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Effects of Chain Length of an Amphipathic Polypeptide Carrying the Repeated Amino Acid Sequence $(LETLAKA)_n$ on α-Helix and Fibrous Assembly Formation

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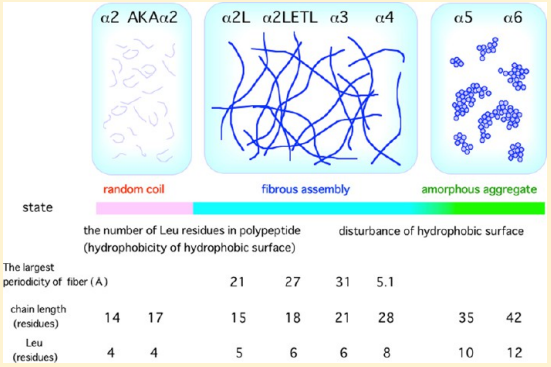
Supporting Information

ABSTRACT: Polypeptide α3 (21 residues), with three repeats of a seven-amino-acid sequence $(LETLAKA)_3$, forms an amphipathic α-helix and a long fibrous assembly. Here, we investigated the ability of α3-series polypeptides (with 14−42 residues) of various chain lengths to form α-helices and fibrous assemblies. Polypeptide α2 (14 residues), with two same-sequence repeats, did not form an α-helix, but polypeptide α2L (15 residues; α2 with one additional leucine residue on its carboxyl terminal) did form an α-helix and fibrous assembly. Fibrous assembly formation was associated with polypeptides at least as long as polypeptide α2L and with five leucine residues, indicating that the C-terminal leucine has a critical element for stabilization of α-helix and fibril formation. In contrast, polypeptides α5 (35 residues) and α6 (42 residues) aggregated easily, although they formed α-helices. A 15−35-residue chain was required for fibrous assembly formation. Electron microscopy and X-ray fiber diffraction showed that the thinnest fibrous assemblies of polypeptides were about 20 Å and had periodicities coincident with the length of the α-helix in a longitudinal direction. These results indicated that the α-helix structures were orientated along the fibrous axis and assembled into a bundle. Furthermore, the width and length of fibrous assemblies changed with changes in the pH value, resulting in variations in the charged states of the residues. Our results suggest that the formation of fibrous assemblies of amphipathic α-helices is due to the assembly of bundles via the hydrophobic faces of the helices and extension with hydrophobic noncovalent bonds containing a leucine.



The α-helix, which is frequently detected in the three-dimensional structures of protein molecules, is one of the fundamental structural units of proteins.[1−3] In the α-helix structure, the main chain of the polypeptide molecule is folded into a spiral, in which 3.6 amino acid residues produce one turn with a pitch of 5.4 Å.[4] Amphipathic α-helices associate to form a left-handed coiled-coil structure. The parts of the coiled-coil structure consist of repetitions of a characteristic amino acid sequence of seven amino acid residues (**abcdefg**),[5] in which specific positions are occupied by similar kinds of amino acids: for example, positions **a** and **d** are occupied by hydrophobic amino acids and other positions by hydrophilic amino acids. The coiled-coil is stabilized among the amphipathic α-helices through a hydrophobic interaction at the **a** and **d** positions.[3,6−19] The coiled-coil is further stabilized by salt bridges that are formed between charged amino acids at

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positions **e** and **g**.[6,7,19−26] The association state of the coiled-coil comprises dimer to heptamer,[1−3,6,8,18] and the orientation of the α-helices is either parallel or antiparallel.[1,2,6]

Amphipathic α-helix structures of various lengths exist as secondary structures in protein molecules.[1,2,27] Generally, α-helix structures comprise fewer than about 26 amino acid residues in globular proteins[28] but are composed of relatively long coiled-coil structures in fibrous proteins such as tropomyosin (284 residues)[29] and myosin rods (over 1000 residues).[30] In many studies of artificially designed polypeptides that take typical α-helix structures,[16,31−33] the chain length of the polypeptide, as well as the constitution and arrangement of the amino acid residues, affects the formation of the α-helix structure. For example, Su et al. studied the coiled-coil-forming ability of different-sized polypeptides containing various numbers of hydrophobic amino acid residues, especially leucine and isoleucine.[32] In examining the coiled-coil-forming ability of different-sized amphipathic peptides, they found that polypeptides larger than 23 residues and containing five hydrophobic amino acid residues were able to form stable coiled-coil structures. Stability of the structure against heat or denaturating agents increased as the length of the polypeptide increased. Any of these amphipathic polypeptides were able to form a coiled-coil structure, providing that its length was more than two or three times the number of repeats of seven-amino-acid units. Furthermore, several groups have reported various artificially designed polypeptides that form fiber assemblies comprising α-helices, in which the type of amino acids, the pH effect, and metal ions binding have been discussed.[34−62]

We also previously characterized amphipathic α-helix-forming polypeptides prepared by gene engineering. Polypeptide α3 (Figure 1, Tables 1 and 2), which comprises three

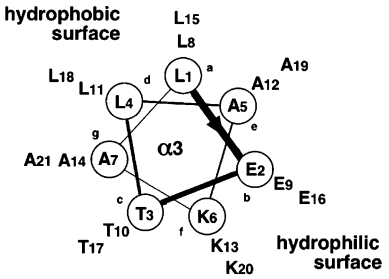

Figure 1. Helical wheel and positions **a** to **g** of polypeptide α3.

Table 1. Amino Acid Sequences of Polypeptides with Different Numbers of Repeats of the Seven-Amino-Acid Sequence LETLAKA

polypeptide name	amino acid sequence	number of amino acid residues
α2	LETLAKA LETLAKA	14
α3	LETLAKA LETLAKA LETLAKA	21
α4	LETLAKA LETLAKA LETLAKA LETLAKA	28
α5	LETLAKA LETLAKA LETLAKA LETLAKA LETLAKA	35
α6	LETLAKA LETLAKA LETLAKA LETLAKA LETLAKA LETLAKA	42

repeats of a sequence of seven amino acids (LETLAKA) positioned in an α-helical wheel (**abcdefg**), was designed artificially in our laboratory.[63] The hydrophobic surface comprises leucine residues (**a**, **d**) and alanine residues (**e**, **g**),

Table 2. Amino Acid Sequences of Polypeptides Longer than α2 but Shorter than α3

polypeptide name	amino acid sequence	number of amino acid residues			
		residues	L	E	K
α3	LETLAKA LETLAKA LETLAKA	21	6	3	3
α2	LETLAKA LETLAKA	14	4	2	2
α2L	LETLAKA LETLAKA L	15	5	2	2
α2LET	LETLAKA LETLAKA LET	17	5	3	2
α2LETL	LETLAKA LETLAKA LETL	18	6	2	3
Aα2	A LETLAKA LETLAKA	15	4	2	2
AKAα2	AKA LETLAKA LETLAKA	17	4	2	3
LAKAα2	LAKA LETLAKA LETLAKA	18	5	2	3
LAKAα2L	LAKA LETLAKA LETLAKA L	19	6	2	3

and the hydrophilic surface comprises threonine (**c**), lysine (**f**), and glutamic acid (**b**) residues when the polypeptide forms an amphipathic α-helix in an aqueous solution containing KCl. The α-helix of polypeptide α3 is stabilized by intramolecular salt bridges that are formed between the lysine and glutamic acid residues on the hydrophilic surface. The amino acid sequence of polypeptide α3 characteristically has alanine residues at the **e** and **g** positions in the α-helix structure. We have further found serendipitously that the α3 helix assembles autonomously into thick and long fibrous structures in a buffer solution containing KCl.[34−36,63] For the formation of fibrous assemblies of α-helices, it is important that assembly can occur via the leucine zipper formed on the hydrophobic surfaces. We have therefore studied the characteristics of analogues in which the leucine residues at positions **a** and **d** have been replaced by aliphatic amino acids other than leucine.[36] Formation of both an amphipathic α-helix and fibrous assemblies depended on the hydrophobicity of the substituted amino acid residues. Subtle differences in the shapes of the fibrous assemblies were observed by electron microscopy in some cases; these differences may reflect differences in the states of the assemblies.

Here, we studied the range of chain lengths of polypeptides required to form the α-helix structure and fibrous assemblies and the mechanism of association of amphipathic helical polypeptides. We prepared a series of polypeptide analogues of polypeptide α3, with various chain lengths, and analyzed their physical characteristics by using circular dichroism (CD) spectra, Fourier transform infrared (FTIR) spectra, electron-microscopic observation, and X-ray diffraction. Our study revealed that the arrangement of hydrophobic residues in our polypeptide plays a critical role in the formation of fibrous assemblies.

EXPERIMENTAL DETAILS

Preparation of Polypeptides by Gene Engineering. Polypeptides consisting of fewer than 28 amino acid residues were prepared by means of a gene engineering method.[36] The polypeptides were expressed as proteins fused with the N-terminal part of porcine adenylate kinase (ADK) by using the expression vector pMKAK3, which is capable of expressing large amounts of ADK as inclusion bodies in *Escherichia coli*.[64] We inserted the DNA segment encoding the designed polypeptide downstream of the ADK sequence in pMKAK3, using the sequence ATG encoding methionine as a linker. The

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presence of the methionine residue in the final product enabled us to precisely cut the designed polypeptide from the fused protein product by means of cyanogen bromide (BrCN) treatment. The DNA sequences encoding the polypeptides consisted of the most suitable codons in *E. coli*. Complementary DNA sequences were designed. DNA strands were synthesized by Nissinbo Co., Ltd. (Tokyo, Japan), and double-stranded DNAs were prepared by annealing.

Each chemically synthesized double-stranded DNA encoding a designed polypeptide was inserted between the restriction sites *Eco*RI and *Bam*HI of the cloning vector pUC18. After the DNA sequence was verified, the DNA was treated with three enzymes: *Eco*RI, *Pst*I, and the Klenow fragment of DNA polymerase I. The DNA fragments thus obtained were purified and inserted between restriction sites *Sma*I and *Pst*I of the expression vector pMKAK3 to prepare the expression vector pMK peptide used in this study.[64]

Escherichia coli strain JM109, which was transformed with pMK peptide, was incubated at 37 °C for 18 h in Luria−Bertani medium containing ampicillin. Cells were collected by centrifugation at 2000*g* for 10 min at 4 °C. The harvested cells were washed twice with TE (10 mM Tris-HCl, pH 8) buffer containing 1 mM ethylenediaminetetraacetic acid (EDTA). The washed cells were suspended in TE buffer containing 1 mM EDTA and then disrupted by sonication on ice. The fused protein was collected as inclusion bodies by centrifugation at 8000*g* for 10 min at 4 °C. To remove the N-terminal section containing the ADK peptide from the designed polypeptide, the inclusion bodies were incubated in 70% formic acid, 2% BrCN, and 1% 2-mercaptoethanol at 37 °C for 48 h; they were then dialyzed against Milli-Q water at 4 °C for 8 h and then dialyzed against 10 mM Tris-HCl buffer (pH 8.5) at 4 °C overnight. The precipitate was removed by centrifugation at 8000*g* for 10 min at 4 °C. The dialyzed solution was applied to a diethylaminoethyl (DEAE) cellulose (DE 52) column (ø 3 × 10 cm) (Whatman, Tokyo, Japan) and then washed with 200 mL of 0.01 M Tris-HCl buffer (pH 8.5) at a flow rate of 1 mL/min. The designed polypeptides were eluted with 200 mL of 0.5 M NaCl in 0.01 M Tris-HCl buffer (pH 8.5). The polypeptide-containing fractions were further purified by reverse-phase high-performance liquid chromatography (HPLC) (Gilson, USA or France) on a C4 column (C4 P-300−5, ø 4.6 × 150 mm; Tokyo Kasei Co., Ltd., Tokyo, Japan). A linear gradient of solvents A and B was applied for 60 min. Solvent A consisted of 0.1% trifluoroacetic acid (TFA) (v/v) in Milli-Q water, and solvent B consisted of 0.1% TFA (v/v) and 80% acetonitrile (v/v) in Milli-Q water. The polypeptide-containing fractions were collected and lyophilized. Each polypeptide was dissolved in Milli-Q water and then purified by reverse-phase HPLC on a C18 column (L-column octadecyl silyl, ø 4.6 × 150 mm; Chemicals Inspection & Testing Institute, Tokyo, Japan). The solvents and gradient conditions were the same as those used in the case of the C4 column. The purified polypeptide was lyophilized, dissolved in Milli-Q water, and stored in a deep freezer at −30 °C.

Preparation of Polypeptides by Chemical Synthesis. Polypeptides that were larger than 35 amino acid residues and not able to be prepared by means of gene engineering were synthesized by using the standard 9-fluorenyl-methoxycarbonyl (Fmoc) solid-phase method in a Kokkusan peptide synthesizer (Kokusankagaku Co., Ltd., Tokyo, Japan); the Fmoc amino acids and all other reagents were purchased from Kokusankagaku Co., Ltd. Fmoc amino acid coupling reactions were performed for 1.5 h at room temperature in *N*,*N*-dimethylformamide (DMF) containing *N*-hydroxybenzotriazole and *N*,*N*′-diisopropylcarbodiimide. The Fmoc-group removal reaction was performed in a 20% piperidine−DMF solution for 3 min at room temperature. After all the reactions had been performed, the resin was washed with methanol and then dried with an aspirator. The polypeptides were split from the resin by incubation for 2 h at room temperature with the reaction solution, which consisted of TFA containing thioanisol, 1,2-ethanedithiol, and *m*-cresol. TFA was removed from the reaction solution by evaporation, and then the reaction solution was mixed with cold diethylether. The crude polypeptides were collected as a white precipitate on a glass filter and were then purified by size-exclusion chromatography on G-10 gel (Pharmacia, Sweden). The polypeptide-containing fractions were purified further by reverse-phase HPLC (Gilson, USA or France), as described in the preceding section on the preparation of polypeptides by means of gene engineering. The fractions containing the polypeptides were lyophilized, and the purified polypeptides were stored at −30 °C until use.

Determination of Polypeptide Concentrations. The concentration of each polypeptide was determined by amino acid composition analysis with an amino acid analyzer L-8500 (Hitachi, Japan) after hydrolysis in 20% (v/v) HCl at 110 °C for 24 h under a vacuum.

CD Measurements. The CD spectra of the polypeptides were measured with a model J-720 CD spectrophotometer (Jasco, Japan). The light-path length of the quartz cell was 1 mm. To investigate the relationship between α-helix formation and the length of the polypeptides, measurement was performed under several concentrations of polypeptides and pH values (i.e., 2, 6, or 12), including 1 M KCl.

FTIR Measurements. Infrared spectra were measured with an FTIR spectrophotometer model 8400 (Shimadzu, Japan). The cell used was a KRS-5 (Shimadzu, Japan), which contained a mixture of thallium bromide and thallium iodide. The light-path length of the cell was 0.05 mm. D_2O was used as the sample solvent. The measurements were performed in 10 mM phosphate buffer (pD 6) containing 1 M KCl. The polypeptide concentration was 5 mg/mL.

Electron-Microscopic Observation. Fibrous association of the polypeptides was observed under a transmission electron microscope. An aliquot of a solution comprising 25 μM to 2 mM polypeptide sample in 10 mM citrate buffer (pH 2 or 6) or 10 mM borate buffer (pH 12) containing 0.1 or 1 M KCl was mounted on a grid with a film of carbon-coated parlodion, and then the grid was washed with the same buffer as used for the sample. The excess solution was removed with filter paper. The grid was then negatively stained with 2.5% uranyl acetate. The sample-mounted grid was examined under a transmission electron microscope (JEM 1010; JEOL, Japan) at an accelerating voltage of 80 kV. The electron micrographs were recorded on Fuji FG electron image film (11.8 × 8.2 cm) (Fuji Film, Japan).

X-ray Diffraction Experiment. Polypeptide (20 mg) was dissolved in 1 mL of Milli-Q water. Then the insoluble matter was removed from the polypeptide solution by ultracentrifugation at 540000*g* for 10 min at 4 °C in an Optima MAX ultracentrifuge (Beckman) using the rotor TLA-100.2. Appropriate buffer solution (1 mL) was added to the supernatant containing the polypeptides, and the solution was left to stand for 60 min at room temperature to allow fibrous association to occur. The conditions for formation of the

fibrous assemblies for polypeptides α3 and α4 were 10 mM phosphate buffer (pH 6) containing 0.1 M KCl; the conditions for polypeptide α2LETL were 10 mM citrate buffer (pH 2) containing 1 M KCl; and the conditions for polypeptide α2L were 10 mM citrate buffer (pH 2) containing 1 M KCl. The fibrous assemblies of the polypeptides were collected as precipitates after ultracentrifugation (540000*g* for 60 min) at 4 °C. The collected precipitate was loaded into a quartz capillary (Müllar, Germany), ø 0.6 to 0.7 × 70 mm. The capillary was placed in a magnetic field of 13.5 T for magnetic orientation of the liquid crystals.[65] The state of the liquid crystals was observed by using polarizing microscopy. X-ray diffraction patterns were recorded with an FR-D X-ray generator (Rigaku, Japan) and an R-AXIS-IV imaging plate detector (Rigaku, Japan). The exposure time was 12 h, and the specimen-to-detector distance was 250 mm, which was calibrated by using powder diffraction of $CaSO_4 \cdot 2H_2O$.

RESULTS

α-Helix Formation of Polypeptides Longer than α2 but Shorter than α6. We previously designed polypeptide α3, which consists of three repeats of a seven-amino-acid sequence, LETLAKA (Figure 1, Tables 1 and 2), and found that it formed amphipathic α-helices and fibrous assemblies.[34−36,63] To determine the range of chain lengths of the polypeptides forming stable α-helix structures, we measured the CD spectra of polypeptides carrying different numbers of repeats of the amino acid sequence LETLAKA (polypeptides α2 to α6; 14−42 residues, Table 1). Examination of the CD spectra (at pH 6 in 1 M KCl) indicated that polypeptide α2 did not form an α-helix, but polypeptides α3, α4, α5, and α6 did form α-helix structures (Figure 2A,B). The helical structures in polypeptides

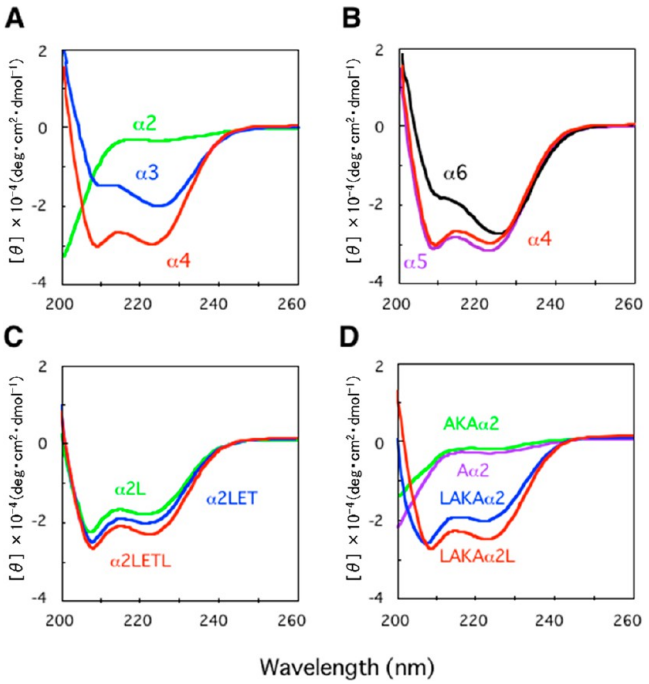

Figure 2. Circular dichroism spectra of polypeptides (150 μM) with different chain lengths (α2 to α6). Buffer conditions in panels A and B are 1 M KCl and 10 mM phosphate buffer (pH 6) at 30 °C. Buffer conditions in panel C are 1 M KCl and 10 mM citrate buffer (pH 2) at 30 °C. Buffer conditions in panel D are 1 M KCl and 10 mM borate buffer (pH 12) at 30 °C. [θ], molar ellipticity.

α4 and α5 were more stable than that in α3, because the troughs at 222 nm in polypeptides α4 and α5 were deeper than that of polypeptide α3. Analysis of the CD spectra of polypeptides α2 to α6 under various pH conditions (pH 2, 6, and 12) showed that the α-helix profiles changed little with changes in pH (Figure S1, Supporting Information).

α-Helix Formation by Polypeptides Longer than α2 and Shorter than α3. To determine the minimum length of polypeptides required for α-helix formation and to examine the characteristics of the amino acids in the polypeptides, we prepared samples by adding an extension at the carboxyl (C)-terminal or the amino (N)-terminal, or both, of polypeptide α2 (α2-based polypeptides; Table 2). Polypeptide α2L (15 residues) was the minimum size for α-helix formation at 10 mM citrate buffer (pH 2) in 1 M KCl (Figure 2C), although polypeptide Aα2 (15 residues) did not form an α-helix. Polypeptides α2LET and α2LETL also formed α-helices (Figure 2C). These helical formations were most stable at pH 2 (Figure S2, Supporting Information). In addition, the α-helical structure of α2LETL was more stable than that of α2LET (Figure 2C). The CD spectra of polypeptides Aα2 (15 residues) and AKAα2 (17 residues; Figure 2D) showed patterns of random coils, but polypeptides LAKAα2 and LAKAα2L showed two troughs, at wavelengths of 222 and 208 nm; they were able to form α-helix structures at 10 mM borate buffer (pH 12) in 1 M KCl (Figures 2D and S2, Supporting Information). Therefore, the number of leucine residues available to cause hydrophobic interactions was important for α-helix formation. In addition, the stability of the α-helix in relation to the pH value depended critically on the components of the amino acids.

Confirmation of Secondary Structures by FTIR Measurements. FTIR spectrum analysis is used to study the secondary structures of protein solutions that contain insoluble materials such as amyloid fiber.[66] To confirm the secondary structures of solutions containing fibrous assemblies of different chain lengths or white turbidity (polypeptides α2LETL, LAKAα2L, α3, α4, α5, and α6), we measured the FTIR spectra of all six polypeptides and found a main absorption band at wavenumber 1650 cm^{-1}, representing the α-helix structure (Figure S3, Supporting Information).[39,67−69] These results led us to the same conclusions as those derived from our examination of the CD spectra. The only exception was the patterns of the CD spectra from polypeptides α3 and α6: the absolute values of [θ] (molar ellipticity) at about 208 nm were lower than those of the other polypeptides (see Figure 2), although this was probably due to white turbidity formation. A small band at 1670 cm^{-1} was derived from the trifluoroacetic acid used in the purification steps.[69]

Electron-Microscopic Observations of Amphipathic Polypeptides of Various Lengths. To examine fibrous assembly formation from different-chain-length polypeptides, we observed polypeptide associations under an electron microscope. Electron microscopic observations were performed at polypeptide concentrations of 25 μM to 2 mM. For each of the polypeptides, the shapes of the fibrous assemblies and the shape changes with changes in pH were the same with different polypeptide concentrations, although the observed quantity of fibrous assemblies increased with increasing polypeptide concentration (data not shown). Polypeptide α2 (14 residues) was not observed to form a fibrous assembly (data not shown). Fibrous assemblies with 50 μM of other polypeptides were clearly observable (Figures 3−5). Amphipathic polypeptide α3

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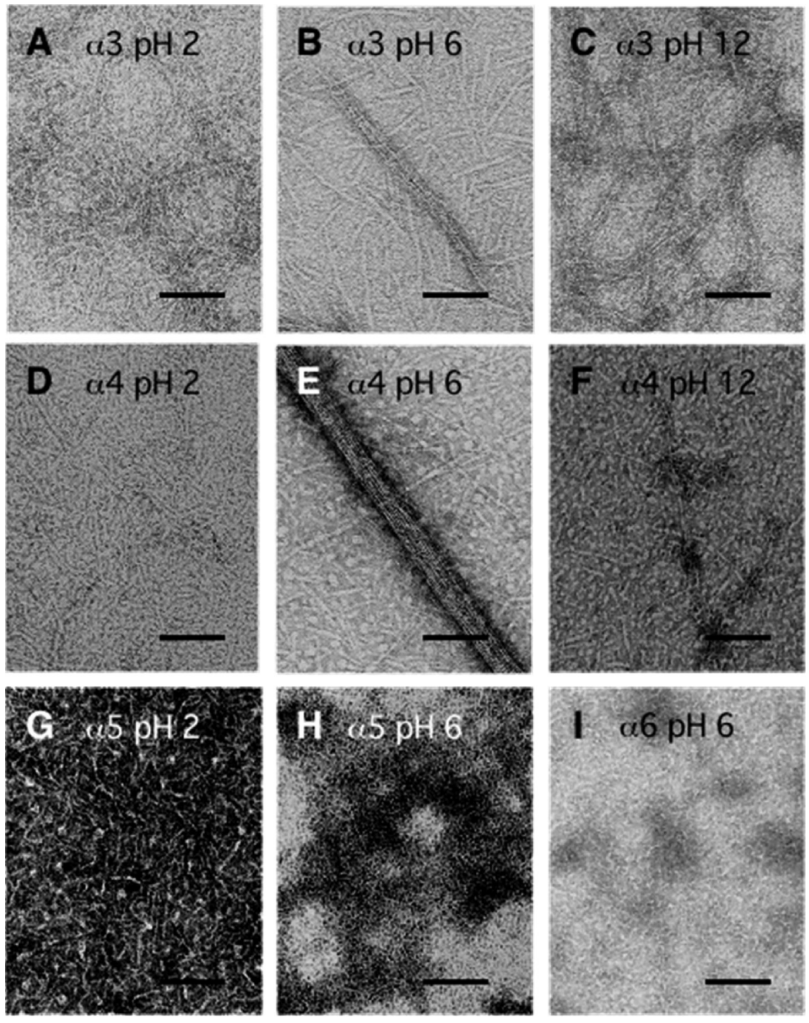

Figure 3. (A–I) Electron micrographs of polypeptides of various chain lengths (α3 to α6). The buffer conditions are 1 M KCl and 10 mM citrate buffer (pH 2), 1 M KCl and 10 mM citrate buffer (pH 6), and 1 M KCl and 10 mM borate buffer (pH 12). The polypeptide concentration was 50 μM. Fibrous assemblies of polypeptides were stained with a 2.5% uranium acetate solution. Scale bars represent 50 nm.

(21 residues) was assembled into fibers in the presence of 1 M KCl at pH 2, 6, or 12 (Figure 3A–C). At pH 2 or 12 in 1 M KCl, the flexible fibrous assemblies resembled a mesh, and very few of them associated (Figure 3A,C). Polypeptide α4 formed fibrous assemblies under all conditions (Figure 3D–F); these observations were very similar to those for polypeptide α3 (Figure 3A–C). In contrast, in the case of polypeptides as long as polypeptide α5 (35 residues), or longer, amorphous aggregations were observed under almost all buffer conditions (Figure 3G–I), particularly under the conditions of 1 M KCl and pH 6 (see Figure 3H). The fibrous assemblies of polypeptide α5 resembled a mesh at any pH; fibrous assemblies appeared at pH 2, but amorphous aggregates appeared at pH 6 (Figure 3G) and pH 12 (data not shown). In the case of polypeptide α6, amorphous aggregates appeared at any pH (Figure 3H and data not shown). Therefore, although polypeptides α5 and α6 were able to take on α-helix structures, they also formed amorphous aggregates.

In the case of polypeptide α3, thin fibers about 20 Å thick (20.4 ± 0.3 Å) assembled into many thicker, linear fibers at pH 6 (Figure 3B). The length of longest thick fibers was around 15 μm. In addition, the fibrous assemblies at pH 2 and pH 6 were about 40 Å wide (at pH 2, 40.3 ± 0.4 Å; at pH 6, 41.7 ± 0.5 Å) (Figure 4). Some of the fibrous assemblies 40 Å wide had a line in the center of the fiber or a dotted line in the fibrous assemblies (Figure 4). Therefore, we predicted that the fibrous

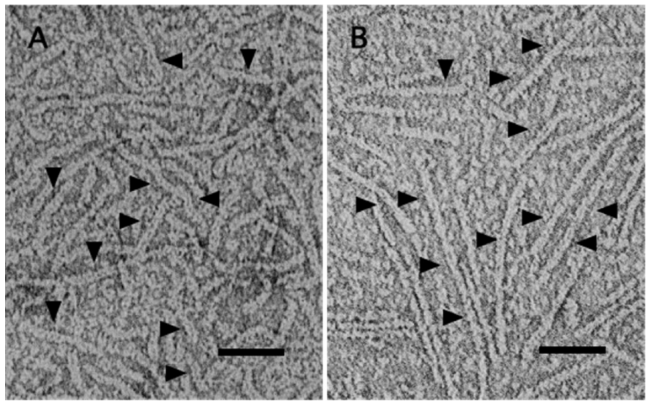

Figure 4. Electron micrographs of fibrous assemblies of polypeptide α3. The buffer conditions in panel (A) are 1 M KCl and 10 mM citrate buffer (pH 2) and panel (B) in 1 M KCl and 10 mM citrate buffer (pH 6). The polypeptide concentration was 50 μM. Fibrous assemblies of polypeptides were stained with a 2.5% uranium acetate solution. Scale bars represent 25 nm. Arrowheads show fibrous assemblies 40 Å thick in which lines or dotted lines can be seen.

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assemblies 40 Å wide were composed of the two thinnest fibrous assemblies. Such interaction of intermolecular α-helix bundles has been reported in the transmission electron microscope and scanning transmission electron microscope observation studies of Kajava et al.[48] and the cryo-electron microscope observation studies of Dong et al.[58] Recently, Sharp et al. investigated packaging of α-helices and their interaction in the fibers of self-assembling fiber peptides by using cryo-electron microscopy.[45]

Polypeptides that were longer than α2 but shorter than α3 were also measured by using electron microscopy (Figure 5).

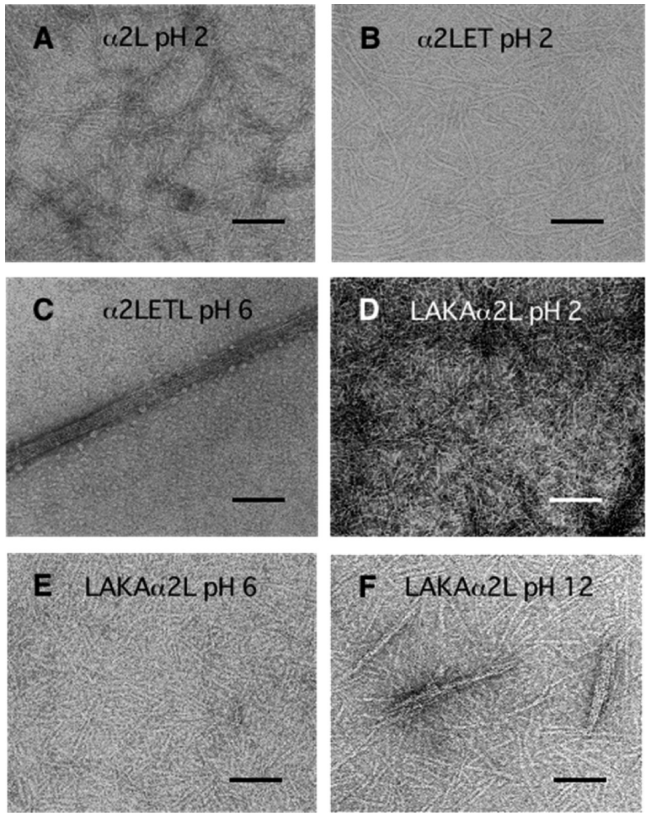

Figure 5. (A–F) Electron micrographs of polypeptides longer than polypeptide α2 but shorter than polypeptide α3. The buffer conditions are 1 M KCl and 10 mM citrate buffer (pH 2), 1 M KCl and 10 mM citrate buffer (pH 6), and 1 M KCl and 10 mM borate buffer (pH 12). The polypeptide concentration was 50 μM. Fibrous assemblies of polypeptides were stained with a 2.5% uranium acetate solution. Scale bars represent 50 nm.

The shortest polypeptide, α2L (15 residues), which was able to form an α-helix, formed fibrous assemblies at pH 2 in the presence of 1 M KCl (Figure 5A). Polypeptide AKAα2, which does not form an α-helix, did not form fibrous assemblies (data not shown). In the case of α2LET, an α-helix was formed only in 10 mM citrate buffer (pH 2) containing 1 M KCl; fibrous assemblies that resembled a mesh were observed under the same conditions (Figure 5B). In contrast, in the case of LAKAα2, fibrous assemblies were not observed under any of the buffer conditions (data not shown), although LAKAα2 can form an α-helix (Figure 2D). The results for LAKAα2 suggest that the formation of fibrous assemblies depends on the nature of the amino acid sequences at both termini. In the cases of polypeptides α2LETL and LAKAα2L, the shapes of the fibrous assemblies varied with the solution conditions (Figure 5C–F). For α2LETL, networks of fibers were observed at pH 2 (data not shown), but thick fiber-like assemblies were often observed at pH 6 (Figure 5C). For LAKAα2L, networks of fibers were observed at pH 2 and 6 (Figure 5D,E), but thick fiber-like assemblies, about 100 nm long, were often observed at pH 12 (Figure 5F). The thickness of the scattered fibrous assemblies of polypeptides longer than polypeptide α2 but shorter than polypeptide α3 was approximately 40 Å, and many 20 Å wide fibrous assemblies were observed in the thick fibers (data not shown). For example, the thicknesses of the scattered fibrous assemblies and the thinnest fibrous assemblies of α2LETL in 1 M KCl at pH 6 were 41.1 ± 0.5 Å and 20.9 ± 0.4 Å, respectively. The thickness of the scattered fibrous assemblies of α2L in 1 M KCl at pH 2 was 40.9 ± 1.0 Å.

X-ray Fiber Diffraction of Fibrous Assemblies of Amphipathic Polypeptides. X-ray fiber diffraction studies of the α-helices in fibrous assemblies of designed polypeptides have been performed by several groups.[37,38,46,52] Notably, Papapostolou et al. found that the association states of the polypeptides they designed followed a hexagonal-packing model.[38] To examine the arrangement of α-helices in the fibrous assemblies, we analyzed different-chain-length polypeptides (α2L, α2LETL, α3, and α4) by using X-ray fiber diffraction.

As the α-helix structure of a polypeptide chain shows diamagnetic anisotropy due to the orientation of the peptide bonds parallel to the α-helix axis,[70] we considered that the fibrous assemblies of α-helical polypeptide molecules would be oriented well in a strong magnetic field. Transparent pellets of amphipathic polypeptide samples were obtained from polypeptide solutions and were loaded into quartz capillaries. Their liquid crystalline states were then observed by polarization microscopy before and after magnetic orientation. A pellet of polypeptide α3 in the capillaries, observed in the diagonal and extinction positions before magnetic orientation, showed a mosaic pattern of interference colors, indicating that the pellet was composed of small liquid crystalline domains that were randomly oriented (Figure 6A,B). After the capillary sample had been left in a superconducting magnet with a field strength of 13.5 T for a few days with the capillary axis parallel to the direction of the magnetic field, the interference color became a homogeneous yellow green in the diagonal position and was totally dark in the extinction position (Figure 6C,D). These results indicated that the liquid crystalline domains were oriented and fused into a single, large domain in which the α-helix structures were oriented parallel to the magnetic field. In the case of a sample of polypeptide α4, the liquid crystalline state and response to the magnetic field were similar to those shown by polypeptide α3 (data not shown). In contrast, liquid crystalline samples of α2L and α2LETL did not show clear responses to the magnetic field (data not shown).

X-ray diffraction patterns from these liquid crystalline samples were recorded with the capillary axis vertical (Figure 7). In the equatorial direction, all the diffraction patterns showed a relatively strong but diffuse intensity with a spacing of about 20 Å and a weak, more diffuse intensity with a spacing of approximately 10 Å. The diffuse reflection with a spacing of 20 Å probably reflected the lateral packing of α-helical coiled-coils in the fibrous structure. The size of the lateral packing of the α-helix was close to the diameters of the thinnest fibrous assemblies observed under the electron microscope.

In contrast, the diffuse reflection with a spacing of 10 Å was likely due to the packing of α-helices within each α-helical

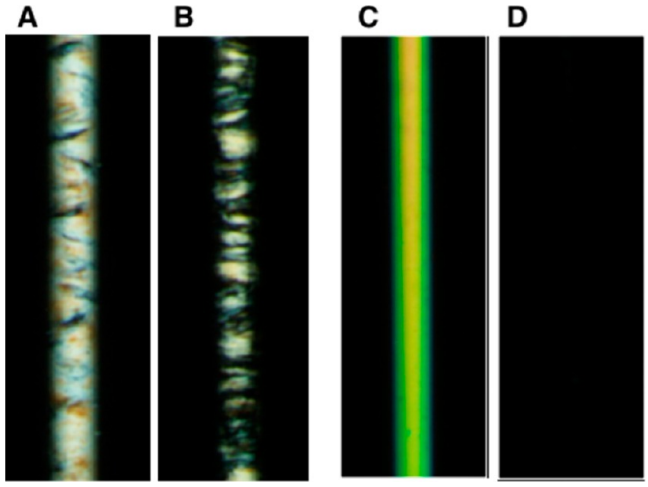

Figure 6. Polarizing micrographs of the state of liquid crystals of polypeptide α3. In panels (A) and (B), polypeptide α3 precipitate was loaded onto a quartz capillary and photographs were taken under a polarizing microscope. In panels (C) and (D), polypeptide α3 precipitate was loaded onto a quartz capillary and left in a 13.5-T magnetic field for a few days. The photographs were taken under a polarizing microscope. Polarizing micrographs (A) and (C) were taken in the diagonal position, and polarizing micrographs (B) and (D) were taken in the extinction position.

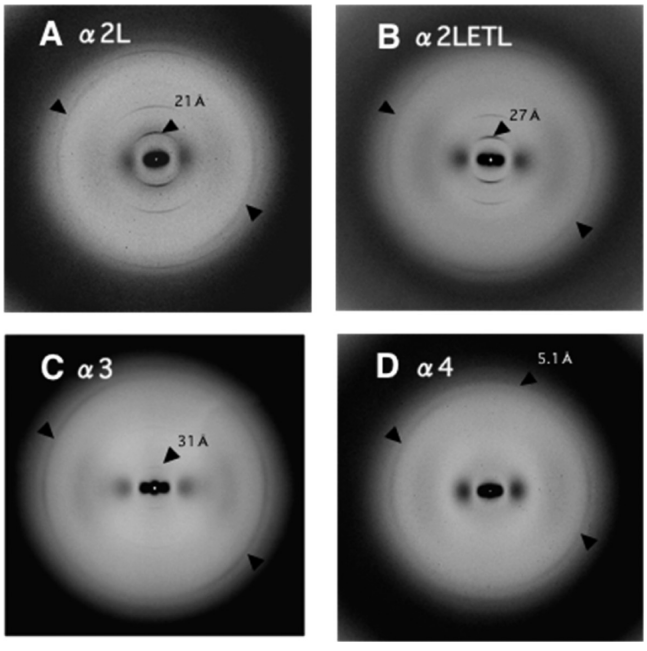

Figure 7. (A–D) Fiber diffraction patterns of polypeptides of different chain lengths. Arrowheads indicate the longest periodicity in the X-ray fiber diffraction patterns, with the fibrous assemblies along the fibrous axis. The arc-shaped diffraction patterns at the upper left and lower right of the diffraction images which indicate that arrowheads are attributed to the Mylar membrane used as a window material for the He path.

coiled-coil, indicating that the α-helices were oriented more or less parallel to the capillary axis, although the disorientation angle was relatively large. In the meridional direction, the α2L and α2LETL polypeptides showed a series of sharp reflections, indicating that the axially repeating structures in the fibrous assemblies of these two polypeptides were very well ordered. On the other hand, those of polypeptide α3 were weak, indicating that the axial periodicity was less regular. Furthermore, no such reflections were observed for polypeptide α4, indicating that the fibrous assembly had no regular axial periodicity. The spacings of these meridional reflections represented the axial periodicities in the fibrous structures: 21 Å for α2L; 27 Å for α2LETL; and 31 Å for α3. The axial periodicity corresponded to the length of the α-helix formed by each polypeptide, indicating that the α-helices formed by these three polypeptides were self-assembled into fibrous structures with their axes oriented parallel to the fibers. The meridional reflection observed at 5.1 Å resolution, which was indicative of a typical α-helical coiled-coil structure, was sharp in the case of α2L, less so for α2LETL, and diffuse for α3 and α4 (Figure 7A–D). These features also reflected the regularity of the axial periodicity. However, the presence of the meridional 5.1 Å reflection, albeit weak and diffuse, and the two diffuse equatorial reflections in the diffraction pattern from polypeptide α4 indicated that α4 also formed α-helical coiled-coils that were assembled in a bundle structure to form the fibrous assembly, as was also observed by electron microscopy (see Figure 3D–F). The absence of axial periodicity in the fibrous assembly formed by polypeptide α4 might have been due to the copresence of multiple structures with different axial staggers between the two chains upon formation of the α-helical coiled-coils; this would have disturbed the regularity of the axial periodicity upon formation of fibrous assemblies. The longer the chain of a polypeptide, the more easily aggregation occurred.

DISCUSSION

To elucidate the chemical requirements for α-helix and fibrous assembly formation by amphipathic polypeptides of the α3 series, we prepared a series of polypeptides of different sizes and investigated their characteristics. The results are summarized in Figure 8. Polypeptide α2 [$(LETLAKA)_2$, 14 residues] did not form an α-helix or fibrous assemblies. However, polypeptide α2L [$(LETLAKA)_2L$, 15 residues] was able to form not only an α-helix, but also fibrous assemblies. Helix formation was coupled with fibrous assembly formation under the same conditions. Although AKAα2 [AKA-$(LETLAKA)_2$] comprised 17 residues, with the three-amino-acid sequence AKA added to the amino terminus, the peptide formed neither an α-helix nor fibrous assemblies. Consideration of these results and those for other polypeptides suggests that the minimum size of our designed amino acid sequences for formation of an α-helix was 15 residues, but five leucine residues were required. Formation of the amphipathic α-helix depended on an increase in the number of leucine residues rather than on the chain length of the polypeptide, suggesting that leucine residues on the hydrophobic surface play an important role in amphipathic α-helix formation. This result agrees with the experimental results obtained with different kinds of polypeptides by Su et al.[32] In addition, our results suggest that polypeptide α2L has the shortest chain length among all of the reported artificially synthesized polypeptides that form α-helices and fibrous assemblies: for example, polypeptides SAF-1 and SAF-2 (28 residues), reported by Pandya et al.;[37] polypeptide αFFP (34 residues), reported by Potekhin et al.;[46] polypeptide KIA13 (20 residues), reported by Boon et al.;[49] polypeptide VW19 (26 residues), reported by Pagel et al.;[55] and the polypeptide designed by Dong et al. (21 residues).[58]

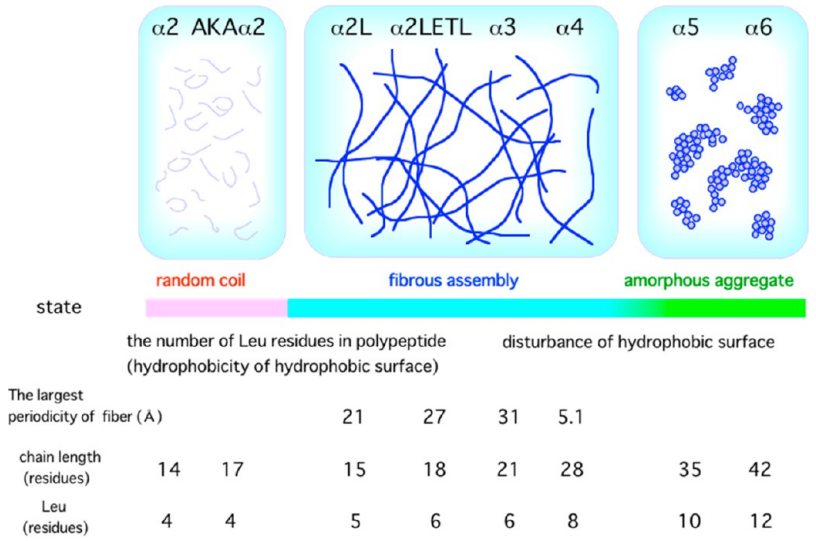

Figure 8. Relationships of chain length and number of leucine residues with fibrous assembly formation.

Polypeptides α5 and α6 were soluble in water; however, these polypeptides aggregated to yield a white precipitate in buffer solutions containing KCl. Electron microscopic observation revealed that polypeptides that were the length of α5 (35 residues) or longer showed amorphous association under almost all the buffer conditions examined, although fibrous assemblies were observed occasionally. In the case of a polypeptide chain as long as α5, or longer, the degree of disorder on the hydrophobic surface would have been increased, and the various states of polypeptide molecules would have appeared, so that the polypeptide chains would not have been able to form fibrous assemblies and would thus have tended to form aggregates. Thus, in the case of amphipathic polypeptides with a repeating unit of seven amino acid residues that had a definite sequence (i.e., LETLAKA), the peptides formed an α-helix structure and associated autonomously to form fibrous assemblies within a certain chain-length range (15 to about 35).

To analyze the structures of fibrous assemblies of polypeptide α3, we grew homogeneous liquid crystals of these fibers in a strong magnetic field and recorded the X-ray fiber diffraction patterns. Because the fibers of polypeptide α3 were oriented well in the strong magnetic field, the α-helix was expected to be oriented parallel to the fiber axis.[70] Examination of the X-ray fiber diffraction patterns revealed that the repeat distances of the filaments agreed well with the lengths of the α-helices formed by each polypeptide. The X-ray fiber diffraction results showed that the longer the polypeptide, the weaker the diffraction intensity corresponding to the basic repeat of the filament (although these phenomena were not observed in the case of polypeptide α4 (Figure 7D)). The results suggest that the longer the polypeptide, the more disordered the location of each molecule in the fiber structure. It was difficult for the long polypeptides—α5 (35 residues) and α6 (42 residues)—to form fibrous assemblies, and they tended to aggregate easily (see Figure 3H,I). These results might have been due to a further increase in disorder at the location of each molecule. As the hydrophobic surfaces of our designed polypeptides were composed of the hydrophobic amino acids leucine and alanine, the longer the chain length, the greater the disorder in the axial direction. This suggestion was confirmed by the fiber diffraction patterns described above: the diffraction intensity indicating the regularity of the location of the α-helix became fainter with an increase in the chain length of the polypeptide molecule (α2L, α2LETL, α3), and diffraction patterns corresponding to the basic building blocks of the filament were not observed in the diffraction image of polypeptide α4.

In the case of native proteins (e.g., GCN4), the coiled-coil structure (a parallel homodimer of α-helices) is supported only by the hydrogen bonding of one asparagine residue on the hydrophobic surfaces of the two respective α-helices.[71] In a study of "Peptide Velcro," O'Shea et al. reported an experiment that was designed for GCN4-p1 and Fos/Jun.[72] If one asparagine residue at one of the **a** positions on the hydrophobic surface was replaced by a leucine residue, the α-helices in the mutant peptide formed a hetero tetramer in which the orientations of the α-helices were heterogeneous, although the heat stability of the α-helix was increased.[73] In these experiments, polypeptides in which both the **a** and **d** positions on the hydrophobic surface of the α-helix were occupied by only leucine residues were heterogeneous in their tertiary structures. The replacement of a leucine by an asparagine residue on the hydrophobic surface in "Peptide Velcro" played an important role in the maintenance of homogeneity of the tertiary structure of the helix bundle. From these experimental results, Lumb and Kim deduced that the coiled-coil-forming polypeptides form assemblies with heterogeneous tertiary structures when the hydrophobic surface comprises only leucine residues.[73]

Although it has been reported in many papers that a hydrophobic interaction is the most important factor for the formation of an amphipathic α-helix structure, our experiments here showed that, in the case of the polypeptides with short chains, the α-helix-forming ability increased dramatically with an increase in the number of hydrophobic amino acid leucine residues. In the case of the amphipathic polypeptide α3 series, the chain length at which fibrous assembly is possible only through hydrophobic interaction appears to be around 35 amino acid residues.

In contrast, several designed polypeptides that are longer than 35 residues form fibrous assemblies. For example, the lengths of the polypeptides YZ1 and TZ1H, designed by Zimenkov et al., are 42 and 41 residues,[52,53] respectively. Papapostolou et al. designed polypeptide SAF-p1-ext and SAF-

p2a-ext pairs with 35 residues.[38] Notably, Palapostolou group[38] and Zimenkov group[52] focused on the role of asparagine residues on the hydrophobic surface of GCN4; they succeeded in designing SFA peptide and YZ1 peptide, which formed fibrous assemblies.

Our electron-microscopic observations revealed that the minimum thickness (approximately 20 Å) of fibers corresponded to a bundle of several α-helices. It was therefore likely that several polypeptide molecules forming the α-helix structure were assembled into a bundle, and then the bundles were built into a filament through appropriate noncovalent bonding with neighboring bundles. The SAF peptides reported by Woolfson et al. are specifically designed to give sticky ended dimers that act as building blocks for fiber assembly;[37] on the other hand, the systems reported by Kajava et al. and Conticello et al. are described as having mismatches that lead to shorter ragged ends that promote fibrillogenesis.[46,52] The CD spectra and electron-microscopic observations showed that a change in pH altered the stability of the α-helix and the shape of the fibrous assemblies (e.g., the width and length). These changes probably result in variation in the charged states of the glutamic acid and lysine residues. Notably, addition of one leucine to the C-terminus of polypeptide α2 or LAKAα2 led to dramatic changes in α-helix and fibrous assembly formation. Our findings suggested that the structure of both the N- and the C-terminus of the polypeptide was critical for the formation of fibrous assemblies. In addition, appropriate interaction of the polypeptides was critical for forming ordered fibrous assemblies. We therefore conclude that the arrangement of hydrophobic residues, such as leucine, in polypeptides plays a critical role in the fibrous assemblies. Kajava et al successfully discussed the number of bundles per building blocks.[46] On the basis of the proposals and the similarity of the amino acid residues located in the hydrophobic surfaces, it is plausible to propose that the block of the peptide α3 fiber reported here are composed of three helices, and shorter ragged ends might be critical for fiber assembly. It should be noted that Woolfson et al have successfully described α-helical fibers, leading to better designs.[38,45] More detailed knowledge of the tertiary atomic structures of the polypeptide fibrous assemblies reported here could open up a new era for the construction of artificial proteins, including the reconstitution of hair, skin, and other highly ordered biomaterials.

CONCLUSION

We examined α-helix and fibrous assembly formation by polypeptide α3 $(LETLAKA)_3$ series of various chain lengths. Fibrous assembly was coupled with helix formation in the case of polypeptides at least as long as polypeptide α2L and with five leucine residues. We found that the C-terminal leucine in polypeptides plays an essential role in stabilization of the α-helix and fibril formation. Furthermore, electron microscopy and X-ray fiber diffraction experiments indicated that the α-helix structures were orientated along the fiber axis and assembled into a bundle. Our findings suggest that the leucine residue plays a key role in amphipathic α-helix and fibril formation through the assembly of bundles and extension with hydrophobic noncovalent bonds.

ASSOCIATED CONTENT

Supporting Information

CD spectra of the polypeptides at various pH values (Figures S1 and S2) and the FTIR spectra of the polypeptides (Figure S3) are shown. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

ADK, adenylate kinase; C-terminal, carboxyl terminal; CD, circular dichroism; DMF, *N*,*N*-dimethylformamide; Fmoc, 9-fluorenyl-methoxycarbonyl; FTIR, Fourier transform infrared; HPLC, high-performance liquid chromatography; N-terminal, amino terminal; TFA, trifluoroacetic acid

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