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Requirement of Ala Residues at g Position in Heptad Sequence of α-Helix-forming Peptide for Formation of Fibrous Structure

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One feature of the α3-peptide, which has the amino acid sequence of (Leu-Glu-Thr-Leu-Ala-Lys-Ala)$_3$, that distinguishes it from many other α-helix-forming peptides is its ability to form fibrous assemblies that can be observed by transmission electron microscopy. In this study, the effects of Ala→Gln substitution at the e (5th) or g (7th) position in the above heptad sequence of the α3-peptide on the formation of α-helix and fibrous assemblies were investigated by circular dichroism spectral measurement and atomic force microscopy. The 5Qα3-peptide obtained by Ala→Gln substitution at the e position of the α3-peptide was found to form very short fibrils with long-elliptical shape, whereas the 7Qα3-peptide with Gln residues at the g position lost its ability to form such assemblies, in spite of α-helix formation in both peptides; the stabilities of both peptides decreased. These results indicate that Ala residues at the g position in the heptad sequence of the α3-peptide are key residues for the formation of fibrous assemblies, which may be due to hydrophobic interactions between α-helical bundle surfaces.

Key words: α-helix, atomic force microscopy, circular dichroism spectra, fibre formation, heptad sequence.

Abbreviations: AFM, atomic force microscopy; TEM, transmission electron microscopy.

The α-helix is a secondary structure of proteins that contributes to the stability and folding of proteins. Its sequence-stability relationship, as well as several interactions between side chains and the intrinsic helix-forming tendency of amino acids, has been extensively studied (*1–19*). We previously designed and synthesized an amphipathic 21-residue peptide (α3-peptide) with three repeats of the seven-residue (heptad) sequence Leu-Glu-Thr-Leu-Ala-Lys-Ala, anticipating that it will form an α-helical bundle structure through hydrophobic interactions between Leu residues. We found that the α3-peptide exhibits a concentration-dependent stabilization of its α-helix, suggesting the formation of oligomers (*20*). Unexpectedly, we demonstrated that the α3-peptide form fibrous assemblies that can be observed by transmission electron microscopy (TEM) (*21*). To our knowledge, this might have been the first report on the formation of fibrous assemblies by a *de novo*-designed α-helical short peptide, in contrast to many reports on the formation of amyloid mainly composed of β-sheets (*22–31*). Thus, we have synthesized several variants of the α3-peptide to investigate the relationship between the sequence, α-helix stability and formation of fibrous assemblies. When the sequence of the α3-peptide was reversed, the resultant r3-peptide formed a very stable α-helix and long fibres (*32*). Since the α-helix, which does not form fibrous assemblies, is generally destabilized by sequence reversal through electrostatic repulsion with an intrinsic dipole of the α-helix, the stabilization of the α3-peptide by sequence reversal may be specific to only fibre-forming peptides. On the other hand, the α-helix and fibrous assemblies of the α3-peptide are destabilized by substitutions of Leu residues on the hydrophobic surface with less hydrophobic amino acids, possibly owing to the decrease in the degree of hydrophobic interactions (*33*). This is a general feature of peptides that form multimeric α-helical bundle structures.

In this study, we focused on Ala residues at the e (5th) and g (7th) positions in the heptad sequence of the α3-peptide. Since peptides with charged residues (Glu and Lys) at these two positions form a two-stranded coiled-coil structure (*11*, *14*), it is suggested that Ala residues at both or either position in the α3-peptide are required for the formation of fibrous structures. Thus, we substituted these Ala residues of the α3-peptide with less hydrophobic Gln residues, since Gln is a polar amino acid with the strongest α-helix-forming tendency among non-charged and non-hydrophobic amino acids.

α3-peptide variants with Gln residues at the e or g position in the heptad sequence of the α3-peptide, namely the 5Qα3- and 7Qα3-peptides (Fig. 1), respectively, were chemically synthesized and purified using reverse-phase HPLC with an acetonitrile gradient in 0.1% trifluoroacetic acid. Their concentrations were determined by amino acid composition analysis after hydrolysis with 5.7 N HCl at 110°C for 24 h *in vacuo*.

The CD spectra of the 5Qα3- and 7Qα3-peptides were measured using a JASCO J-720 spectropolarimetre in a neutral pH buffer at 30°C to investigate the effects of Ala→Gln substitutions at the e or g position in the

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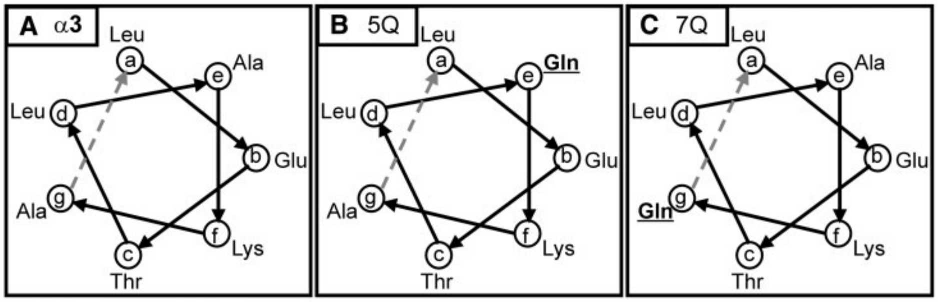

Fig. 1. **Helical wheel representations of (A) α3-, (B) 5Qα3- and (C) 7Qα3-peptides.** The 5Qα3- or 7Qα3-peptide was produced by substituting Ala residues at the e (5th) or g (7th) position in the heptad sequence of the α3-peptide.

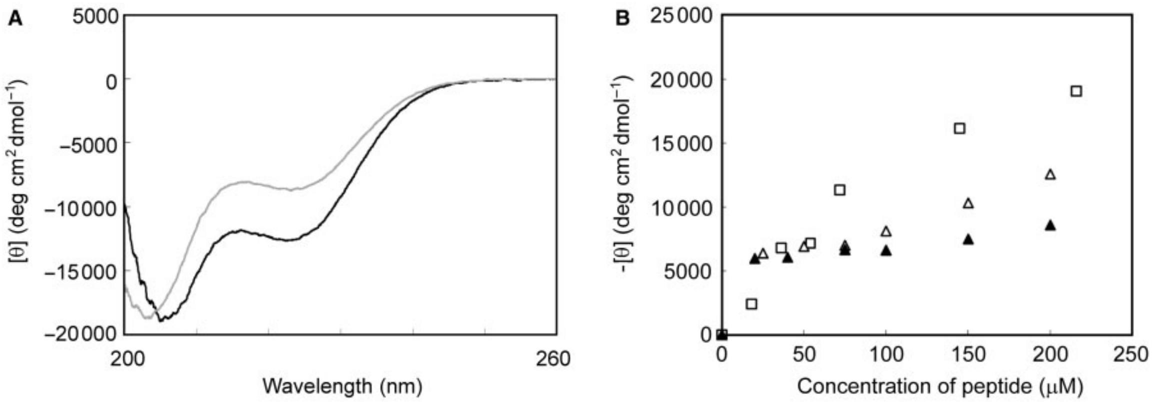

Fig. 2. **(A) CD spectra of 5Qα3-peptide (black line) and 7Qα3-peptide (gray line) at 200 μM and (B) peptide concentration dependence of [θ] at 222 nm of the α3-peptide (open square), 5Qα3-peptide (open triangle) and 7Qα3-peptide (filled triangle) in 10 mM phosphate buffer (pH 6.0) containing 0.1 M KCl.** After the concentrations of the peptides were determined by amino acid composition analyses, the CD spectra at various peptide concentrations were measured at 30°C using a JASCO J-720 circular dichroism spectropolarimetre with a path length of 1 mm; the results are expressed as mean residue molar ellipticity.

heptad sequence of the α3-peptide on the α-helix stability of the α3-peptide. It was found that both peptides showed CD spectra that indicate α-helix formation (Fig. 2A), as in the case of other variants of the α3-peptide. However, since the helix contents of both peptides were lower than that of the α3-peptide, the concentration dependences of [θ] at 222 nm for the two peptides were investigated and compared with that of the α3-peptide.

As shown in Fig. 2B, the [θ] values of the 5Qα3- and 7Qα3-peptides increased as peptide concentration increased, which strongly indicates that the α-helices of these peptides are stabilized by oligomerization, as in the case of other variants of the α3-peptide. However, the α-helix of the α3-peptide was destabilized by the substitution of Ala residues at the e position in the heptad sequence with Gln residues, and much more prominently by the substitution at the g position. As a result, among the peptides examined, the 7Qα3-peptide had the most unstable α-helix. The helix contents of the α3-, 5Qα3- and 7Qα3-peptides at about 200 μM, which were estimated using the equation $[\theta]_{222\,\mathrm{nm}}/\{-40{,}000(1-2.5/n)+100T\}$, where $n=21$ and $T=30$ (*34*), were 59, 39 and 27%, respectively.

Since the α3-peptide was demonstrated to form fibrils 5–10 nm in width and intermediate in length that can be observed by TEM in a neutral pH buffer, we observed such fibrous structures of the α3-peptide in air at 20°C by atomic force microscopy (AFM, JEOL-JSTM-4200D), as well as those of the 5Qα3- and 7Qα3-pepitdes to determine the effects of Ala→Gln substitution in the α3-peptide on the formation of fibrous assemblies. AFM in the tapping mode was performed with Si cantilevers (spring constant: 1.38 N/m, resonance frequency: 74 kHz) on an atomically flat cleaved mica (001) surface immersed in 20 μl of peptide solutions. The typical scan speed was about 3 min/image.

Figures 3A and B–D show AFM images of the α3-peptide at 4 and 50 μM, in phosphate buffer with pHs 5–6. No fibrous structures were observed at pH 2, 7 or 8. It is clear that the α3-peptide at 50 μM formed fibrils as demonstrated by TEM, whereas at 4 μM no such fibrous assemblies were observed. Fibrils were observed between pHs 3 and 6. Each single fibril had a length of >1, 000 nm. At pH 5.5, each fibril had a width of 33.0 ± 0.4 and 2.1 ± 0.4 nm in height.

When the 5Qα3- and 7Qα3-peptides at 40 μM were observed by AFM, no fibrous assemblies were observed. Since the α-helices of these peptides were less stable than that of the α3-peptide, as demonstrated by CD measurements, AFM was carried out at higher peptide

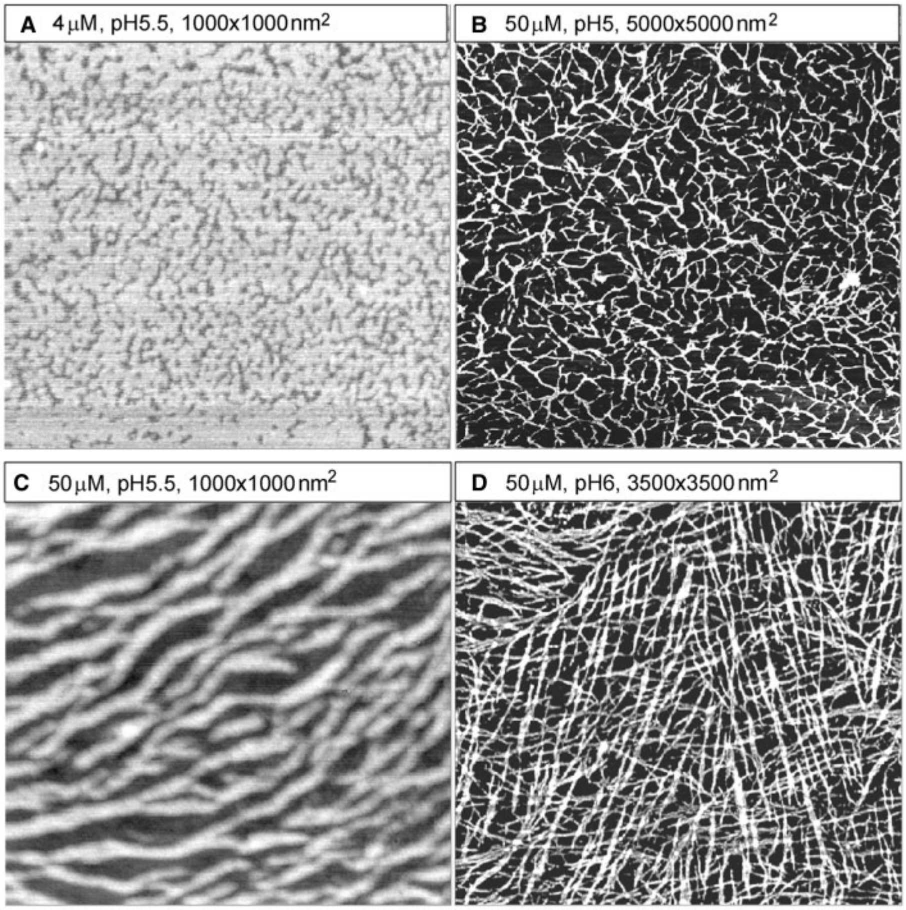

Fig. 3. **AFM images of the α3-peptide from 4 μM solution at pH 5.5 (A) and from 50 μM solution at pHs 5 (B), 5.5 (C) and 6 (D).** A single fibre has a length of >1000 nm.

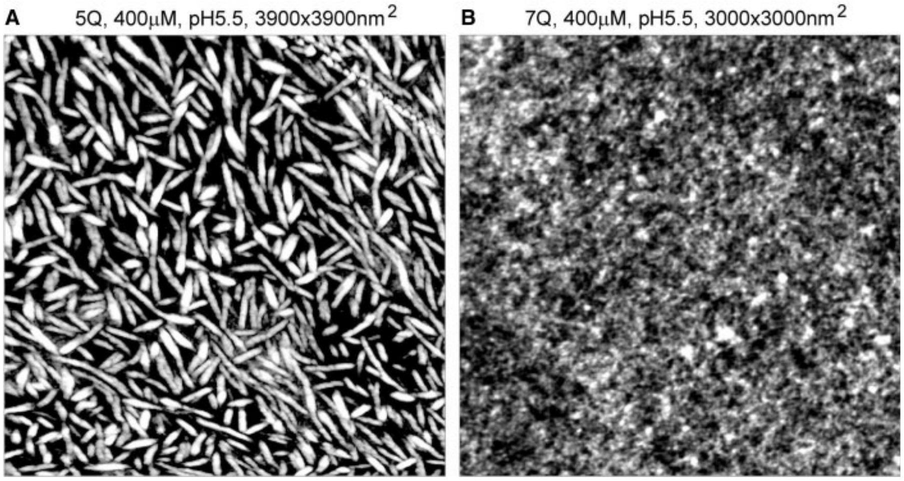

Fig. 4. **AFM images of the 5Qα3-peptide (A) and 7Qα3-peptide (B) from 400 μM solution.**

concentrations. When the concentration of the 5Qα3-peptide was increased up to 400 μM, many very short fibrils with long-elliptical shape were detected, as shown in Fig. 4A. Each single 5Qα3-peptide fibril was 400 ± 100 nm in length, 2.9 ± 0.8 nm in height and 61.7 ± 8.5 nm in width. However, no such structures were observed for the 7Qα3-peptide at the same peptide concentration (Fig. 4B). These results strongly indicate that Ala residues at the g position of the heptad sequence of the α3-peptide largely contribute to the formation of fibrous assemblies.

The destabilization of the α-helix formed by the 5Qα3-peptide is considered to be due to the difference in α-helix formation tendency between Gln and Ala. However, since some degree of hydrophobicity is retained in the 5Qα3-peptide, very short fibrils with long-elliptical shape of this peptide might have been observed by AFM, although a higher peptide concentration was required for such observation. In contrast, the 7Qα3-peptide that also has Gln residues did not form fibrous assemblies. To explain this phenomenon, we considered that the chemical environments around the Gln residues in the helical bundle structure differed between the 5Qα3- and 7Qα3-peptides. From the helical wheel representation (Fig. 1), the surface formed by c-, d- and g-position residues is more hydrophobic than the opposite surface formed by a-, b- and e-position residues, since a Thr residue with methyl and hydroxyl groups is more hydrophobic than a Glu residue with a negative charge. Therefore, the 5Qα3-peptide that retains a more hydrophobic surface is considered to have an ability to form supramolecular assemblies, whereas the 7Qα3-peptide in which the hydrophobicity of the surface formed by c-, d- and g-position residues is weakened by Ala→Gln substitution at the g position seems to lose its ability to form such assemblies. Zeng *et al.* (*35*) have previously produced various e- and g-position mutants of a two-stranded GCN4 leucine zipper by random mutagenesis and have shown that type II mutants that form higher order oligomers commonly have Ala residues at the g position. Their finding is consistent with our results in this study. Therefore, similar mechanisms of forming higher order oligomers may operate in their peptides and ours; however, they provided no explanation for their finding.

It is also demonstrated that a decrease in the hydrophobicity of the helical surface formed by c-, d- and g-position residues of the α3-peptide by Leu→Val substitution at the d position results in the loss of the ability to form fibrous assemblies (*33*). However, in this case, the resultant peptide (4Vα3-peptide) also had no ability to form α-helices; thus, the formation of fibrous assemblies is closely related to α-helix formation. In contrast, the 7Qα3-peptide in this study lost its ability to form fibrous assemblies in spite of retaining its ability for α-helix formation. Therefore, Ala residues at the g position in the α3-peptide are concluded to be the key residues for forming fibrous assemblies. In the future, subsequent analyses using α3-derived peptides with various sequences will clarify the detailed mechanisms of the fibre formation of the α3-peptide.

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