm.uconn.edu/~wwwbiotc/uaf.html>).

Data were fit to a monomer- n -mer equilibrium model using the software HeteroAnalysis (see above for reference). Fixed parameters were as follows: $MW_{\text{monomer}} = 1682$ Da; $v\text{-bar} = 0.7876$ cm³/g (calculated according to Durchschlag and Zipper¹⁸); $d = 1.00674$ g/ml; $\epsilon_{280} = 2520$ cm⁻¹M⁻¹; $\epsilon_{230} = 9680$ cm⁻¹M⁻¹ and $n = 4$. Fitted parameters were as follows: $\ln K_a = 40.9 \pm 1.5$; RMSD = 0.00968 and baseline deviation <0.02.

4.4 Circular dichroism spectroscopy

Wavelength-dependent CD spectra were obtained in triplicate for concentrations of Acid-3Y ranging from 20 μ M to 300 μ M at 25°C in continuous scan mode with data pitch = 0.5 nm, scanning speed = 50 nm/min, response time = 4 s, band width = 0.5 nm and 3 accumulations. The concentration dependence of MRE_{min} was determined by least-squares fitting of the total peptide monomer concentration as a function of experimental MRE using the equation below in Kaleidagraph (Synergy Software, Reading, PA).¹⁹ Fixed parameters were $MRE_{\text{Mon}} = 0$ deg•cm²•dmol⁻¹ and $n = 4$.

$$[\text{Peptide}]_{\text{Total}} = \left\{ \frac{(MRE_{\text{Exp}} - MRE_{\text{Mon}})(1/K_a)}{n(MRE_{\text{Nmer}} - MRE_{\text{Mon}}) \left[1 - \left(\frac{MRE_{\text{Exp}} - MRE_{\text{Mon}}}{MRE_{\text{Nmer}} - MRE_{\text{Mon}}} \right)^n \right]} \right\}^{1/(n-1)}$$

Temperature-dependent CD spectra were taken by monitoring the change in MRE at 212 nm between 5°C and 95°C, using the variable temperature module provided with the instrument, with data pitch = 1°C, delay time = 5 s, temperature slope = 1°C/min, response time = 4 s and band width = 1 nm. The first derivatives of the temperature-dependent CD spectra for various concentrations of Acid-3Y were computed and the T_m values correspond to the temperatures at which maximum $\delta MRE/\delta T$ occur.

4.5 ANS binding studies

Stock solutions of 800, 400, 200, 100, 50, 25, and 12.5 μM Acid-3Y and 20 μM ANS were prepared by serial dilution in buffer A. The peptide solutions were then mixed with the stock ANS solution at a 1:1 ratio (80 μl of each) to give final concentrations of 400, 200, 100, 50, 25, 12.5 and 6.25 μM Acid-3Y and 10 μM ANS. Fluorescence intensity (counts/s) measurements were made using a Photon Technology International (Lawrenceville, NJ) Quantamaster C-60 spectrofluorimeter at 25°C in a 1 cm path length quartz cuvette. The samples were excited with 350 nm light (4 nm slit width) and fluorescence emission was measured at 1 nm intervals between 400 and 600 nm. I/I_0 was calculated by dividing the maximum ANS fluorescence intensity at each concentration of peptide (I) by the fluorescence intensity in the absence of peptide (I_0).

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Fig. 1. (a) Helical net and linear representations of β^3 -peptides discussed in this work and (b) comparison between β^3 -hLeu, β^3 -hVal and β^3 -hIle side chains. Letters represent standard amino acid abbreviations; O = ornithine.

Fig. 2. Self-association of Acid-3Y monitored by sedimentation equilibrium analytical ultracentrifugation (SE-AU) and fit to a monomer- n -mer equilibrium model. Results are shown for [Acid-3Y] = 27.5 μ M. Data points are represented as open circles with colors corresponding to the indicated speeds. Black lines indicate fits to (a) $n = 4$; (b) $n = 3$; (c) $n = 5$; and (d) $n = 8$ models.

Fig. 3.

Biophysical characterization of the Acid-3Y bundle. (a) Wavelength-dependent circular dichroism (CD) spectra of Acid-3Y at concentrations between 20 and 100 μM ; (b) Plot of MRE_{212} versus [Acid-3Y] fit to an ideal monomer-tetramer equilibrium (red line); (c) Plot of $\Delta\text{MRE}/\Delta T$ as a function of T for five different concentrations of Acid-3Y; (d) Ratio of ANS fluorescence (I/I_0) in the presence of the indicated concentration of Acid-1Y (octamer), Acid-VY (tetramer) and Acid-3Y (tetramer). The concentration of ANS was 10 μM .