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Induction of Protein-like Molecular Architecture by Self-assembly Processes

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Abstract—One of the most intriguing self-assembly processes is the folding of peptide chains into native protein structures. We have developed a method for building protein-like structural motifs that incorporate sequences of biological interest. A lipophilic moiety is attached onto an N^{α}-amino group of a peptide chain, resulting in a 'peptide-amphiphile'. The alignment of amphiphilic compounds at the lipid–solvent interface is used to facilitate peptide alignment and structure initiation and propagation. Peptide-amphiphiles containing potentially triple-helical structural motifs have been synthesized. The resultant head group structures have been characterized by circular dichroism and NMR spectroscopies. Evidence for a self-assembly process of peptide-amphiphiles has been obtained from: (a) circular dichroism spectra and melting curves characteristic of triple-helices, (b) one- and two-dimensional NMR spectra indicative of stable triple-helical structure at low temperatures and melted triple-helices at high temperatures, and (c) pulsed-field gradient NMR experiments demonstrating different self-diffusion coefficients between proposed triple-helical and non-triple-helical species. The peptide-amphiphiles described here provide a simple approach for building stable protein structural motifs using peptide head groups.

Introduction

The creation of protein-like molecular architecture is desired for studies of protein folding and stability and the design of proteins geared for specific functions. Many initial studies focused on the ability of peptides to self-assemble and form protein-like structures. For example, in 1968 Sakakibara et al. demonstrated synthetic peptides of the sequence $(Pro\text{-}Pro\text{-}Gly)_n$, where $n = 10$ or 20, could self-assemble to form collagen-like triple-helices.[1] Peptide self-assembly has since been used to create α-helical bundle and β-sheet proteins.[2]

Unfortunately, peptide self-assembly is not a particularly easy process to regulate. Nondesired molecular assemblies may occur (such as formation of a mixture of α-helical dimers, trimers, tetramers, etc.) or peptide chains may simply form large aggregates of unknown order. Controlling peptide interaction could minimize these difficulties. One approach for controlled peptide assembly is to incorporate moieties on the end of peptide chains, and use properties of these moieties to drive peptides to interact in a specific fashion. The advantages of this approach are several. The proper number of peptides will associate, eliminating undesired aggregates. The moieties themselves may act as templates, which could initiate and stabilize structure formation. For example, lattice statistical mechanics has predicted that surfaces (i.e., templates) would enhance secondary structure formation by minimizing unfolded states.[3,4] Surfaces have been shown to induce peptide secondary structures, either α-helical or β-sheet.[5–7] The template effect has been documented for synthetic four α-helical bundles, as the α-helicity of the individual peptide chains was greatly enhanced upon incorporation onto a template containing several other α-helical peptide sequences.[8–10] Thus, one method for creating distinct protein-like structures is to use directed self-assembly to create a peptide-template.

One well documented controlled self-assembly approach has utilized chelators attached to peptides and appropriate metals to initiate interstrand association. A triple α-helical bundle protein has been created by incorporating 5-carboxy-2,2′-bipyridine onto the 15-residue peptide Gly-Glu-Leu-Ala-Gln-Lys-Leu-Glu-Gln-Ala-Leu-Gln-Lys-Leu-Ala-NH_2 and using Co(II), Ni(II), or Ru(II) complexation.[11] The trimeric bundle was confirmed by size-exclusion chromatography and mass spectrometry (MS). The circular dichroism (CD) spectrum of the Ru(II) complexed-peptide indicated a left-handed supercoiled α-helical structure. GdnHCl denaturation experiments gave free energy of folding values of $\Delta G(H_2O)_{Ru} = -1.86$ kcal/mol and $\Delta G(H_2O)_{Ni} = -1.93$ kcal/mol. A triple α-helical bundle protein has

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also been created by incorporating 2,2′-bipyridine-4,4′-dicarboxylic acid onto the 15 residue peptide Ala-Glu-Gln-Leu-Leu-Gln-Glu-Ala-Glu-Gln-Leu-Leu-Gln-Glu-Leu-NH_2 and complexing with Fe^{2+}.[12] The individual strands, designated 'pepy', were 35% α-helical, while the resultant protein [$Fe^{II}(pepy)_3$] was 85% α-helical. $Fe^{II}(pepy)_3$ was found to exist in four stereoisomeric states.[13] A Ru(II)-complexed four α-helix bundle has been constructed by incorporating a pyridyl functionality onto the N-terminus of the 15 residue helix-forming peptide Gly-Leu-Ala-Gln-Lys-Leu-Leu-Glu-Ala-Leu-Gln-Lys-Ala-Leu-Ala-NH_2.[14] The assembled four-helix bundle was confirmed by size-exclusion chromatography and MS. The complex was >90% α-helical by CD spectroscopy. GdnHCl-induced denaturation of the bundle was highly cooperative, with $\Delta G(H_2O) = -5.6$ kcal/mol.

Rather than using chelators and metal complexation, we envisioned the construction of a novel peptide-amphiphile for directed self-assembly. A peptide 'head group' would have the propensity to form a distinct structural element, while a hydrophobic 'tail' would serve to align the peptide strands and induce secondary and tertiary structure formation as well as providing a hydrophobic surface for self-association. Our initial efforts have focused on this noncovalent, self-assembly approach for building a collagen-like structural motifs.[15–19] The present article describes the synthesis and characterization of peptide-amphiphile collagen-like molecular architecture.

Results and Discussion

Synthesis of peptide-amphiphiles

The design of peptide-amphiphiles requires that long chain lipophilic moieties associate by hydrophobic forces to drive structure initiation in the peptide portion of the molecule and/or stabilize protein-like structure. We initially examined the use of dialkyl ester chains for creating peptide-amphiphiles (Fig. 1). As described by Kunitake,[20] design of synthetic lipids requires consideration of four building blocks: tail, linker/connector, spacer, and head group. Our design featured C_{12}, C_{14}, and C_{16} dialkyl tails, a Glu linker, a $-(CH_2)_2-$ spacer, and a collagen-model peptide head group.[15,16] Construction of tail compounds first involves the acid-catalyzed condensation of Glu with the appropriate fatty acid alcohol to form the dialkyl ester of Glu (*p*-toluenesulfonate salt).[15] The free amino group of Glu is then treated with succinic anhydride and triethylamine to create a free carboxylic acid. The dialkyl ester tail may then be acylated to the resin-bound peptide using *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HBTU)[16,17] or converted to a 4-nitrophenyl ester and used for coupling.[15]

The peptide-amphiphile solid-phase method was based upon 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. The resin-bound peptide composition (before addition of the lipophilic tail) was confirmed by Edman degradation sequence analysis.[21] Couplings involving dialkyl ester tails did not appear to be particularly sterically hindered; a typical reaction required 4 h. Mild peptide-resin cleavage conditions using trifluoroacetic acid (TFA) did not affect the tail integrity. Following cleavage, care was required during ether extraction of scavengers, as long chain dialkyl ester peptide-amphiphiles could enter the ether layer. Purification of dialkyl ester peptide-amphiphiles was best accomplished by C_4 reversed-phase high performance liquid chromatography (RP-HPLC) using a gradient of H_2O–isopropanol containing 0.05% TFA.[16] Peptide-amphiphile homogeneity was evaluated by C_{18} RP-HPLC using a gradient of H_2O–acetonitrile containing 0.05% TFA.[15] Final characterization of the purified peptide-amphiphile was achieved readily by NMR spectroscopy[15] and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry.[16]

Subsequent studies have used monoalkyl chains to create peptide-amphiphiles (Fig. 1). Synthesis is simplified greatly, as commercially available monoalkyl acids are coupled directly to peptide-resins.[18] These peptide-amphiphiles have no linker or spacer per se. We have created a series of C_6, C_8, C_{10}, C_{12}, C_{14}, and C_{16} monoalkyl peptide-amphiphiles.[19] Monoalkyl peptide-amphiphiles are less prone to losses during either the ether extraction step or RP-HPLC due to the decreased hydrophobicity of this class of compounds compared to dialkyl ester peptide-amphiphiles.

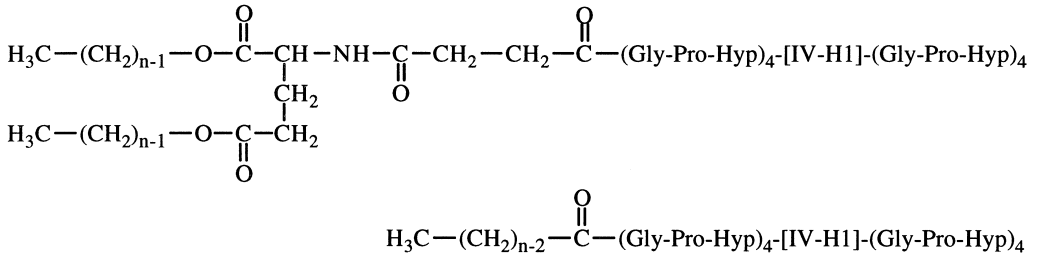

Figure 1. General structures of dialkyl ester and monoalkyl peptide-amphiphiles.

Our peptide-amphiphiles have been constructed using mono- or dialkyl ester tail compounds. Other research groups have created peptide-amphiphiles using tail compounds such as dialkyl amides,[22,23] phospholipids,[24–27] fluorescent phospholipids,[28] and tripalmitoyl-Cys [$(Pam)_3$Cys].[29] Lipophilic compounds have typically been incorporated by solution-phase[23–25,28–30] or chemoselective ligation[26,27] methods. To our knowledge, only one laboratory has reported the incorporation of a branched lipid (a 1,2-dimyristoyl-*sn*-glycerol derivative) onto a peptide by solid-phase methods.[31] The hydrophobic nature of peptide-amphiphiles has resulted in a variety of approaches being utilized for compound purification and characterization. These include traditional purification methods, such as Sephadex LH-20 gel filtration chromatography,[24,29,31] silica gel medium pressure liquid chromatography,[23] normal phase liquid chromatography,[26] or extraction/recrystallization.[28,30] Peptide-amphiphiles containing short peptide head groups (5–7 amino acids) have been purified by HPLC using cyanopropyl, C_8, or C_{18} columns.[25,31,32] We found that for longer peptide head groups and either dialkyl or monoalkyl tails, C_4 RP-HPLC purification was effective. The mobile phase required isopropanol instead of acetonitrile used with other peptide-amphiphiles.[25,32]

Peptide-amphiphiles have been characterized primarily by fast-atom bombardment (FAB) MS and/or NMR spectroscopy.[23,27,29–32] While we have found NMR spectroscopy to be an effective characterization method,[16] our peptide-amphiphiles were too large for FAB-MS analysis. Instead, MALDI-MS was found a reproducible characterization tool.[16,19]

Protein-like structure of peptide-amphiphiles

To evaluate the head group structures formed by peptide-amphiphiles, several different biophysical approaches were utilized. The overall structure of the peptide-amphiphile was examined by CD spectroscopy, the thermal stability of peptide-amphiphiles was determined by CD melting curves, one- and two-dimensional NMR spectroscopy of peptide-amphiphiles was used to evaluate the environment of specific residue side-chains, and pulsed-field gradient (PFG) NMR experiments were used to determine peptide-amphiphile diffusion coefficients. For our studies, the α1(IV) 1263–1277 collagen sequence Gly-Val-Lys-Gly-Asp-Lys-Gly-Asn-Pro-Gly-Trp-Pro-Gly-Ala-Pro (IV-H1) was combined with alkyl chains to create potentially collagen-like peptide-amphiphiles (Fig. 1).[16,17]

CD analysis was based upon well-known properties of the collagen triple-helix. The collagen triple-helix consists of three polypeptide chains, each in an extended, left-handed polyPro II-like helix, which are staggered by one residue and then supercoiled along a common axis in a right-handed manner.[33,34] Collagens in triple-helical conformation exhibit a CD spectrum similar to a poly-Pro II helix, with positive ellipticity from $\lambda = 215$–240 nm.[35] At 25 °C, (Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$ was found to exhibit this characteristic CD spectrum.[16,17] For (Gly-Pro-Hyp)$_4$-[IV-H1] and [IV-H1]-(Gly-Pro-Hyp)$_4$ a small magnitude of positive ellipticity at $\lambda = 225$ nm was observed, while the IV-H1 peptide did not show any positive ellipticity at this wavelength.[16,17] Of the C_{12} dialkyl chain peptide-amphiphiles, $(C_{12})_2$-Glu-C_2-[IV-H1] displayed a CD spectrum similar to that of IV-H1 (no positive ellipticity at $\lambda = 225$ nm), while $(C_{12})_2$-Glu-C_2-(Gly-Pro-Hyp)$_4$-[IV-H1], $(C_{12})_2$-Glu-C_2-[IV-H1]-(Gly-Pro-Hyp)$_4$, and $(C_{12})_2$-Glu-C_2-(Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$ showed a large magnitude of positive ellipticity at $\lambda > 220$ nm.[16,17] Similarly, the monoalkyl peptide-amphiphile C_6-(Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$ exhibited a CD spectra typical of triple-helical conformation at 25 °C.[19] The ellipticity per residue values indicate a maximal ordered structure for $(C_{12})_2$-Glu-C_2-(Gly-Pro-Hyp)$_4$-[IV-H1], $(C_{12})_2$-Glu-C_2-[IV-H1]-(Gly-Pro-Hyp)$_4$, $(C_{12})_2$-Glu-C_2-(Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$, and C_6-(Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$. It appears that all residues in these peptide-amphiphiles are in triple-helical conformation.

A triple-helical assembly can be distinguished from a simple, non-intercoiled poly-Pro II structure by its thermal denaturation behavior. Triple-helical melts are highly cooperative.[35] The thermal transitions of peptides and peptide-amphiphiles were monitored by measuring molar ellipticity at $\lambda = 225$ nm as a function of increasing temperature. Amongst the peptides, only (Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$ gave a typical sigmoidal transition associated with the transformation of triple-helical to single-stranded structure. The first derivative of the melting curve gave a melting temperature (T_m) value of 35.6 °C (Table 1).[16,17] [IV-H1]-(Gly-Pro-Hyp)$_4$ showed a small magnitude of positive ellipticity which decreased nearly linearly from 5–20 °C then flattened out, as did (Gly-Pro-Hyp)$_4$-[IV-H1].[16,17] These two peptides do not appear to be in a triple-helical conformation. Thermal denaturation studies of the dialkyl peptide-amphiphiles showed molar ellipticities that decreased very gradually between 10–30 °C, then more markedly starting at around 30–40 °C, with some traces of positive CD detectable up to 80 °C.[16,17] The midpoint of the transition (T_m) for $(C_{12})_2$-(Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$ was 71.2 °C (Table 1). The melting curve was fully reversible upon cooling. Although the changes in ellipticity were large, thermal transitions for

Table 1. T_m values for peptide and peptide-amphiphile triple helix $\Longleftrightarrow$ coil transitions

Peptide or peptide-amphiphile	T_m(°C)
[IV-H1]	NTH[a]
(Gly-Pro-Hyp)$_4$-[IV-H1]	NTH[a]
[IV-H1]-(Gly-Pro-Hyp)$_4$	NTH[a]
(Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$	35.6
C_6-(Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$	42.2
C_8-(Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$	45.6
C_{10}-(Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$	51.3
C_{12}-(Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$	55.0
C_{14}-(Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$	63.1
C_{16}-(Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$	69.8
$(C_{12})_2$-Glu-C_2-(Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$	71.2
$(C_{12})_2$-Glu-C_2-(Gly-Pro-Hyp)$_4$-[IV-H1]	$\sim 50 \pm 5$
$(C_{12})_2$-Glu-C_2-[IV-H1]-(Gly-Pro-Hyp)$_4$	$\sim 50 \pm 5$

[a]NTH = not triple-helical.

the dialkyl peptide-amphiphiles were broad. The thermal stability of C_n-(Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$ monoalkyl peptide-amphiphiles, where $n = 6$, 8, 10, 12, 14, or 16, were similarly studied by monitoring ellipticity at $\lambda = 225$ nm as a function of increasing temperature.[19] All peptide-amphiphiles gave sharp sigmoidal transitions associated with the transformation of triple-helical to single-stranded structure. The melting curves were fully reversible upon cooling. T_m values were determined from the first derivative of the melting curves, and found to increase with monoalkyl tail chain length (Table 1). These observations suggest that the peptide-amphiphile structures consist of triple-helically packed polyPro II-like helices, and that increasing lipid tail chain length enhances the stability of this assembly.

^{1}H NMR spectroscopy was utilized to further characterize peptide and peptide-amphiphile structure. The Pro and Hyp spin systems in total correlation spectroscopy (TOCSY) were identified by the lack of amide protons and reference to the chemical shifts of the side-chain protons from other collagen-like peptides (Fig. 2).[36–38] The chemical shift of the Pro and Hyp side-chain protons is sensitive to their conformation.[36–38] For our peptides and peptide-amphiphiles, there are Pro residues that are found both surrounding and within the IV-H1 sequence while Hyp residues surround the IV-H1 sequence. At 10 °C, the relatively few cross peaks found in the Pro/Hyp region of the ^{1}H NMR spectra indicate that the Pro and Hyp residues of (Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$ are in a limited number of conformations (Fig. 2),[16] as expected for a compound with an ordered structure. The cross peaks found at 4.6 ppm[16] are comparable to those observed for the triple-helical, template bound peptide Kemp triacid-[Gly-(Gly-Pro-Hyp)$_3$-NH$_2$]$_3$.[39] The spectra of (Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$ at 50 °C shows additional cross peaks at 4.85 ppm, indicating less ordered conformation at higher temperature.[16] Some of these additional cross peaks are consistent with the multiple states that exist for the Pro residues within the IV-H1 sequence when in a non-triple-helical conformation.[40] After the (Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$ peptide is lipidated with a C_{12} tail, similar NMR spectra are obtained.[16] For example, $(C_{12})_2$-Glu-C_2-(Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$ at 25 °C shows a few well defined cross peaks, indicating ordered conformation of the peptide-amphiphile. Consistent with our CD observations, the NMR spectra of $(C_{12})_2$-Glu-C_2-(Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$ at 80 °C indicates more disorder than at 25 °C.[16] Additional cross peaks are seen at 4.85 ppm, in similar fashion to (Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$ at 50 °C. Overall, the CD and NMR spectra of the (Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$ peptide and the $(C_{12})_2$-Glu-C_2-(Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$ peptide-amphiphile suggest that both can spontaneously form a well-ordered poly-Pro II-like triple-helical structure.

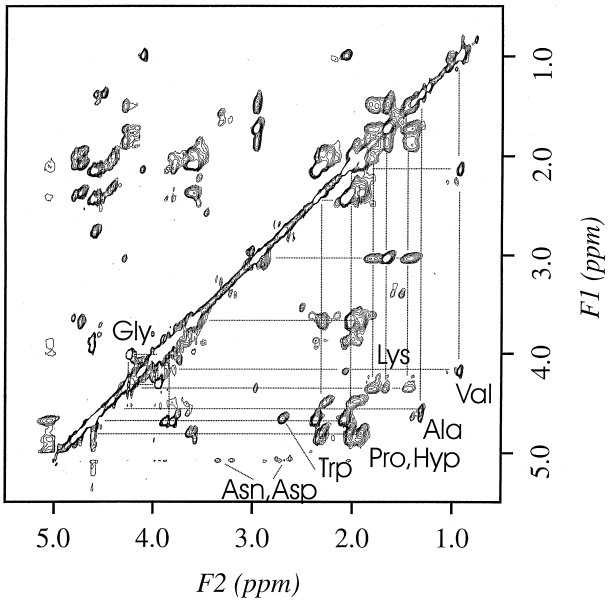

Figure 2. TOCSY spectrum of (Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$ in D_2O at 10 °C. Peptide concentration was 5 mM. For the preliminary assignment of amino acid residues present in the peptide, data from additional TOSCY experiments (D_2O:H_2O,1:9) and NOESY experiments were used. The Pro/Hyp region was identified from the lack of an amide ^{1}H signal and the shown connectivity pattern in TOCSY. Reprinted with permission from *J. Am. Chem. Soc.* **1996**, *118*, 12518, American Chemical Society.[16]

Additional one-dimensional NMR experiments were performed in D_2O solution. The resonance lines from labile protons are replaced in D_2O, so that the ^{1}H NMR spectrum will contain only the resonance lines of the carbon-bound protons.[41] In the case of Trp residues, resonances in the 7.0–8.0 ppm region result from the protons attached to the 2, 4, 5, 6, and 7 positions of side-chain indole ring.[41] Thus, spectra of the investigated peptides and peptide-amphiphiles in the 7.0–8.0 ppm region will result from a unique Trp residue found within the IV-H1 sequence. The ^{1}H NMR spectrum of (Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$ at 40 °C showed prominent resonances at ~7.70, 7.68, 7.51, 7.50, 7.30, 7.26, and 7.18 ppm.[19] The CD spectrum indicates a non-triple-helical conformation. However, the ^{1}H NMR spectrum of (Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$ at 10 °C is substantially different than that at 40 °C. In particular, there are prominent signals in the 7.71–7.78 ppm and 7.33–7.40 ppm regions.[19] These resonances are not due to a simple change in temperature, as the 10 °C spectrum of [IV-H1]-(Gly-Pro-Hyp)$_4$ does not contain such signals. Since the CD data indicate that (Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$ is triple helical at 10 °C, the additional resonances appear to be derived from the Trp side-chain in a triple-helical conformation. Increasing the temperature to 25 °C, which results in a decrease in triple-helicity of (Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$, causes a decrease in the intensities of the 7.71–7.78 ppm and 7.33–7.40 ppm signals. The ^{1}H NMR spectrum peaks that decrease with increasing temperature may thus represent Trp within a triple-helical conformation.

The ^{1}H NMR spectra of C_6-(Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$ at 10 and 25 °C exhibit resonance lines at similar chemical shifts as that of (Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$ at 10 °C.[19] Comparing the peptide-amphiphile to (Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-

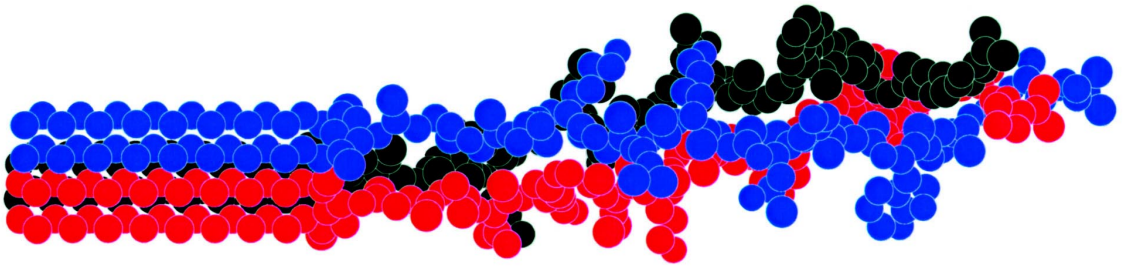

Figure 3. Model of a triple-helical peptide-amphiphile. Individual peptide strands are represented by red, blue, or black. In this model, the dialkyl chains are tightly packed due to hydrophobic interactions, while the peptide strands initiate triple-helix formation in the proper orientation and register. At the present time, it cannot be determined if triple-helical folding or dialkyl chain association is the initial event, or if both occur simultaneously. It seems most likely that triple-helical folding occurs first, as (1) triple-helices nucleate in a zipper-like fashion from the C- to N-terminus[48,49] and (2) this would allow for the proper register of the individual chains.

Pro-Hyp)$_4$ at the same temperatures (either 10 or 25 °C) indicates that the peaks corresponding to triple-helical conformation are much stronger in the former compound. The peaks at 7.64–7.71 ppm, which correspond to monomeric conformation, are very weak in the NMR spectrum of C_6-(Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$ at 10 °C.[19] This result suggests that at 10 °C C_6-(Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$ is primarily triple-helical. The thermal transitions evaluated from the NMR spectra and the CD melting curves are in agreement.

The CD and ^{1}H NMR experiments have indicated that some NMR spectral signals arising from the side chain of Trp are associated with triple-helical conformational changes. If this is the case, than self-diffusion coefficients calculated from triple-helical peaks are expected to be smaller than self-diffusion coefficients calculated from monomeric peaks.[37] The self-diffusion coefficient (D) was subsequently measured using PFG NMR spectroscopy and varying the gradient strength. D was initially measured for the peaks occurring at 7.74 ppm, potentially corresponding to triple-helical conformation, and 7.70 ppm, potentially corresponding to monomeric conformation.[19] The D values calculated at 7.74 and 7.70 ppm are 0.69×10^{-6} cm^2/sec and 1.02×10^{-6} cm^2/sec, respectively.[19] Thus, D is lower for the triple-helical peak compared with the monomeric peak.

Conclusions

CD and NMR spectroscopic studies have provided strong evidence that (1) peptide-amphiphiles are in a predominantly triple-helical conformation at temperatures below 30 °C, (2) the [IV-H1] region within peptide-amphiphiles are in a triple-helical environment, and (3) there exist two peptide-amphiphile states, one at low temperature and one at high temperature, of different diffusion coefficients. The evidence for triple-helicity is based upon (1) CD spectra characteristic of triple-helices, (2) sigmoidal melting curves for the transition from triple-helix to monomeric state, (3) two-dimensional NMR spectra of the Pro and Hyp side-chains within and/or surrounding the [IV-H1] site correlating cross-peaks with melting transitions observed by CD spectroscopy, (4) one-dimensional ^{1}H NMR spectra correlating shifts in Trp proton signals with melting transitions observed by CD spectroscopy, and (5) PFG NMR determination of self-diffusion coefficients for two distinct species correlated to the melting transitions observed by CD spectroscopy.

The peptide-amphiphiles described herein provide a simple approach for building stable protein structural motifs using peptide head groups. The lipid hydrophobic interactions of peptide-amphiphiles (Fig. 3) exert a significant influence on triple-helical structure formation and stabilization. For example, the difference in the denaturation temperatures between the structured C_n-(Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$ peptide-amphiphiles is about 2.75 °C per $-CH_2-$.[18] The tight alignment of the N-terminal amino acids achieved through the association of the lipid part of the molecule in a monolayer could be a general tool for initiation of peptide folding.

One of the most intriguing features of this system is the possible formation of stable lipid films on solid substrates, or the use of the novel amphiphiles in bilayer membrane systems, where the lipid tail serves not only as a peptide structure-inducing agent but also as an anchor of the functional head group in the lipid assembly. One can mix peptide-amphiphiles with vesicle-forming lipids (such as dilauryl phosphatidylcholine) to form stable mixed vesicles with collagen-model, triple-helical peptide head groups. Vesicles featuring collagen coatings have already been shown to be advantageous for targeted drug delivery.[42] The peptide-amphiphile system potentially offers great versatility with regard to head and tail group composition and overall geometries and macromolecular structures.

Experimental

Synthesis of peptide-amphiphiles

All standard peptide synthesis chemicals and solvents were analytical reagent grade or better and purchased from Applied Biosystems, Inc. (Foster City, CA) or Fisher Scientific (Pittsburgh, PA). Fmoc-4-(2′,4′-dimethoxyphenylaminomethyl)phenoxy resin (substitution level = 0.46 mmol/g) was purchased from Novabiochem

(La Jolla, CA). All Fmoc-amino acid derivatives were from Novabiochem and are of L-configuration. 1-Hydroxybenzotriazole (HOBt) was purchased from Novabiochem, HBTU from Richelieu Biotechnologies (St. Hyacinthe, Quebec), and *N*,*N*-diisopropylethylamine (DIEA) from Fisher Scientific.

Construction of dialkyl tail compounds first involves the acid-catalyzed condensation of Glu with the appropriate fatty acid alcohol to form the dialkyl ester of Glu (*p*-toluenesulfonate salt).[15] For example, hexadecanol (44.85 g, 0.185 mol) and Glu (13.6 g, 0.092 mol) are mixed with 21.0 g (0.102 mol) of *p*-toluenesulfonate in toluene, and the mixture is heated until an equimolar amount of water is recovered in a Dean–Stark trap. The toluene is removed, and the product recrystallized from acetone in ~80% yield. The free amino group of Glu is then treated with succinic anhydride and triethylamine to create a free carboxylic acid. 1′,3′-Dihexadecyl L-Glu (20 g, 26 mmol) and triethylamine (5.5 mL, 39 mmol) are dissolved in tetrahydrofuran:$CHCl_3$ (1:1), and 3.9 g (39 mmol) of succinic anhydride is added under stirring. The mixture is kept for 4 h at 30 °C. The product obtained after removal of the solvent is recrystallized from acetone and ethanol in ~94% yield. The dialkyl tail can then be used directly for solid-phase synthesis, or is converted to a 4-nitrophenyl ester. Dihexadecyl *N*-succinyl-L-Glu (6.90 g, 9.9 mmol) and 4-nitrophenol (1.65 g, 11.9 mmol) are dissolved in CH_2Cl_2, and 2.05 g (9.9 mmol) of *N*,*N*-dicyclohexylcarbodiimide as well as a catalytic amount (80 mg) of (dimethylamino)pyridine is added to the reaction mixture on an ice bath. The reaction is continued for 2 h on the ice bath and for 24 h at room temperature. The formed dicyclohexylurea is filtered off, and the reaction product precipitated with cold dry ethanol in a yield of 85%. The two other dialkyl ester tail precursors, 1′,3′-ditetradecyl *N*-[*O*-(4-nitrophenyl)succinyl]-L-Glu [designated $(C_{14})_2$-Glu-C_2-pNp] and 1′,3′-didodecyl *N*-[*O*-(4-nitrophenyl)succinyl]-L-Glu [designated $(C_{12})_2$-Glu-C_2-pNp], are synthesized as described above for 1′,3′-dihexadecyl *N*-[*O*-(4-nitrophenyl)succinyl]-L-Glu [designated $(C_{16})_2$-Glu-C_2-pNp]. The C_{14} and C_{12} tails are prepared using 1-tetradecyl alcohol and 1-dodecyl alcohol, respectively. The monoalkyl chains hexanoic acid [CH_3-$(CH_2)_4$-CO_2H, designated C_6], octanoic acid [CH_3-$(CH_2)_6$-CO_2H, designated C_8], decanoic acid [$CH_3(CH_2)_8$-CO_2H, designated C_{10}], dodecanoic acid [CH_3-$(CH_2)_{10}$-CO_2H, designated C_{12}], tetradecanoic acid [CH_3-$(CH_2)_{12}$-CO_2H, designated C_{14}], and palmitic acid [CH_3-$(CH_2)_{14}$-CO_2H, designated C_{16}] were purchased from Aldrich.

Peptide-resin assembly is performed by Fmoc solid-phase methodology.[21,43,44] Peptide-resins are characterized by Edman degradation sequence analysis as described previously for embedded (noncovalent) sequencing.[21] Peptide-resins are then lipidated with the appropriate $(C_n)_2$-Glu-C_2[15,16] or CH_3-$(CH_2)_{n-2}$-CO_2H[19] tail. Cleavage and side chain deprotection of peptide-amphiphile-resins proceeds for 1 h using either ethanedithiol:thioanisole:phenol:H_2O:trifluoroacetic acid (TFA) (2.5:5:5:5:82.5) or H_2O:TFA (1:19).[45,46] Peptide-amphiphile cleavage solutions are extracted with methyl *t*Bu ether prior to purification.

Peptide purification and analysis

Preparative RP-HPLC purification is performed on a Rainin AutoPrep System. Peptide-amphiphile purification is achieved using a Vydac 214TP152022 C_4 column (15–20 μm particle size, 300 Å pore size, 250×22 mm) at a flow rate of 10 mL/min. The elution gradient is 55–90% B in 20 min where A is 0.05% TFA in H_2O and B is 0.05% TFA in isopropanol. Detection is at 229 nm. Analytical RP-HPLC is performed on a Hewlett–Packard 1090 Liquid Chromatograph equipped with a Hypersil C_{18} column (5 μm particle size, 120 Å pore size, 200×2.1 mm) a flow rate of 0.3 mL/min. The elution gradient is 0–60% B in 45 min where A is 0.05% TFA in H_2O and B is 0.05% TFA in acetonitrile. Diode array detection is at λ = 220, 254, and 280 nm.

Edman degradation sequence analysis is performed on an Applied Biosystems 477A Protein Sequencer/120A Analyzer. MALDI-MS is performed on a Hewlett–Packard G2025A laser desorption time-of-flight mass spectrometer using a sinnapinic acid matrix.[15,16] Peptide-amphiphile samples were dissolved in either H_2O or H_2O-acetonitrile containing 0.05% TFA.

Circular dichroism spectroscopy

CD spectra were recorded over the range of λ = 190–250 nm on a JASCO J-710 spectropolarimeter using a thermostated 0.1 mm quartz cell. Thermal transition curves are obtained by recording the molar ellipticity ([θ]) in the range of 10–80 °C at λ = 225 nm. The peptide-amphiphile concentration is 0.5 mM in H_2O at 25 °C.

NMR spectroscopy

NMR spectra were acquired on a 500 MHz Bruker AMX-500 spectrometer at 10, 25, 50, and/or 80 °C. Freeze-dried samples for NMR spectroscopy were dissolved in D_2O or D_2O:H_2O (1:9) at peptide and peptide-amphiphile concentrations of 3–5 mM at least 48 h prior to experiments. Two dimensional TOCSY and nuclear Overhauser effect spectroscopy (NOESY) are performed with 256 t1 increment and 1024 complex data points in the t2 dimension. TOCSY spectra are obtained at mixing times of 40–150 ms. NOESY spectra are obtained at mixing times of 60–250 ms. The spectral widths are 6024 Hz in both dimensions. Self-diffusion coefficients (D) were measured by PFG NMR as described by Gibbs and Johnson.[47] Experiments were performed using a PFG duration of 6 ms, 52.4 ms between PFG pulses, and gradient strengths of 4.12, 8.24, 12.36, 16.48, 20.60, 24.72, 28.84, and 32.96 Gauss/cm. Since samples were dissolved in D_2O, water suppression was not required.

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