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The Effects of the Side Chains of Hydrophobic Aliphatic Amino Acid Residues in an Amphipathic Polypeptide on the Formation of α Helix and Its Association

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The polypeptide α3, which was synthesized by us to produce an amphipathic helix structure, contains the regular three times repeated sequence $(LETLAKA)_3$, and α3 forms a fibrous assembly. To clarify how the side chains of amino acid residues affect the formation of α helix, Leu residues, which are located in the hydrophobic surface of an amphipathic helix, were replaced by other hydrophobic aliphatic amino acid residues systematically, and the characters of the resulting polypeptides were studied. According to the circular dichroism (CD) spectra, the Ile-substituted polypeptides formed α helix like α3. However, their helix formation ability was weaker than that of α3 under some conditions. The Val-substituted polypeptides formed α helix only under restricted condition. The Ala-substituted polypeptides did not form α helix under any condition. Thus, it is clear that the order of the α helix formation ability is as follows: Leu ⩾ Ile > Val > Ala. The formation of α helix was confirmed by Fourier Transform Infrared (FTIR) spectra. Through electron microscopic observation, it was clarified that the formation of the α helix structure correlates with the formation of a fibrous assembly. The amphipathic α helix structure would be stabilized by the formation of the fibrous assembly.

Key words: amphipathic α helix, circular dichroism, fibrous assembly, hydrophobic interaction, transmission electron microscopy.

The α helix structure, which is frequently detected in the three-dimensional structures of protein molecules, is one of the fundamental structural units in a protein. In the α helix, the main chain of a polypeptide molecule is folded into a spiral, in which 3.6 amino acid residues produce one turn with a pitch of 5.4 Å (*1*). In order to clarify the principle required for the formation of the α helix structure, we investigated the character of artificially synthesized amphipathic helix–forming polypeptides, which consist of repeats of the designed seven amino acid sequence (*2*).

In many kinds of fibrous proteins, such as myosin (*3*), tropomyosin (*4*), yeast transcription factor GCN4 (*5*), and hemagglutinin in influenza virus (*6*), the amphipathic α helices associate to form a left-handed coiled coil structure. The characteristic amino acid sequences of the coiled coil–forming part in these proteins contain repeats of seven amino acid residues (abcdefg) (*7*), in which definite position are occupied by similar kinds of amino acid residues, for example, positions a and d are occupied by hydrophobic amino acids, and positions e and g by hydrophilic amino acids. The coiled coil structure involves the association of two coils through a hydrophobic interaction at positions a and d (*8–11*). The two helices seem to stabilize each other through association. Furthermore, the coiled coil structure would also be stabilized by the salt bridges formed by the electrically charged amino acid residues at positions e and g (*12–16*).

It is considered that the kinds and arrangements of amino acid residues, which construct the hydrophobic surface of α helix, would determine the state of association. Thus, experimental studies on the characters of artificially constructed amphipathic α helices, produced by changing the arrangement of amino acid residues systematically, are important for understanding the association mechanism of complex proteins. Recently, the amphipathic α helix–forming parts that form the coiled coil structure in protein molecules were investigated in many laboratories, and it has been clarified that hydrophobic amino acid residues are situated at positions a and d for the stable association of coils.

For example, Harbury *et al.* (*17*) prepared a series of mutants with the amino acid residues in the amphipathic helix–forming part of the yeast transcription factor GCN4 replaced, and studied the relationship between the combination of amino acids at positions a and d and the state of helix association by X-ray analysis of the mutant polypeptides. The results revealed that the helix containing Ile at position a and Leu at position d forms a helix dimer, that containing Ile at both positions a and d a helix trimer, and that containing Leu at position a and Ile at position d a helix tetramer.

Furthermore, it has been reported by Wagschal *et al.* (*8*) that replacement of only one amino acid residue at the 19th position on the hydrophobic surface of an artificial

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amphipathic helix–forming polypeptide composed of 38 amino acid residues induced changes in the association of helices and their stability. Like these examples, it is clear that the replacement of amino acids at position a and/or d in a helical wheel induces a change in the helix association.

Using a polypeptide composed of three repeats of the seven amino acid sequence, $(LETLAKA)_3$, designed to form the amphipathic helix, we investigated systematically its character and state of association to clarify the role of the side chains of amino acid residues in the formation of the helical structure. α3 forms a hydrophobic surface comprising Leu and Ala, and a hydrophilic surface comprising Lys and Glu in the amphipathic helix, see Table 1 and Fig. 1. The characteristic amino acid sequence of α3 has Ala residues at positions e and g in a helical wheel. These Ala residues are expected to form a tetramer of helices (*2*).

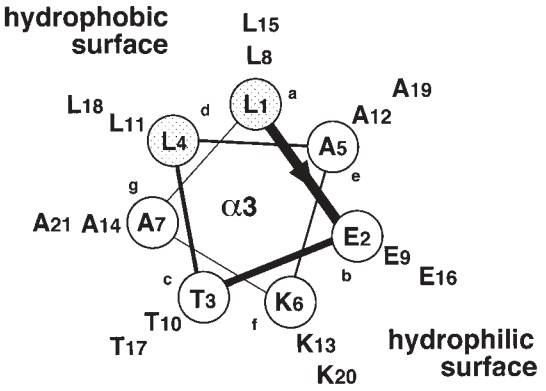

Fig. 1. **Helical wheel and positions a–g of the α3-polypeptide.**

As the formation of the α helix structure of polypeptide α3 depended on its concentration, it was considered that α3 formed an assembly. In fact, this was confirmed by gel filtration and analytical ultracentrifugation experiments, and electron microscopic observations (*2*, *19*). The latter showed fibrous assemblies clearly.

Thus, it is necessary to examine the changes in the formation of α helix and the state of fibrous assembly on amino acid substitutions at positions a and d in the helical wheel of the α3 polypeptide. Three kinds of hydrophobic aliphatic amino acid, Ile, Val and Ala, were selected for the amino acid replacement. Generally, Ile and Val residues are often detected at position a in the helical wheel of the helices in the coiled coil–forming proteins, however, it is also known that Ile and Val residues at position d destabilize the coiled coil structur due to methyl group of the β branch in the side chains of these amino acids (*9*, *10*).

Here, we characterized systematically nine kinds of polypeptides replaced with the hydrophobic aliphatic amino

Table 1. **Amino acid sequences of the synthesized polypeptides in this study**. The amino acid residues typed by italic letters represent the substituted amino acids from α3 (L).

α3	L	E	T	L	A	K	A	L	E	T	L	A	K	A	L	E	T	L	A	K	A
(position)	a	b	c	d	e	f	g	a	b	c	d	e	f	g	a	b	c	d	e	f	g
1Iα3	*I*	E	T	L	A	K	A	*I*	E	T	L	A	K	A	*I*	E	T	L	A	K	A
4Iα3	L	E	T	*I*	A	K	A	L	E	T	*I*	A	K	A	L	E	T	*I*	A	K	A
I14Iα3	*I*	E	T	*I*	A	K	A	*I*	E	T	*I*	A	K	A	*I*	E	T	*I*	A	K	A
1Vα3	*V*	E	T	L	A	K	A	*V*	E	T	L	A	K	A	*V*	E	T	L	A	K	A
4Vα3	L	E	T	*V*	A	K	A	L	E	T	*V*	A	K	A	L	E	T	*V*	A	K	A
1V4Vα3	*V*	E	T	*V*	A	K	A	*V*	E	T	*V*	A	K	A	*V*	E	T	*V*	A	K	A
1Aα3	*A*	E	T	L	A	K	A	*A*	E	T	L	A	K	A	*A*	E	T	L	A	K	A
4Aα3	L	E	T	*A*	A	K	A	L	E	T	*A*	A	K	A	L	E	T	*A*	A	K	A
1A4Aα3	*A*	E	T	*A*	A	K	A	*A*	E	T	*A*	A	K	A	*A*	E	T	*A*	A	K	A

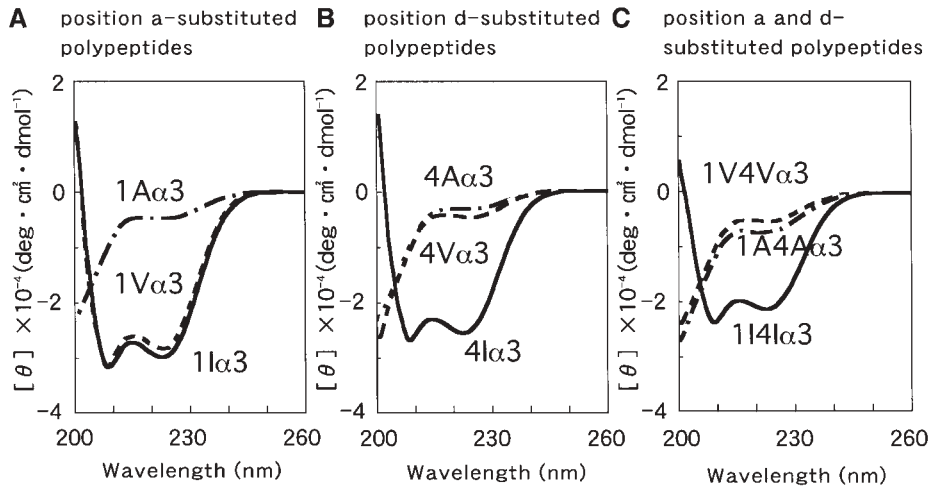

Fig. 2. **The CD spectra of the position a and d–substituted polypeptides.** The CD spectra of polypeptides were measured in a solution comprising 0.15 mM each peptide in 1 M KCl–10 mM citrate buffer (pH 2), at 30°C. The Ile-substituted polypeptides (—), the Val-substituted polypeptides (- - -), and the Ala-substituted polypeptides (-·· -).

acids at positions of a and/or d of polypeptide α3 having Leu residues at positions a and d, as follows: position a–replaced polypeptides, 1Iα3, 1Vα3, and 1Aα3; position d–replaced polypeptides, 4Iα3, 4Vα3, and 4Aα3; position a and d–replaced polypeptides, 1I4Iα3, 1V4Vα3 and 1A4Aα3.

The substituted positions are shown in the helical wheel of polypeptide α3 in Fig. 1 and Table 1.

EXPERIMENTAL PROCEDURES

Preparation of Polypeptides by Means of the Gene Engineering Method—All of the polypeptides used in this study were prepared by means of the gene engineering method (*2*). The polypeptides were expressed as fused proteins with the N-terminal part of porcine adenylate kinase (ADK), as the expression efficiency of the vector constructed for this protein (ADK) was high in *E. coli* (*19*). The DNA strand corresponding to the inner part of ADK was added with the sequence ATG coding Met and thereafter the DNA sequence corresponding to the designed polypeptide in order to cut the polypeptide out from the fused protein product by brome cyan treatment. The DNA chains coding the polypeptides consisted of the most suitable codons in *E. coli* and complementary DNA chains were designed. These DNAs were synthesized by Nissinbo Co., Ltd. The double-stranded DNAs were prepared by annealing.

Each chemically synthesized double-stranded DNA was inserted between restriction sites *Eco*RI and *Bam*HI of the cloning vector pUC18. After checking the base sequence, the DNA was treated with three enzymes, *Eco*RI, *Pst*I, and 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 expression vector pMKAK3′Δ used in this study. Expression vector pMKAK is a circular DNA, which is able to express a great amount of ADK as inclusion bodies in *E. coli* (*2*, *18*).

E. coli strain JM109, which was transformed with pMKAK3′Δ, was incubated in LB medium containing ampicilin at 37°C for 6 h. Cells were collected by centrifugation at 2,000 × *g* for 10 min at 4°C. The harvested cells were washed two times with TE (10 mM Tris-HCl, pH 8.0) buffer containing 1 mM 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 8,000 × *g* for 10 min at 4°C. In order to split off the

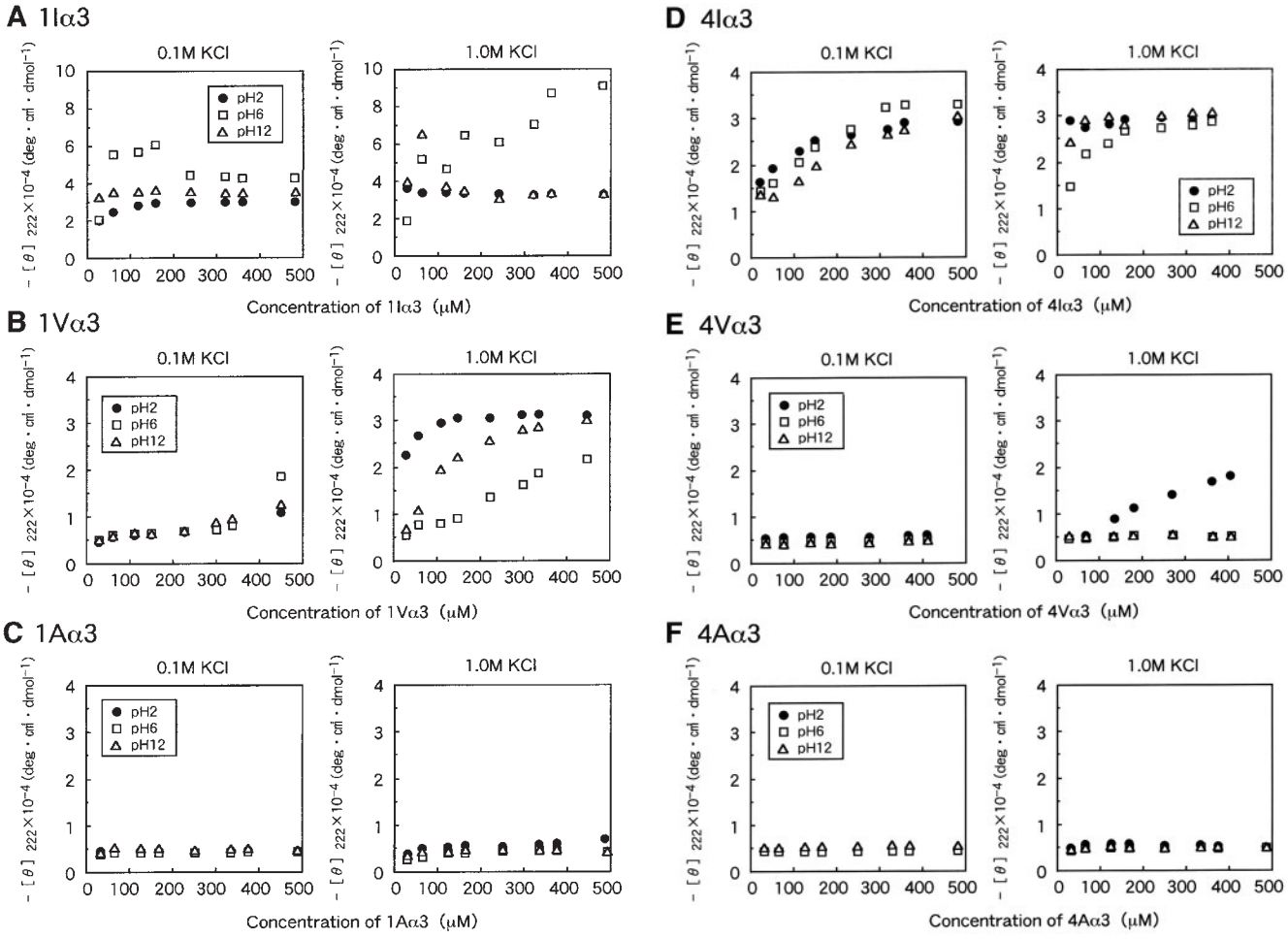

Fig. 3. **The molar ellipticity [θ] value at 222 nm in CD spectra of the position a and/or d–substituted polypeptides under different conditions.**

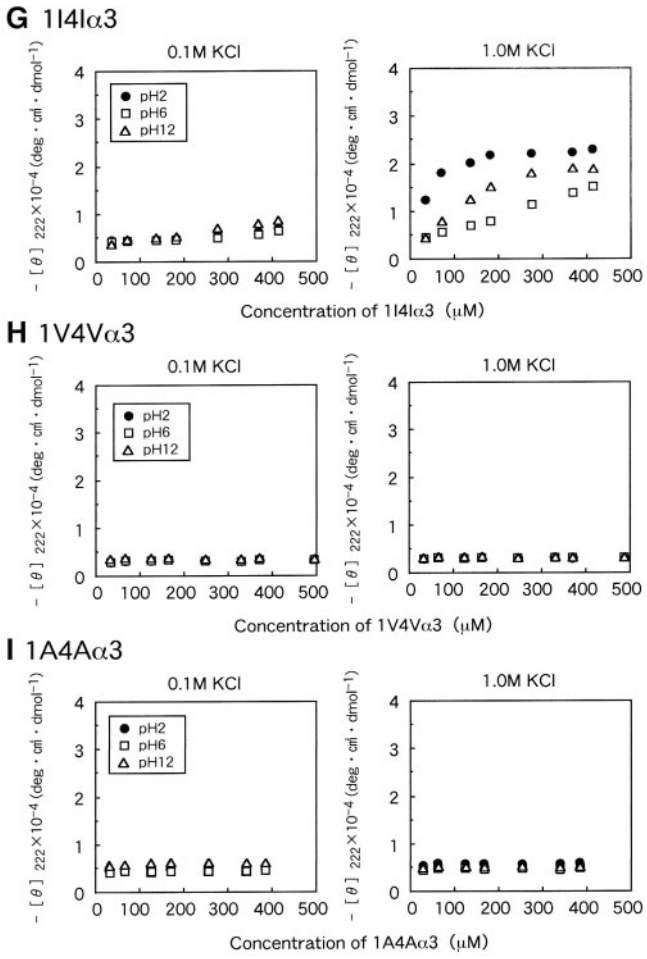

Fig. 3. Continued.

N-terminal part of ADK from the designed polypeptide, the inclusion bodies were incubated in 70% formic acid, 2% BrCN, 1% 2-mercaptoethanol at 37°C for 48 h, and then dialyzed against MilliQ water at 4°C for 8 h and then against 0.01 M Tris-HCl buffer (pH 8.5) at 4°C over night.

The precipitate was centrifuged off at 8,000 × *g* for 10 min at 4°C. The dialyzed solution was applied to a DEAE cellulose column (3 × 10 cm) and then washed with 200 ml 0.01 M Tris-HCl buffer (pH 8.5) at the flow rate of 1 ml/min. The designed polypeptides were eluted with 5 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 HPLC on a C4 column (C4 P-300-5, ϕ 4.6 × 150 mm; Tokyo Kasei Co., Ltd.). A linear gradient of solvents A and B was applied for 60 min. Solvent A comprised 0.1% trifluoroacetic acid (TFA) (v/v) in MilliQ water, and solvent B comprisrd 0.1% TFA (v/v), 80% acetonitrile (v/v) in MilliQ water. The polypeptide-containing fractions were collected and lyophilized. Each polypeptide was dissolved in MilliQ water, and then purified by reverse-phase HPLC on a C18 column (L-column ODS, C18, ϕ 4.6 × 150 mm; Chemicals Inspection & Testing Institute, Japan.). The solvents and gradient conditions were the same as those used in the case of the C4 column. The purified polypeptide was lyophilized and then dissolved in MilliQ water. It was stored in a deep freezer at –30°C.

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

Circular Dichroism (CD) Measurements—CD spectra of the polypeptides were measured with a Circular Dichroism Spectrophotometer J-720 (JASCO). The light path length of the cell was 1mm. The temperature was regulated by circulation of electrostatically controlled water through a jacket surrounding the cell.

Fourier Transform Infrared Spectroscopy (FTIR) Measurements—Infrared spectra were measured with an FTIR Spectrophotometer 8400 (SHIMADZU). The cell was a KRS-5 cell (SHIMADZU), which contained a mixture of thallium bromide and thallium iodide. The light path length of the cell was 0.02 mm. Heavy water (D_2O) was used as the sample solvent. The measurements were performed in 0.01 M phosphate buffer (pD 6) containing 0.1 M KCl. The polypeptide concentration was 5 mg/ml.

Electron Microscopy Observation—The fibrous association of the polypeptides was observed by transmission electron microscopy.

An aliquot of a solution comprising 50 μM polypeptide sample in 0.01 M citrate buffer (pH 6.0) containing 0.1 M or 1 M KCl was mounted on a grid with a film of carbon-coated parlodion, then the grid was washed with the same buffer as that for the sample. The excess solution was removed with filter paper. Then, the grid was negatively stained with 2.5% uranyl acetate.

The sample-mounted grid was examined under a Transmission Electron Microscope JEM1010 (JEOL) at an accelerating voltage of 80 kV. The electron micrographs were recorded on Fuji FG electron image film (11.8 × 8.2 cm) (*19*).

RESULTS

Circular Dichroism (CD) Spectra Measurements—In order to check the secondary structure, CD spectra of the substituted polypeptides were measured in 10 mM citrate buffer (pH 2) containing 1 M KCl at the polypeptide concentration of 150 μM at 30°C, under which conditions α3 forms typical α helix. All of the Ala-substituted polypeptides, *i.e.*, 1Aα3, 4Aα3, and 1A4Aα3, and two of the Val-substituted polypeptide, *i.e.*, 4Vα3 and 1V4Vα3, were not able to form α helix. On the other hand, the CD spectra of 1Vα3 and all of the Ile-substituted polypeptides, *i.e.*, 1Iα3, 4Iα3 and 1I4Iα3, showed two minima at 208 nm and 222 nm, representing α helix, as shown in Fig. 2.

Then, CD spectra of the nine kinds of substituted polypeptides were measured under various conditions, the pH value (2, 6, 12), KCl concentration (0.1 M, 1.0 M), and polypeptide concentration being changed, to determine the optimum conditions for helix formation. The molar ellipticity at 222 nm was plotted against the concentration of each sample under definite conditions, as shown in Fig. 3. As the CD spectra depended on the polypeptide concentration under some conditions, the polypeptide chains seem to associate with each other when a helix is formed. The CD spectrum was measured within 1 h after the sample was solved in the definite solvent. The shape of spectrum was not changed during 1 h.

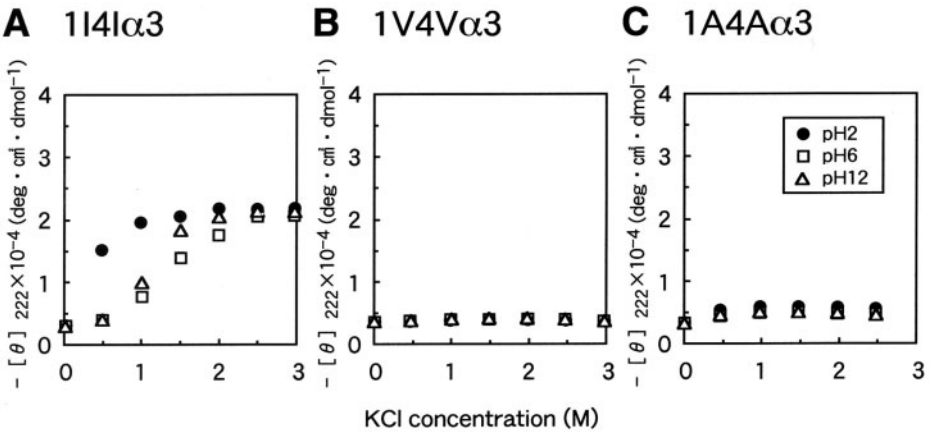

Fig. 4. **The molar ellipticity [θ] value at 222 nm in CD spectra of the position a and d–substituted polypeptides in high KCl concentration (1–3 M).** The CD spectra of polypeptides were measured in a solution comprising 0.15 mM each peptide, at 30°C.

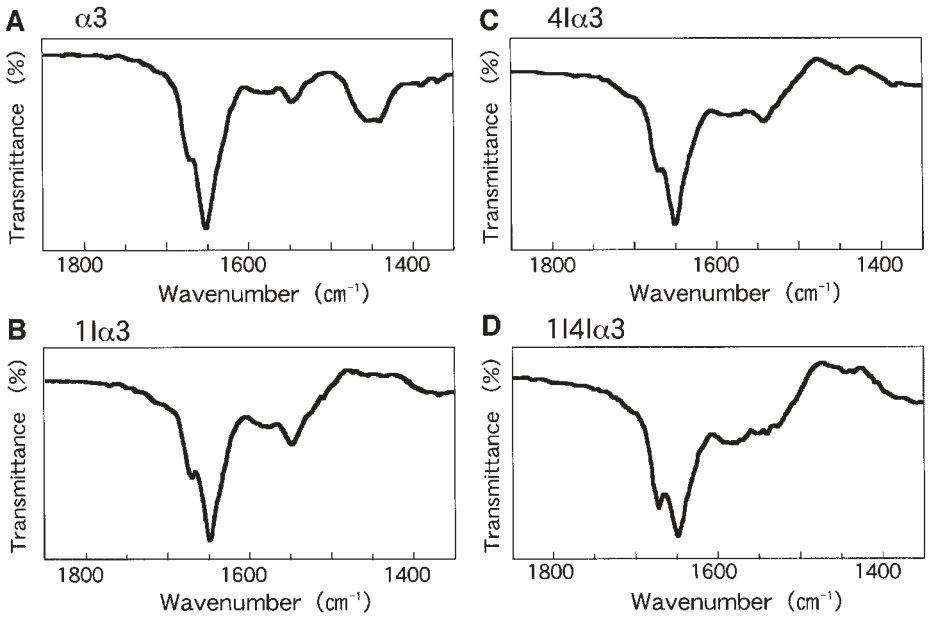

Fig. 5. **Fourier transform infrared spectroscopy (FTIR) spectra of α3 and Ile-substituted polypeptides.** The FTIR spectra of polypeptides were measured in a solution comprising 5 mg/ml each polypeptide in 1 M KCl 10 mM phosphate buffer (pD 6) with D_2O (heavy water), at room temperature.

In the case of 1Iα3 in 1 M KCl, pH 6, the CD spectrum showed the typical α helix structure and the absolute value of mean residue molar ellipticity [θ] at 222 nm was larger than 8×10^4, suggesting that the complete helical chains associate with each other strongly. 1Vα3 was not able to form α helix under 0.1 M KCl conditions, but formed α helix under 1 M KCl conditions and with a high polypeptide concentration. 1Aα3 was not able to form α helix under any conditions.

In contrast to 1Iα3, 4Iα3 did not show strong association under pH 6 conditions although 4Iα3 formed α helical structure at any pH. 4Vα3 only formed α helix in 1 M KCl (pH 2), *i.e.*, it was not able to form α helix under other pH conditions. 4Aα3 was not able to form α helix under any pH or salt conditions.

As shown in Figs. 2 and 3, 1I4Iα3 was not able to form α helix under 0.1 M KCl conditions. However, 1I4Iα3 formed α helix with 1 M KCl and higher concentrations (2 M and 3 M) as shown in Fig. 4. 1V4Vα3 and 1A4Aα3 were not able to form α helix under any pH condition, or with higher KCl concentrations (2 M and 3 M) (Fig. 4).

Fourier Transform Infrared Spectroscopy (FTIR) Spectra Measurements—To confirm the secondary structure of α3 and the three kinds of Ile-substituted polypeptides, *i.e.*, 1Iα3, 4Iα3 and 1I4Iα3, FTIR spectra of these polypeptides were measured in heavy water under the conditions of 1 M KCl, 10 mM phosphate buffer (pD 6) and 5 mg/ml polypeptide concentration. As shown in Fig. 5, the FTIR spectra of α3 and the three Ile-substituted polypeptides exhibited a main absorption band at 1,650 cm^{-1}, so these polypeptides form α helix structure. The FTIR spectrum was measured within 1 h after the sample was solved in the definite solvent. The shape of spectrum was not changed during 1 h.

Observation by Electron Microscopy—Under the α helix–forming conditions, fibrous assembly was observed for α3 and its derivatives in which the Leu residues at positions a and/or d in α3 were substituted by other hydrophobic aliphatic amino acid residues as shown in Figs. 6 to 8. The width of the fibrous assemblies was about 25 Å. At pH 2, the fibrous assemblies of β3, 1Iα3 and 4Iα3 often intertwined to form network of fibers. At pH 6, these polypeptides were observed to form thicker straight strings with a width of 10–100 nm. At pH 12, assemblies of these polypeptides comprised shorter fibers as well as similar sized fibers to those observed in the pH 2 experiments.

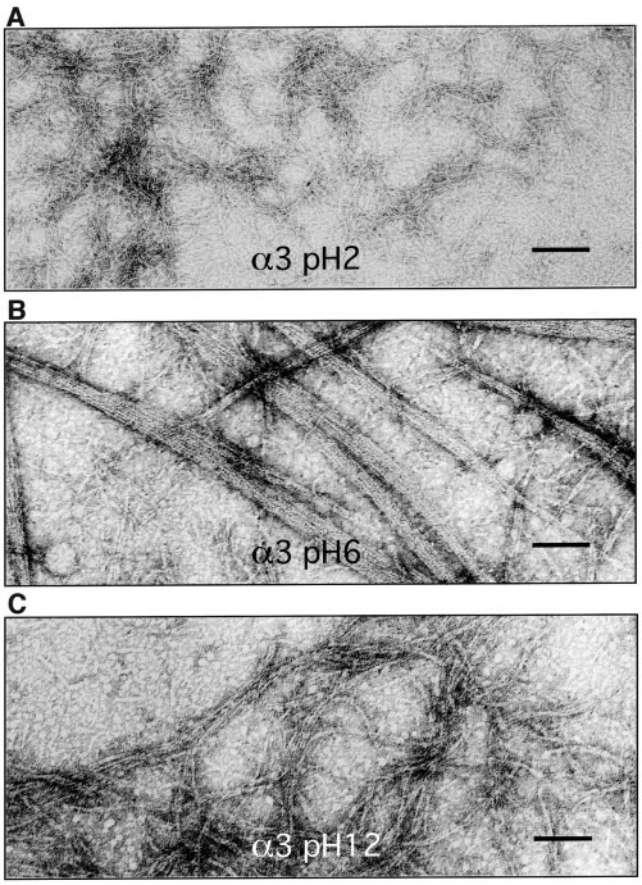

Fig. 6. **Electron micrographs of α3 from the solution containing 1 M KCl.** The scale bar represents 100 nm.

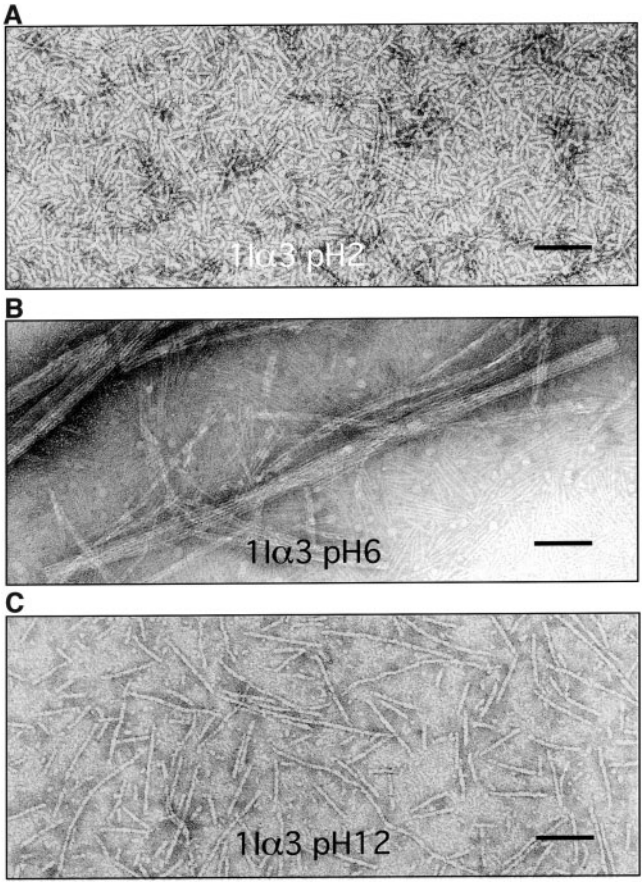

Fig. 7. **Electron micrographs of 1I α3 from the solution containing 1 M KCl.** The scale bar represents 100 nm.

On the other hand, the shape of the fibrous assemblies of polypeptides 1I4Iα3 1Vα3 and 4Vα3, as shown in Figs. 9 to 11, was different from the above-mentioned cases. The fibrous assemblies of 1Vα3 were much shorter than in the case of α3 or 1Iα3 in 1 M KCl at pH 6. In the case of 4Vα3, amorphous association was observed in 1 M KCl and at pH 2. 1I4Iα3 formed large assemblies, in which short fibers associated in random directions under all the buffer conditions. 1V4Vα3 and all the Ala-substituted polypeptides, *i.e.*, 1Aα3, 4Aα3 and 1A4Aα3, which were not able to form α helix, did not form fibrous assemblies under any conditions.

DISCUSSION

The α Helix–Forming Ability of Position a and/or d–Substituted Polypeptides—When the amino acid residues at helical wheel positions a and/or d in amphipathic α helix–forming polypeptide α3, $(LETLAKA)_3$, were changed from Leu to other hydrophobic aliphatic amino acids, the change in the ability of α helix formation was checked mainly by means of CD spectra, solvent, pH and salt concentration conditions being changed. The Ile-substitued polypeptides were able to form α helix as in the case of α3. However, for example, under the same conditions (pH 2, 1 M salt concentration, and 150 μM polypeptide concentration), the absolute values of molar ellipticity [θ] at 222 nm in the CD spectra of α3 and 1I4Iα3 were 2.5×10^4 and 2.2×10^4 deg·cm^2·dmol^{-1}, respectively, so α3 shows a little higher value than 1I4Iα3, revealing that the helical structure of α3 is more stabilized than that of 1I4Iα3.

It has been reported (*21*, *22*) that Ile exhibits a little higher hydrophobicity than Leu, although the side chain of Leu has the same number of carbonate as that of Ile. Accordingly, not only the hydrophobicity, but also the bulkiness of the side chain would affect the helix formation.

In the case of the Val-substituted polypeptides, the α helix structure is only formed in the case of the position a substitution, 1Vα3. In the case of Ala substitution, no polypeptide was able to form the helical structure under any condition.

In the case of the polypeptides forming the α helix structure used in this study, the helical structure was stabilized under the conditions of low pH and a high salt concentration.

These results indicate that the α helix would be destabilized through the electrostatic repulsion at both termini, so that polypeptides should only form the α helix structure under buffer conditions under which the electric repulsion is suppressed (*23*).

From the results and discussion, the following conclusion is drawn for the α helix–forming ability of the positions a and/or d–replaced polypeptides: in polypeptides containing an amino acid carrying an hydrophobic aliphatic chain, higher hydrophobicity is required to form α helix

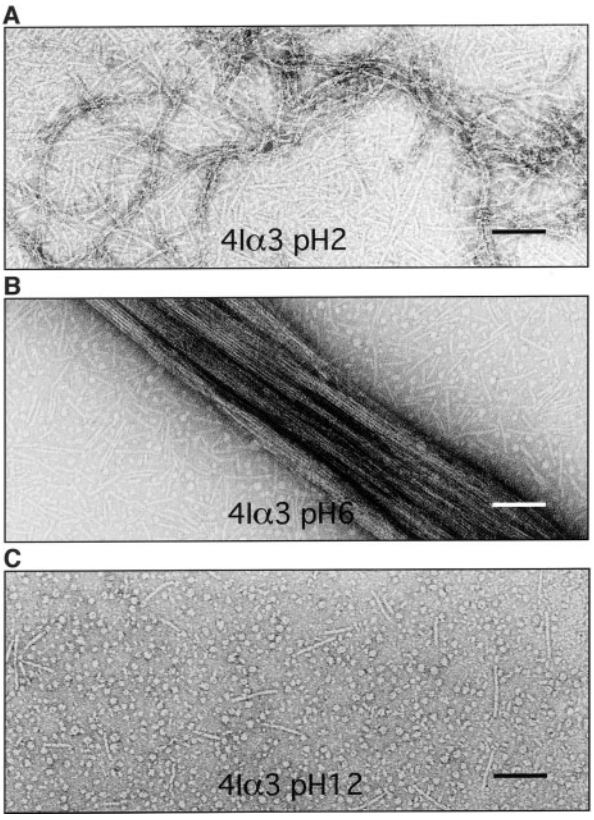

Fig. 8. **Electron micrographs of 4I α3 from the solution containing 1 M KCl.** The scale bar represents 100 nm.

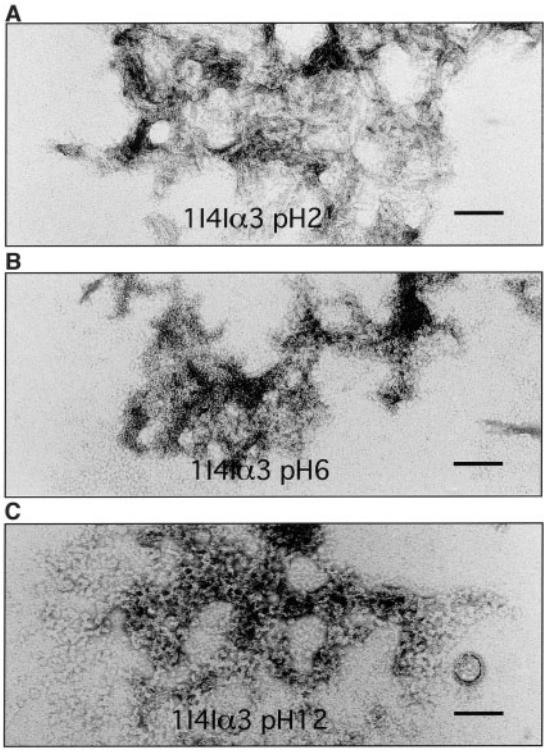

Fig. 9. **Electron micrographs of 1I4I α3 from the solution containing 1 M KCl.** The scale bar represents 100 nm.

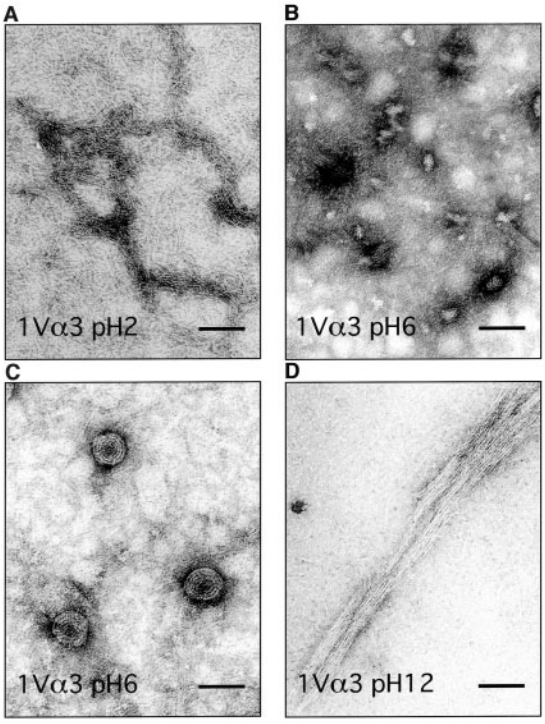

Fig. 10. **Electron micrographs of 1V α3 from the solution containing 1 M KCl.** The scale bar represents 100 nm.

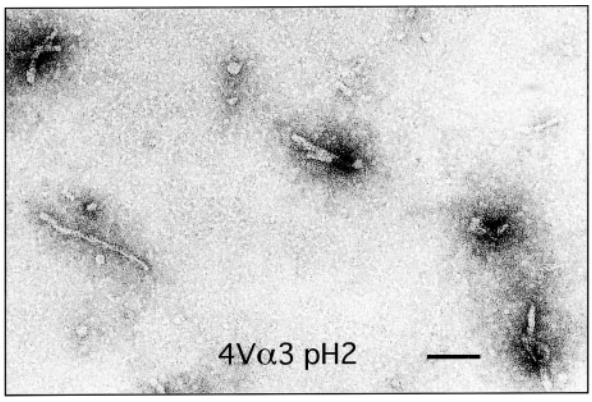

Fig. 11. **Electron micrographs of 4V α3 from the solution containing 1 M KCl.** The scale bar represents 100 nm.

easily and also the bulkiness of the side chain of an amino acid residue affects the ability of helix formation (*9–11*). Accordingly, it is clearly explained that the order of the amino acids at positions a and d in a helical wheel as to the ability of α helix formation is as follows: Leu ⩾ Ile > Val > Ala, as shown by the experimental results.

Relationship between the Abilities of α helix Formation and Fibrous Assembly of Amphipathic Polypeptides. Polypeptide α3 consisting of three repeats of a seven amino acids sequence, LETLAKA, forms an amphipathic helix structure, and it was observed on electron microscopy that the helical molecules assembled into thick and long fibers (*19*). It was expected that Leu residues at the

positions a and d in the helical wheel of α3 were important for formation of the fibrous assemblies. Similar results were obtained when Leu residues were replaced by Ile ones. Furthermore, in the case of the replacement of Leu with Val, fibrous assembly only occurred under the conditions under which α helix was formed. In the case of replacement of Leu residues to Ala ones neither the formation of α helix nor its assembly occurred under any condition. Therefore, the formation of the α helix structure and the fibrous assemblies correlate with each other, and the helical structure should be stabilized by the assemblies.

As the thickness of the minimum fibrous assemblies is approximately 25 Å, it is suggested that four amphipathic helix molecules join together on the hydrophobic surface to produce a bundle or a longer filament.

The Leu residues located at positions a and d in the helical wheel of the α3 polypeptide are important for the assembly of the helices, and when Ile residues replaced the Leu ones the results were the same. Leu residues in α3 are arranged lengthways like a Leu zipper to yield the hydrophobic interface for assembly. However, the lengths and shapes of assembled fibers seemed to be a little different according to the hydrophobicity of the amino acid residues of positions a and d on electron microscopic observation (*24*).

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