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Metal-triggered Nanofiber Formation of His-containing β-Sheet Peptide

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Metal-responsive nanofiber formation was achieved using a designed β-sheet peptide with His residues at the both N- and C-termini. The designed peptide, whose sequence was HPKFKIIEFEPH, was in a random coil in a neutral buffer, and did not show any structural change. However, the peptide underwent the structural transition to a β-sheet conformation in response to metal ions such as Cu^{2+}, Zn^{2+}, Ni^{2+}, or Co^{2+}, and Cu^{2+} was the most effective. The 1-anilinonaphthalene-8-sulfonate (ANS) binding assay suggested that the peptide assemblages in the presence of Cu^{2+} had a well-packed structure. The transmission electron microscopy study revealed that the peptide formed tape-like fibers in the presence of Cu^{2+}, but amorphous flocculates in the presence of other metals. The peptide assemblages were rapidly broken by addition of EDTA. The metal responsiveness was acquired in the peptide assembly and nanofiber formation by attachment of His residues on the both termini of the β-sheet peptide.

Keywords: Nanofiber formation; Amyloid fibrils; β-Sheet peptide; Metal Ion

INTRODUCTION

Molecular self-assembly has been widely applied to construct nanostructures in bottom up approach, and using biomolecules as a building block attracts great attention because of the sophisticated ability in the structure formation [1–9]. Amyloid fibrils are known in the relation to the fatal disease such as Alzheimer's disease and prion disease, and understood as resultant aggregates of misfolded proteins [10–12]. However, in the other aspect, peptide and protein fibrils are successfully self-assembled objects and noticed as potential nanomaterials [13–15] because of the straight fibril structure with a width of 10 nm around, in which numerous β-strands regularly align [16,17]. Recently, fibril or fiber formation based on a β-sheet structure is abundantly found in proteins and peptides derived from both of natural and artificial ones [18–23]. The relationship between the amyloid fibril formation and the amino acid sequence in natural proteins seems to be mostly unrevealed at present. On the other hand, the molecular properties of artificial β-sheet peptides and the self-assembling manner can be adjustable by the amino acid sequence design. In addition, by chemical synthesis, small peptides can be easily prepared accompanied with incorporation of functionality, so that the small peptides can be one of fascinating candidates for the component of nanomaterials. A variety of designed β-sheet peptides have shown that fibril and gel formation properties are affected by circumambient conditions such as pH, salt, and temperature [24–28]. Regulation of fiber formation by environmental stimulus can be one of useful characters in processing and applying the peptide assembly for materials. We are interested in regulation of self-assembled structures of β-sheet peptides by the molecular design [29–33]. Recently, we have reported the fabrication of peptide fibers using 10-residual β-sheet peptides [29]. The designed peptides dissolved in an aqueous solution spontaneously formed a uniform fiber structure with a width of 100 nm around. Based on these peptides, we developed here the peptide fibers with environmental responsiveness. As an environmental factor, we supposed that metal ions potentially regulate the fiber formation by forming coordination complexes as well as affecting electrostatic interactions.

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Actually, metal ions are frequently employed in natural proteins to generate the specific structures and functions, and also the effects of metal ions on natural amyloid fibrils are currently argued [34,35]. To develop metal-responsive fiber formation, we arranged His residues at the both N- and C-termini of the previously designed β-sheet peptides. The peptide designed here lost the ability of spontaneous β-sheet and fiber formation that the original peptides had, but it was activated to assemble and form the fiber structure in response to metal ions, suggesting that the responsiveness for metal ions was successfully incorporated in the fiber formation by the peptide design.

MATERIALS AND METHODS

Peptide Synthesis

The peptide was synthesized manually by the 9-fluorenylmethyloxycarbonyl (Fmoc) solid-phase method [36], using following Fmoc-amino acid derivatives: Fmoc-Glu(O*t* Bu)-OH, Fmoc-Ile-OH, Fmoc-Lys(Boc)-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-His(Trt)-OH (*t* Bu, *tert*-butyl; Boc, *tert*-butyloxycarbonyl; Trt, trityl). The first amino acid derivative was attached on a 2-chlorotrityl chloride resin as follows. Fmoc-His(Trt)-OH (0.6 equiv) dissolved in dry dichloromethane (DCM) containing a small amount of dimethylformamide (DMF) was added to the resin and then *N*,*N*-diisopropylethylamine (DIPEA, 1.5 equiv) was added. After agitation for 1 h, methanol was added to end-cap any remaining reactive trityl groups. After removal of Fmoc group using 5% piperidine (PPD) in DCM/DMF (1:1), the peptide chain was subsequently assembled on the resin, using Fmoc-amino acid derivatives (3 equiv), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, 3 equiv), DIPEA (6 equiv), 1-hydroxybenzotriazole hydrate (HOBt·H2O, 3 equiv) in *N*-methylpyrrolidone (NMP) for coupling, and 20% PPD/NMP for Fmoc removal. Cleavage of the peptide from the resin and deprotection of amino acid side chains were achieved by treatment with trifluoroacetic acid (TFA)/triisopropylsilane/water (95:2.5:2.5). The peptide was precipitated with diethyl ether. The resultant peptide was purified by semi-preparative reversed-phase HPLC on a COSMOSIL 5C18-AR-300 packed column (10 × 250 mm) using a linear gradient of acetonitrile/0.1% TFA at a flow rate of 3.0 ml min^{-1}. The peptide was identified in satisfactory results by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS) and amino acid analysis. MALDI-TOFMS was performed on a Shimadzu KOMPACT MALDI III mass spectrometer using 3,5-dimethoxy-4-hydroxycinnamic acid as a matrix. Amino acid analysis was carried out using a Wakopak WS-PTC column (4.0 × 200 mm) after hydrolysis in 6 M HCl at 110°C for 24 h in a sealed tube and labeling by phenylisothiocyanate (PITC).

Preparation of Peptide Solution

As a peptide stock solution, the peptide **His-FI** was dissolved at a concentration of 2 mM in 15% ethanol/water containing 0.1% TFA. The peptide stock was diluted to a concentration of 100 μM with 20 mM Tris·HCl buffer (pH 7.4), and the peptide solutions were allowed to stand at room temperature. Stock solutions of metal ions were prepared by dissolving the chloride salts of metal ions, Cu^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+}, at a concentration of 10 mM.

Circular Dichroism Spectroscopy

Circular dichroism (CD) measurements were performed on a J-720WI spectropolarimeter at 25°C using a quartz cell with 1.0 mm pathlength. Peptide solutions were prepared by diluting the peptide stock with 20 mM Tris·HCl buffer (pH 7.4) containing appropriate concentrations of metal ions. The measurement was started immediately after the dilution.

Fluorescence Spectroscopy

Fluorescence emission measurements were performed on a Shimadzu RF-5300PC spectrofluorophotometer at 25°C with excitation at 390 nm. A stock solution of 1.24 mM ANS was prepared, and an appropriate aliquot of the ANS stock was added to the peptide solutions incubated sufficiently for more than 7 days. Final concentrations of the peptide and ANS were 90 μM and 124 μM, respectively.

Transmission Electron Microscopy

Collodion-coated copper EM grids were placed coated side down onto the peptide solution, and excess solution was removed by blotting with filter-paper. The grids were washed by floating on water and water was removed by blotting. The sample was negatively stained with 2% (w/v) phosphotungstic acid for 30 s. The grids were blotted and allowed to dry gradually at room temperature. All images were taken using a Hitachi H-7500 electron microscope operating at 100 kV.

RESULTS

Peptide Design and Synthesis

We have previously demonstrated that the peptides spontaneously take a β-sheet structure and form

fibers with a 100 nm-width in a neutral buffer [29]. Based on these peptides, the metal responsive peptide was designed by additionally placing His residues at the both N- and C- termini. His residues were expected not only to work as metal ligands but also to increase the peptide solubility by the hydrophilic nature and potential charge repulsion to prevent its β-sheet formation by self-association. The designed peptide **His-FI**, whose sequence is HPKFKIIEFEPH, was synthesized by the Fmoc solid phase synthetic method. The resultant peptide was purified by reversed-phase HPLC and identified by MALDI-TOFMS and amino acid analysis. The peptide was dissolved at 2 mM in 15% ethanol/water containing 0.1% TFA for the stock solution. The stock solution was diluted by 20 mM Tris·HCl buffer (pH 7.4) for each measurement. The peptide solutions were allowed to stand at room temperature for each time period.

Fiber Formation Induced by Copper Ion

Circular dichroism (CD) studies revealed that the designed peptide **His-FI** was in a random coil in the buffer (pH 7.4) at peptide concentrations less than 400 μM. The peptide did not show any structural change during at least 7 days, suggesting that His residues placed at the both N- and C-termini made the peptide unable to form a β-sheet structure spontaneously. Therefore, the subsequent experiments were performed using the peptide at a concentration of 100 μM. On addition of Cu^{2+}, **His-FI** dramatically changed the structure to a β-sheet, suggested by the CD spectra with a negative maximum at 223 nm and a positive maximum at 201 nm (Fig. 1a and b). The peptide at the concentration of 100 μM was predominantly in a random coil shortly after the addition of Cu^{2+}, except for a slight β-sheet form in the presence of 50 μM Cu^{2+}. Then, the peptide obviously underwent the structural transition to a β-sheet form within 24 h at Cu^{2+} concentrations from 100 μM to 1 mM, although the slight β-sheet form initially observed at 50 μM Cu^{2+} did not change. The CD signals at 201 nm after 4 days were plotted as a function of metal ion concentrations (Fig. 1c). The signal increase reflecting the β-sheet formation was dependent on Cu^{2+} concentrations, and reached to plateau at 0.5-1.0 mM. The β-sheet structure induced by Cu^{2+} was completely unfolded to a random coil by addition of EDTA (Fig. 2). To the solution of β-sheet peptide composed of **His-FI** (100 μM) and Cu^{2+}(500 μM) incubated for ca. 7 days, added EDTA decreased the β-sheet content, and the destruction to a random coil was accomplished at the EDTA concentration of 500 μM.

To investigate the assemblages of **His-FI** formed by addition of Cu^{2+}, 1-anilinonaphthalene-8-sulfonate (ANS) binding assay was carried out. ANS binds to exposed hydrophobic regions present in a partially folded protein, accompanied with an increase in the fluorescence emission and a blue shift of the maximum [37]. Amyloid fibril formation can be examined by ANS, where the emission of ANS is weak for matured fibrils but strong for a partially folded intermediate [38,39]. ANS was added to the peptide solutions which were incubated sufficiently for more than 7 days, and the fluorescence emission was measured. The fluorescence intensity of ANS significantly changed depending on the Cu^{2+} concentrations (Fig. 3). In the presence of 50 μM Cu^{2+}, the fluorescence emission particularly increased to 1.7 times with the blue shift of emission maximum from 514 nm to 470 nm, when it was compared with the spectrum of ANS alone (Fig. 3b). This change was significant because addition of each

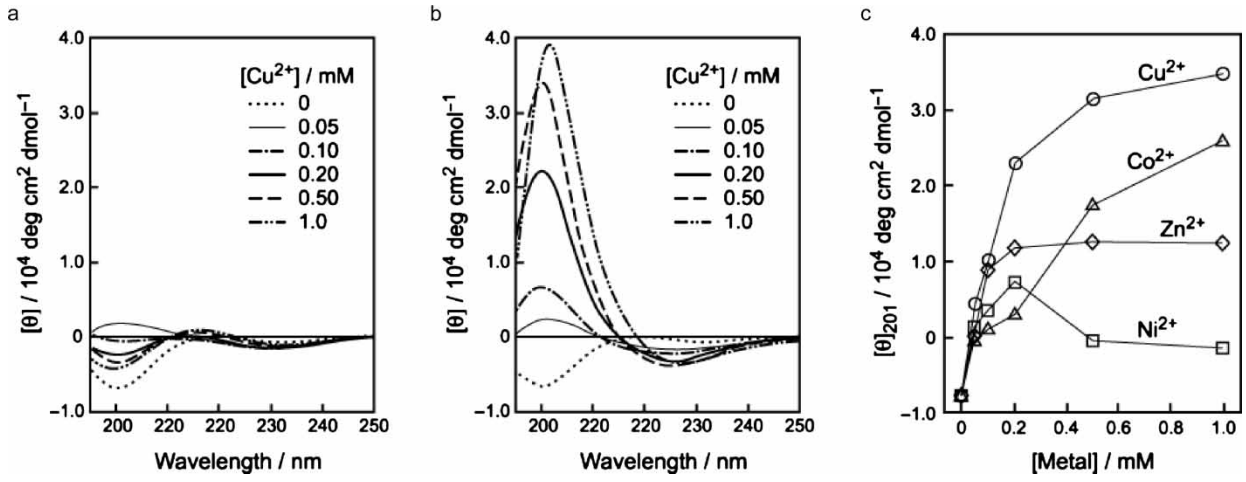

FIGURE 1 The β-sheet formation of **His-FI** induced by metal ions. CD spectra of **His-FI**, shortly (a) and 1 day (b) after addition of various concentrations of Cu^{2+}. (c) Change of the molar ellipticity at 201 nm depending on metal ions after incubation for 4 days. Peptide concentration was 100 μM in 20 mM Tris·HCl buffer (pH 7.4). The peptide solutions were incubated at room temperature and measured at 25°C.

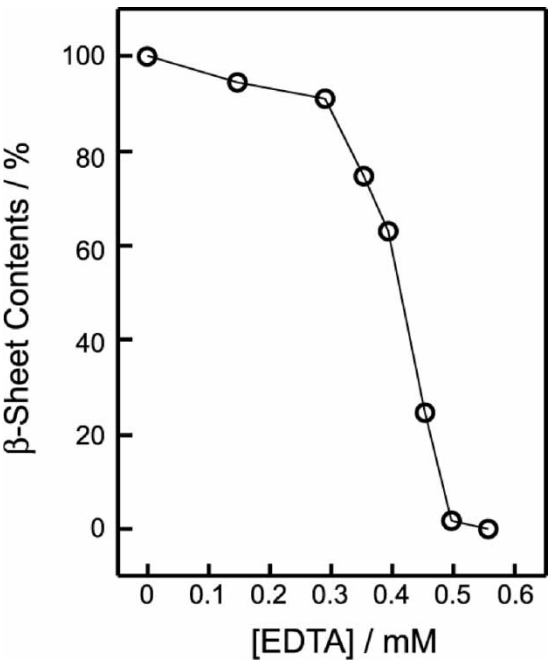

FIGURE 2 EDTA-induced decay of the β-sheet structure of **His-FI** formed in the presence of Cu^{2+}. The apparent β-sheet contents were defined using the molar ellipticity at 201 nm as follows; the molar ellipticity of **His-FI** (100 μM) in the presence of Cu^{2+}(500 μM) after sufficient incubation time was regarded as 100% β-sheet, and the ellipticity of **His-FI** in the absence of Cu^{2+} was 0% β-sheet (random coil). EDTA was added to the peptide solutions incubated sufficiently (**His-FI**; 100 μM, Cu^{2+}; 500 μM in 20 mM Tris·HCl buffer, pH 7.4), and then CD measurement was immediately carried out at 25°C. The CD spectra did not change for 7 days after addition of EDTA.

of **His-FI** or metal ions to ANS solution affected the fluorescence emission in the range of only less than 1.1-fold. Furthermore, addition of more than 100 μM of Cu^{2+} reduced the fluorescence intensity close to the spectrum without Cu^{2+}. As described above, CD studies showed that the peptide was in a β-sheet conformation in the presence of Cu^{2+}, and the signals of the β-sheet increased with the increment of Cu^{2+} concentrations. Therefore, the weak fluorescence emission at higher Cu^{2+} concentrations (above 100 μM) was assumedly attributed not to the unfolded structure but to the tightly-packed structure. This estimation was supported by the results that the increase of ANS emission completely disappeared by addition of EDTA, accompanied with the peptide structural change from a β-sheet to a random coil (data not shown).

The peptide assemblages in the presence of Cu^{2+} were subjected to transmission electron microscopy (TEM). Different morphology of the peptide assemblages was observed depending on Cu^{2+} concentrations (Fig. 4a–c). In the presence of Cu^{2+} at concentrations over 200 μM, the peptide formed tape-like fibers with a width of around 30 nm (Fig. 4a). The tape-like fibers inclined to reduce as Cu^{2+} concentration became lower, and were not found at 50 μM Cu^{2+} (Fig. 4b and c). Instead, the thin and small fibrils with a width of several nanometers and their flocculates were observed (Fig. 4c). The coexistence of tape-like fibers and thin fibrils at 100 μM Cu^{2+} (Fig. 4b) indicated that sufficient Cu^{2+} such as over 200 μM for the peptide (100 μM) was required for the complete formation of tape-like fibers. As described above, ANS emission was significantly weak at such higher concentrations of Cu^{2+}. Therefore, the tape-like fibers were assumed to be well-packed assemblages of the β-sheets lacking exposed hydrophobic regions for the ANS binding. In contrast, the small fibrils observed at 50 μM Cu^{2+} were indicated to be partially folded structure, suggested by the strong ANS emission and the weak

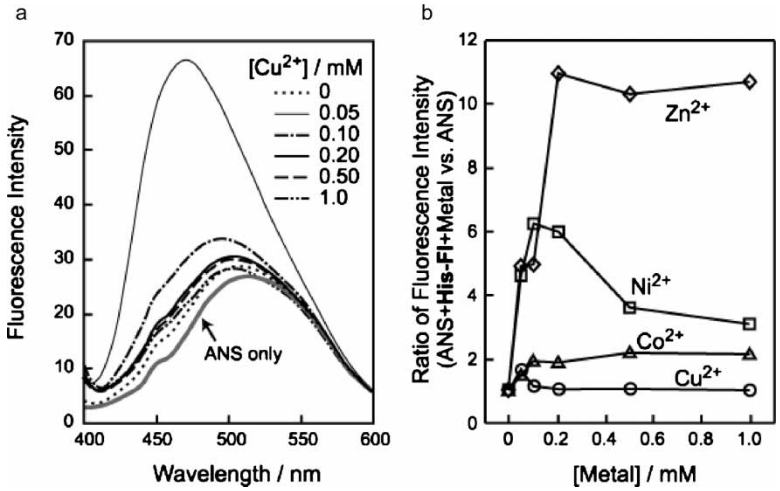

FIGURE 3 ANS binding assay for the peptide assemblages of **His-FI** in the presence of metal ions. (a) Fluorescence emission spectra of ANS in the presence of **His-FI** and various concentrations of Cu^{2+}. (b) Relative fluorescence emission intensity of ANS at 515 nm in the presence of **His-FI** and various concentrations of metal ions compared to that of ANS alone. ANS was added to the peptide solutions incubated sufficiently for more than 7 days. Fluorescence emission was measured at 25°C with excitation at 390 nm. Concentrations of peptide and ANS were 90 μM and 124 μM, respectively.

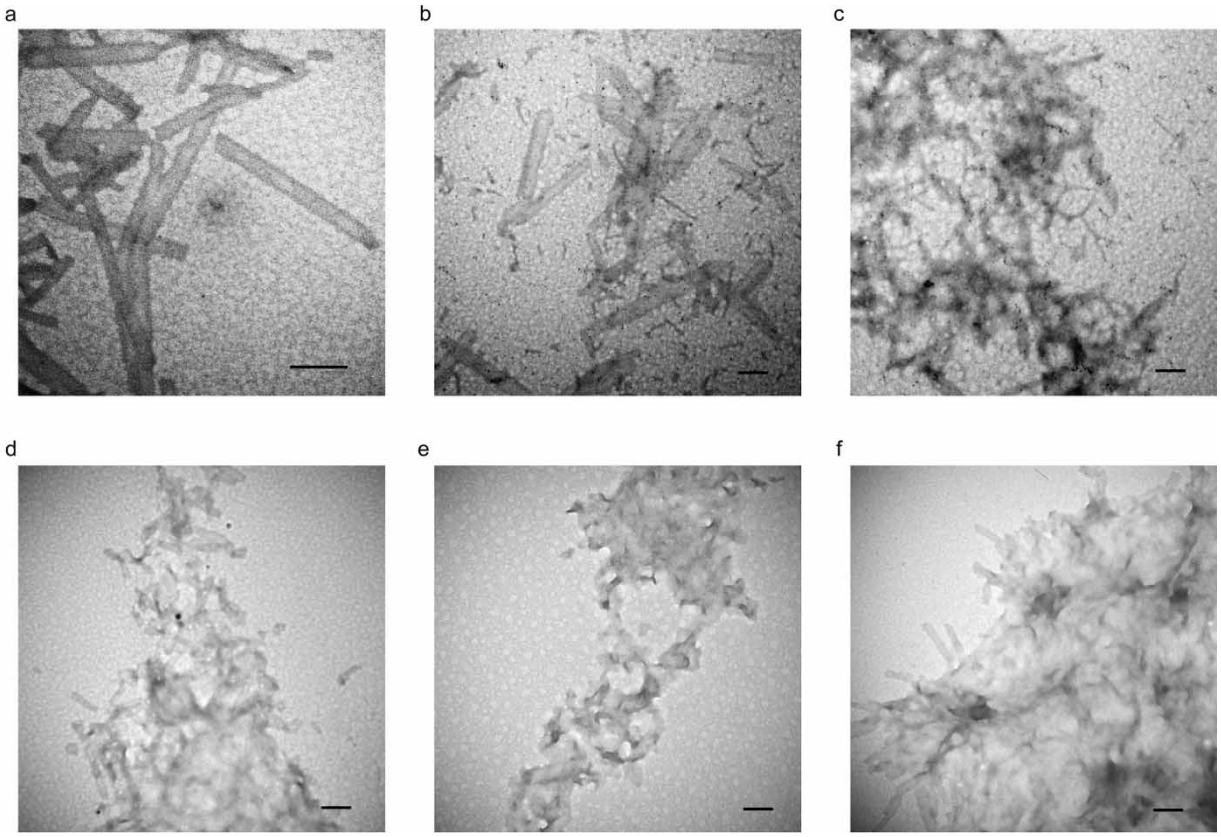

FIGURE 4 TEM images of the peptide assemblages induced by metal ions. Cu^{2+} concentrations were (a) 200, (b) 100, and (c) 50 μM. The results at Cu^{2+} concentrations more than 200 μM were similar to (a). The images of (d), (e), and (f) were in the presence of 200 μM of Ni^{2+}, Zn^{2+}, and Co^{2+}, respectively, resembling the images obtained at other metal concentrations. Before TEM observation, the peptide solutions were sufficiently incubated for more than 7 days. Scale bars; 100 nm.

CD signal of a β-sheet structure. The amount of Cu^{2+} smaller than the peptide monomers may induce rapid aggregation of the peptides around Cu^{2+}. Supposedly, the aggregated peptides form a β-sheet structure to some extent, however, cannot extend the β-sheets to regular fibers due to the structural defects (Fig. 1a and b), resulting in the formation of random flocculates (Fig. 4c). In the presence of Cu^{2+} at the higher concentrations such as an equimolar or more to the peptide, the peptide monomers were initially dispersed to some extent, and gradually aggregated to form a β-sheet structure, resulting in the sufficient extension of the β-sheets (Fig. 1a and b) and organization of the higher ordered structure of tape-like fibers (Fig. 4a) [24]. The tape-like fibers were easily broken by addition of EDTA. Thus, the random coil state of the peptide can be controlled to form the well-regulated fiber in response to Cu^{2+}, apparently demonstrating the regulation of fiber formation by Cu^{2+} using the designed peptide containing His residues.

Peptide Assembly Induced by Other Metal Ions

The structural transition to a β-sheet was also induced by addition of other metal ions of Co^{2+}, Ni^{2+}, or Zn^{2+}, but Cu^{2+} had the highest ability of inducing the β-sheet structure (Fig. 1c). The induced structural transition reached a plateau at Zn^{2+} concentrations over 200 μM, whereas the CD signal of a β-sheet structure was a half of the one in the presence of Cu^{2+}. Ni^{2+} had a lower ability to make a β-sheet, and higher concentrations of Ni^{2+} were indeed inappropriate for the β-sheet formation. Co^{2+} showed a strange behavior, that is, β-sheet structure was slightly induced at the concentrations less than 200 μM, but effectively induced over 500 μM. In the cases of these metal ions, Zn^{2+}, Ni^{2+}, and Co^{2+}, although the β-sheet contents were low, the aggregated objects were visually observed in the solutions, similar to the case of Cu^{2+}.

The fluorescence emission of ANS bound to the peptide in the presence of Zn^{2+}, Ni^{2+}, and Co^{2+}, was much stronger than the corresponding one in the presence of Cu^{2+} (Fig. 3b). The strongest fluorescence emission of ANS was observed in **His-FI** + Zn^{2+} up to 11-fold increase, in spite of the moderate β-sheet formation. The emission was also strong in **His-FI** + Ni^{2+} up to 6.3-fold, although the β-sheet content was low. In the case of **His-FI** + Co^{2+}, the emission was relatively weak, and did not largely change in the range around 2-fold at Co^{2+} concentrations from 100 μM to 1 mM, although in such Co^{2+} concentrations the CD signals

of β-sheet particularly increased (Fig. 1c). These phenomena reflect the inherent ANS binding behaviors to proteins, that is, ANS preferably binds to partially folded proteins and not to both completely unstructured and folded proteins [37]. Overall, the stronger ANS emission corresponded to the weaker CD signals of β-sheet, except for the cases at higher concentrations of Ni^{2+} in which the peptide scarcely showed a β-sheet structure (Figs. 1c and 3b). The stronger emissions of ANS in the cases of **His-FI** + Zn^{2+} and **His-FI** + Ni^{2+} was considered to be attributed to the exposed hydrophobic regions due to the disordered assembly of β-sheets. The alignment of the peptide monomers induced by these metal ions would have a certain irregularity within. Such structural defects might prevent the β-sheets from aligning regularly, and as a result the well-packed higher ordered assembly was not formed. In contrast, in the case of **His-FI** + Cu^{2+}, the significantly weak ANS emission (less than 1.7-fold increase) accompanied with the high β-sheet contents was remarkable, indicating that Cu^{2+} specifically acted on arranging β-strands in a regular manner appropriately to the β-sheet extension and the well-packed assemblages as tape-like nanofibers.

The difference of the peptide assemblages formed by Co^{2+}, Ni^{2+}, or Zn^{2+} compared with those by Cu^{2+} was clearly shown by TEM images. In the presence of Co^{2+}, Ni^{2+}, or Zn^{2+}, neither of tape-like fibers and thin fibrils was found, and only amorphous aggregates were observed (Fig. 4d–f). The fine images were not appeared inside the aggregates even at Co^{2+} concentrations above 200 μM, where the peptide apparently had a β-sheet structure. Considering the results of CD and ANS experiments, Co^{2+} appeared to have a relative high ability in inducing the peptide assembly, but the peptide assemblage induced by Co^{2+} was not sufficient to form isolated tape-like fibers. The Co^{2+}-induced assembly is an intermediate case between those with Cu^{2+} and other metal ions. The aggregates observed in Co^{2+}, Ni^{2+}, or Zn^{2+} were attributed to the indiscriminate alignment of the peptides, suggested by the results of lower β-sheet contents and stronger ANS binding for exposed hydrophobic regions. Thus, it was indicated that to make up regulated nano-sized structures required the fine assembly of β-sheets based on the regular alignment of β-strands, and that Cu^{2+} specifically induced such an assembly leading to tape-like nanofibers.

DISCUSSION

The designed peptide **His-FI** could not form a β-sheet structure by itself, suggesting that His residues located at the both N- and C-termini prevented the peptide self-association by the hydrophilic nature,

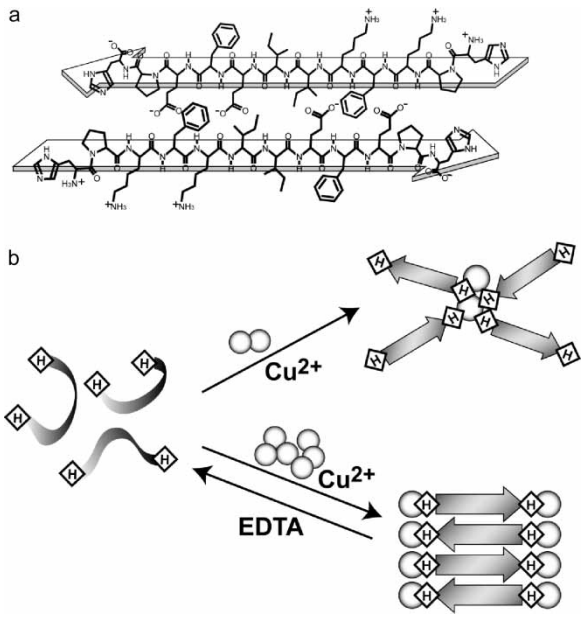

FIGURE 5 Schematic illustration of the β-sheet (a) and fiber formation (b) of **His-FI** induced by Cu^{2+} and the destruction by EDTA. The small amount of Cu^{2+} induced a non-ordered assemblage (flocculated) with a partially folded structure, but an excess amount of Cu^{2+} made tape-like fibers with the ordered assembly.

potential charge repulsion and structural hindrance. Added metal ions compensated such effects of His residues and induced the β-sheet formation. Cu^{2+} had appropriate effects on the β-sheet formation and specifically induced the well-packed assemblages as tape-like fibers (Fig. 5). One suggestive information of how many Cu^{2+} bound to the peptide was obtained by the experiment of the addition of EDTA to the peptide assemblages consisting of **His-FI** and Cu^{2+} (Fig. 2). EDTA was added to the solutions of the peptide assemblages of tape-like fibers which were formed from 100 μM of **His-FI** in the presence of Cu^{2+} at 500 μM by sufficient incubation of ca. 7 days. The apparent β-sheet content was maintained by addition of EDTA at concentrations up to 300 μM, however, it was clearly decreased toward a random coil by further addition of EDTA. The turning point was at a concentration of EDTA around 300 μM, indicating that two His residues in the peptide **His-FI** reacted with Cu^{2+} (ca. 200 μM) in the 1/1 stoichiometric molar ratio, and that the binding of Cu^{2+} to both of His residues at the N- and C-termini was required to form the tape-like fibers. The Cu^{2+} binding sites might involve N-terminal amine or C-terminal carboxylate group as well as imidazole of His side chains. The turning point of metal concentration at 200 μM was roughly shown in the profiles of β-sheet formation not only in the presence of Cu^{2+} but also other metal ion of Co^{2+}, Ni^{2+}, or Zn^{2+} (Fig. 1c). These metal ions might also react with both of His residues at the N- and C-termini. However, it was remarkable that the most effective

metal ion was Cu^{2+} on the β-sheet formation, and that well-packed β-sheet assemblages were specifically formed to give tape-like fibers. It is unlikely that the electrostatic interaction derived from cationic nature of metal ions particularly contributed to the β-sheet induction. The distinctive behaviors induced by Cu^{2+} might be attributed to the greater affinity of imidazole for Cu^{2+} than those for Co^{2+}, Ni^{2+}, or Zn^{2+}[40]. However, the affinity of imidazole for metal ions seems unable to fully account for the characteristic behaviors demonstrated here. Actually, the induction of the β-sheet formation by Ni^{2+} was the least effective in spite of the affinity of imidazole for Ni^{2+} being stronger than those for Co^{2+} and Zn^{2+}. Although the stability constants of imidazole complexes for Co^{2+} and Zn^{2+} are similarly lower compared to those for Cu^{2+} or Ni^{2+}, each of peptide assembly induced by Co^{2+} or Zn^{2+} resulted in much different β-sheet content and ANS binding property, respectively. These phenomena indicated that other characters of metal ions including the coordination geometry and the size of metal ions also affected the peptide assembly. Although the details should be investigated, it would be sure that the metal coordination effectively confined the peptide arrangement. Consequently, only Cu^{2+} had special abilities to control the order of the β-sheet peptides in regular manner to form well-packed tape-like nanofibers.

CONCLUSION

Metal-responsive nanofiber formation was achieved by the designed β-sheet peptide with His residues. The designed peptide completely lost the ability of the spontaneous β-sheet formation by His residues placed at the both peptide termini, and maintained the random coil state in a neutral buffer. However, addition of metal ions induced the β-sheet formation and the higher ordered assembly leading to tape-like nanofibers. The formed fibers were rapidly broken by addition of EDTA. The interaction between Cu^{2+} and the peptide including His residues was required to regulate the peptide assembly for making the well-packed fiber structure. The environmental responsiveness will make the peptide available in processing and functionalizing peptide materials. For example, controlling the induction from the mixture of several kinds of peptide monomers to the fiber structure will expand potentials of designing strategies for fabrication of modified and functionalized nanofibers. The metal-responsive peptide will create a new design of molecular assembly for generating peptide-based materials.

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