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Effects of His mutations on the fibrillation of amyloidogenic Vλ6 protein Wil under acidic and physiological conditions

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SUMMARY

Recently, we showed that the recombinant (r) Vλ6 protein Wil exhibits a more disrupted residual structure and a longer lag time for fibril formation than the rVλ6 protein Jto under highly unfolding conditions at pH 2. Here, we focused on the roles of three histidine residues specific for Wil, which are positively charged at pH 2 and could repel one another. Heteronuclear relaxation experiments revealed that a mutant Wil with H34Q, H53Q and H93S mutations (3HmutWil) had larger R_2 values only in the region of residues 22–55 and formed fibrils much earlier than Wil at pH 2. 3HmutWil also showed a decrease in ThT fluorescence intensity compared with Wil in fibrillation experiments at pH 7.5. The present results suggest that these three histidine residues play important roles in the fibrillation of Wil at both pH 2 and pH 7.5.

Introduction

A number of human diseases such as Alzheimer's, Parkinson's and Creutzfeldt–Jacob disease originate from the accumulation of misfolded and aggregated proteins [1–3]. Although toxic proteins related to these diseases do not exhibit special characteristics in terms of their three-dimensional structures or amino acid sequences [4], all are known to form fibrillar aggregates, termed amyloid fibrils. However, many non-disease-related proteins are also able to form amyloid-like fibrils under appropriate conditions, suggesting that amyloid fibrillation is a generic property of polypeptide chains [5]. AL amyloidosis is a monoclonal plasma cell disorder associated with the overproduction and extracellular deposition of light chains in the form of insoluble fibrils [6,7]. Although other amyloidogenic proteins may be wild-type or linked to a single hereditary mutation, an abundance of mutations is characteristic of AL amyloidosis. It has been found that the λ6 subtype of light chains is preferentially associated with AL amyloidosis [8], resulting in interest in the characterization of λ6 light chains. The crystal structures of two recombinant light chain variable domains belonging to the λ6 subtype (rVλ6), the amyloidogenic Wil and nonamyloidogenic Jto, have been reported [9]. Wil and Jto are acidic proteins composed of 111 amino acid residues and contain a single disulfide bond between Cys23 and Cys88 (Fig. 1A). It has been reported that Wil is thermodynamically less stable and consequently forms fibrils with a shorter lag time than Jto at pH 7.5 [10]. In addition, Wall et al. showed that a long-range electrostatic interaction between Asp29 and Arg68, which is present in Jto but not in Wil, contributes to the increased stability and decreased amyloidogenicity of Jto [11]. However, the mechanisms behind these differences between Jto and Wil are not fully understood.

Recently, we showed that Jto and Wil have non-random residual structures in regions that include some hydrophobic residues and a single disulfide bond at pH 2 and 37 °C [12]. The residual structure of Wil is more disrupted than that of Jto. Interestingly, Wil required a longer time to form fibrils than Jto under the same conditions with constant stirring. These results suggest that the maintenance of a residual structure is necessary for fibrillation of rVλ6 proteins at pH 2. Since a lag time in the fibrillation process was reported to correspond to the formation of fibril nuclei [13,14], the residual structure is likely to be particularly important for nucleation in the fibrillation process. The differences in the residual structure and fibrillation can be attributed to the sequence of Wil, which differs from the sequences of λ6a and Jto at 10 and 18 positions, respectively (Fig. 1A). In particular, Wil has three additional histidine residues, His34, His53 and His93, which would be positively charged at pH 2. Therefore, it is possible that these positively charged residues repel one another, thereby resulting

Abbreviations: CD, circular dichroism; EM, electron microscopy; HSQC, heteronuclear single quantum coherence; r, recombinant; $R_2 = (T_2)^{-1}$, transverse relaxation rate; ThT, thioflavine T; V_L, light chain variable domain; Vλ6, lambda 6 variable domain

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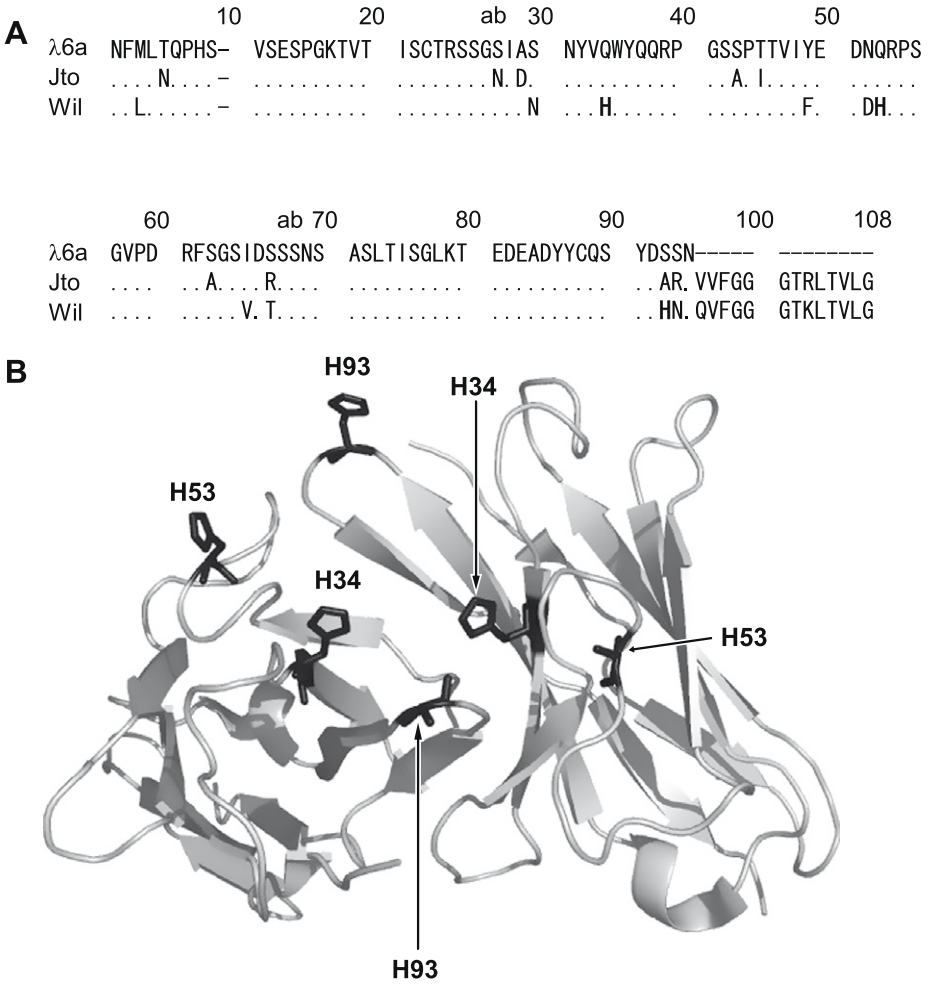

Fig. 1. (A) Comparisons of the amino acid sequences of the Vλ6 proteins Jto and Wil with the predicted amino acid sequence of the Vλ6 germline gene (λ6a). (B) Crystal structure of Wil (PDB ID: 2CD0). The positions of the mutated H34, H53 and H93 residues are shown. This figure was generated with PyMOL.

in disruption of the residual structure and prevention of fibrillation of Wil at pH 2. In the present study, these three histidine residues of Wil were mutated to the corresponding λ6a residues to investigate the roles of these histidine residues in the residual structure and fibrillation of Wil at pH 2. Moreover, the effects of the histidine residues were also examined at pH 7.5.

Materials and methods

Materials. ^{15}N-ammonium chloride and ^{13}C-glucose were obtained from Shoko Co. Ltd. (Tokyo, Japan). Thioflavin T (ThT) was purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals used were of the highest quality commercially available.

Preparation of rVλ6 proteins. Site-directed mutagenesis of Wil was performed by PCR according to previously described methods [15]. Mutations were confirmed by DNA sequence analysis. Wil, Jto and 3HmutWil were expressed and purified as described [12].

CD measurements. CD spectra were collected with a J-720 spectropolarimeter (Jasco, Tokyo, Japan) between wavelengths of 190 and 250 nm. The buffer was 50 mM glycine (pH 2) or 50 mM phosphate (pH 7.5) containing 10 μM protein samples at 37 °C.

NMR experiments. NMR samples were prepared to contain 0.1 mM (^{1}H–^{15}N HSQC, relaxation experiments) or 0.5 mM (3D-NMR) protein in HCl at pH 2 in 95% H_2O/5% D_2O. NMR experiments were performed at 37 °C using a Varian Inova 600 MHz spectrometer equipped with a triple-resonance pulse-field gradient probe with an activity shielded *z* gradient and a gradient amplifier unit as described [12].

In vitro fibril formation assays. Fibrillation of 100 μM rVλ6 protein in HCl at pH 2 and 37 °C was induced by constant stirring using a Teflon-coated microstir bar (3 × 3 mm). Samples were incubated at 37 °C in disposable tubes (12 × 75 mm). At various time points, a 5 μL aliquot was taken from each reaction tube and mixed with 995 μL of 20 μM ThT in phosphate-buffered saline (PBS; 150 mM NaCl, 10 mM Na_2HPO_4, 10 mM NaH_2PO_4, pH 7.5). Fibrillation of 3 μM rVλ6 protein in 50 mM phosphate buffer at pH 7.5 and 37 °C was induced using a Cross Ultrasonic Protein Auto Activating Instrument (ELESTEIN NP070-GOT; ELEKON SCIENCE Co. Ltd., Tokyo, Japan) based on a previous report [16]. The volume of the water bath was about 12 L. The frequency of the instrument was 17–20 kHz, and the power output was set to deliver a maximum of 350 W. The reaction mixtures were ultrasonicated from three directions for 1 min and then incubated for 9 min without sonication, a process that was repeated during incubation at 37 °C. At various time points, a 50 μL aliquot was taken from each reaction tube and mixed with 950 μL of 20 μM ThT in PBS. In vitro fibril formation was monitored by the fluorescence of ThT. The excitation wavelength was fixed at 450 nm and the emission was collected at 485 nm.

TEM analysis. The aggregates of rVλ6 proteins were checked by TEM. The stirred protein solutions were sampled at the end of fibril

formation assays. Each sample was negatively stained with 2% uranyl acetate. The grids were examined using an H-7000E electron microscope (Hitachi High-Technologies Corporation, Tokyo, Japan) at 75 kV.

Urea denaturation experiments. The solutions of rVλ6 proteins (20 μg/mL) were prepared in 10 mM phosphate buffer (pH 7.5) containing various concentrations of urea (0–7 M). The samples were incubated overnight at 25 °C. Fluorescence was measured with excitation at 280 nm and emission at 350 nm. Details of the analysis were as described [17].

Results and discussion

Effects of His mutations on the residual structure and amyloid fibrillation at pH 2

The residual structures of polypeptide chains in highly unfolded states have been identified using various NMR experiments [18–23]. We have studied the residual structures of hen egg-white lysozyme [23,24] and rVλ6 proteins [12] by heteronuclear relaxation experiments. In a previous report involving ^{15}N NMR transverse relaxation experiments, we found that rVλ6 proteins had non-random structures and that the non-random structure of Wil was more markedly disrupted than that of Jto. Fibrillation experiments showed that Wil had a longer lag time for fibril formation than Jto. These results suggest that the residual structure in a highly unfolded state plays an important role in amyloid fibrillation. To investigate the mechanisms behind the differences between Wil and Jto, we focused on the His residues of Wil, which would be positively charged at pH 2.

Initially, we prepared a mutant Wil termed 3HmutWil, which contained mutations of the three additional histidine residues of

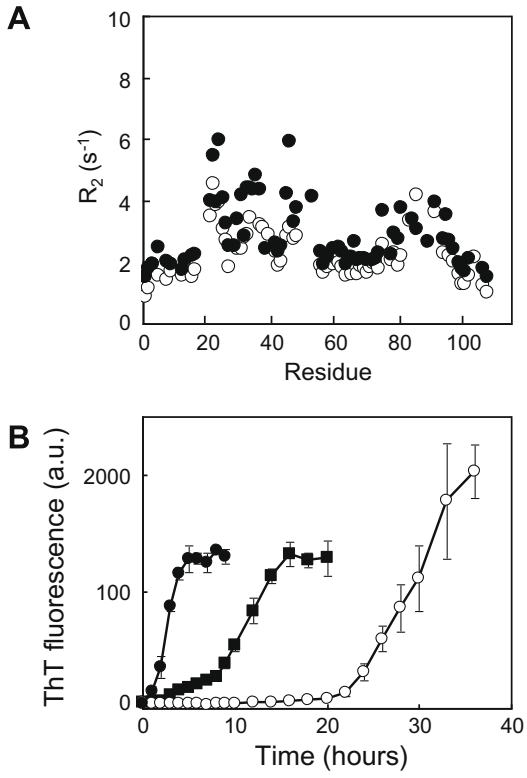

Fig. 3. (A) Residue-specific R_2 rates of the rVλ6 proteins Wil (○) and 3HmutWil (●) at pH 2 and 37 °C. (B) Amyloid fibrillation of the rVλ6 proteins Jto (■), Wil (○) and 3HmutWil (●) at pH 2 and 37 °C. Fibril formation was monitored by ThT emission at 485 nm after excitation at 450 nm. The data for Jto and Wil were taken from Ref. [12].

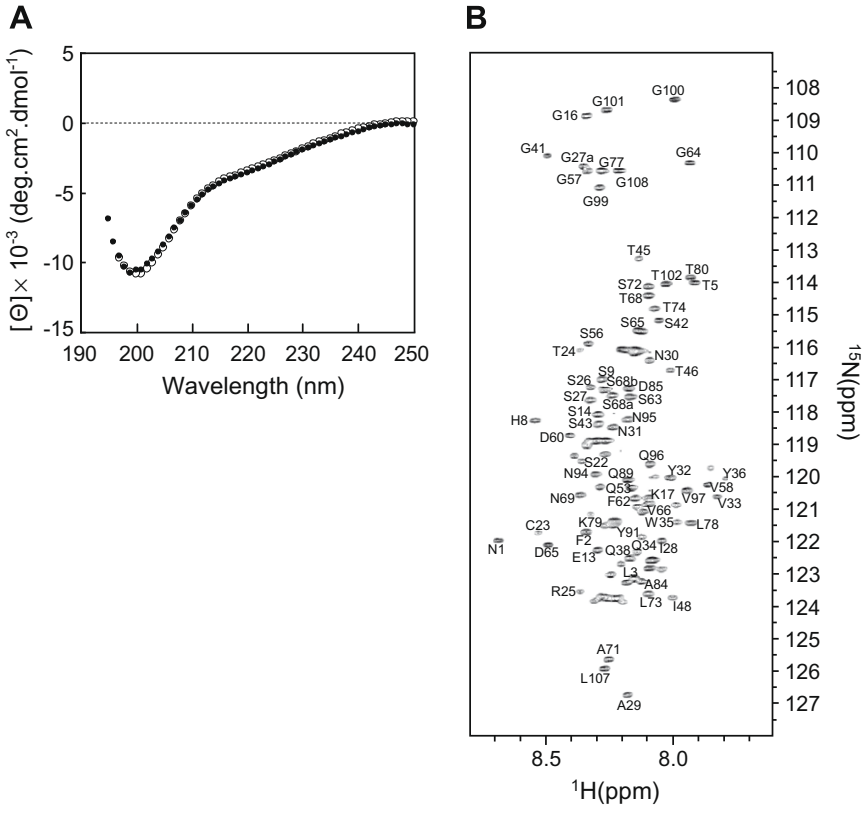

Fig. 2. (A) Far-UV CD spectra of the rVλ6 proteins Wil (○) and 3HmutWil (●) at pH 2 and 37 °C. The data for Wil were taken from Ref. [12]. (B) ^1H–^{15}N HSQC spectra of the rVλ6 protein 3HmutWil in HCl at pH 2 in 95% H_2O/5% D_2O at 37 °C. Assignments are indicated in the figure.

Wil (H34Q, H53Q and H93S). The residual structure of 3HmutWil was examined under the same conditions used in our previous study [12]. The far-UV CD spectrum of 3HmutWil showed a negative peak at around 200 nm similar to that of Wil (Fig. 2A), indicating that 3HmutWil was also highly unfolded at pH 2 and 37 °C. The HSQC spectra of ^{15}N-labeled rVλ6 proteins were measured at pH 2 and 37 °C (Fig. 2B). The spectra showed narrow chemical shift dispersions typical of unfolded proteins. Assignments of the ^{1}H and ^{15}N resonances were performed using a series of triple-resonance experiments as previously described [12]. ^{15}N relaxation experiments at pH 2 and 37 °C revealed that the R_2 values of 3HmutWil exhibited broad variation (Fig. 3A), indicating the presence of a non-random residual structure in the polypeptide chain. 3HmutWil showed larger R_2 values in the regions of residues 22–55 and 78–95, especially for Cys23 and Cys88, which form a disulfide bond, and hydrophobic residues, similar to the case for Jto and Wil. The R_2 values of Wil were smaller than those of Jto throughout the sequence, including the regions of residues 22–55 and 78–95 [12]. Interestingly, the region containing residues 22–55 of 3HmutWil showed larger R_2 values than the corresponding region of Wil, whereas the region containing residues 78–95 of 3HmutWil showed similar R_2 values to the corresponding region of Wil (Fig. 3A). Even though both regions contained mutated sites, H34Q and H53Q in the former and H93S in the latter, changes in the R_2 values were only observed in the region containing residues 22–55 of 3HmutWil compared with the corresponding region of Wil. These results suggest that the mutations of the three histidine residues would disrupt the N-terminal region of the residual structure in Wil.

Amyloid fibrillation of 3HmutWil was induced by constant stirring at pH 2 and 37 °C. Based on time-course analyses of ThT fluorescence emission, we previously reported that Jto forms fibrils earlier than Wil at pH 2 and 37 °C [12]. When the three histidine residues of Wil were mutated, the lag time of its fibrillation was drastically reduced and even shorter than those of not only Wil but also Jto (Fig. 3B). These results suggest that the three histidine residues have important roles for not only the maintenance of the residual structure but also the fibrillation of Wil. Therefore, it is suggested that maintenance of the N-terminal region of the residual structure is a key for amyloid fibrillation of Wil. In contrast, the

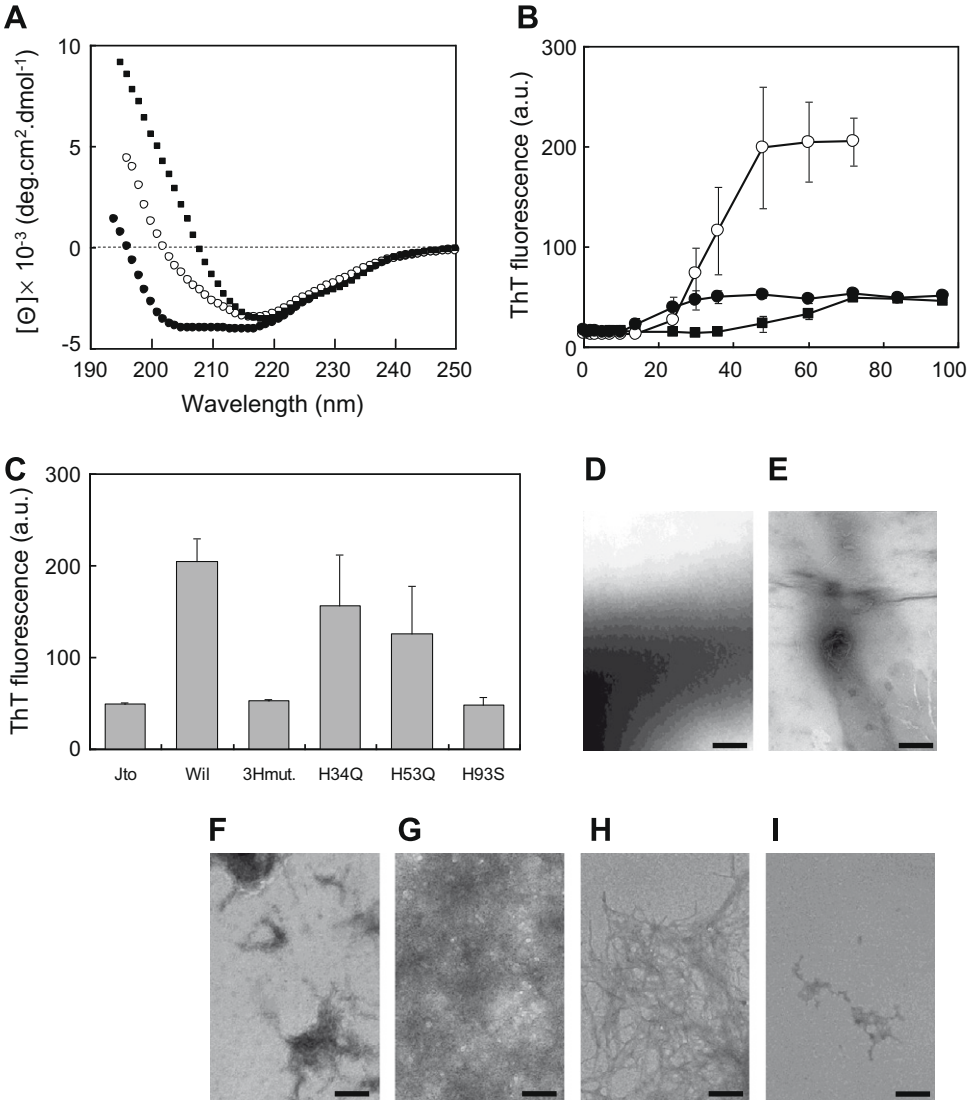

Fig. 4. (A, B) Far-UV CD spectra (A) and amyloid fibrillation (B) of the rVλ6 proteins Jto (■), Wil (○) and 3HmutWil (●) at pH 7.5 and 37 °C. The far-UV CD spectra for Wil and Jto were taken from Ref. [12]. Fibril formation was monitored by ThT emission at 485 nm after excitation at 450 nm. (C) ThT fluorescence of the rVλ6 proteins after 72 h of incubation at pH 7.5 and 37 °C. (D–I) Electron micrographs of fibrils formed by Jto (D), Wil (E), 3HmutWil (F), H34Q-Wil (G), H53Q-Wil (H) and H93S-Wil (I) at pH 7.5 and 37 °C. Scale bars represent 500 nm.

C-terminal region of the residual structure seems to be less important than the N-terminal region under acidic conditions. Since histidine residues are positively charged at pH 2, these residues are likely to repel one another in Wil, resulting in disruption of the N-terminal region of the residual structure and delaying the amyloid fibrillation of Wil.

Effects of His mutations on amyloid fibrillation at pH 7.5

Next, we focused on the effects of the histidine residues on amyloid fibrillation of Wil at pH 7.5. In the native state, immunoglobulin light chain variable regions have a β-sandwich fold typical of proteins belonging to the immunoglobulin superfamily (Fig. 1B). The histidine residues are located on the surface of Wil. In our previous report, Jto was found to show an extension of a single negative peak at around 215 nm, which is characteristic of a β-sheet structure. On the other hand, Wil showed a negative peak at 215 nm with a shoulder from 205 to 215 nm, suggesting that Wil has a β-sheet structure and contains a random-coil structure to some extent [12]. 3HmutWil also exhibited a single negative peak with a weaker shoulder, indicating the presence of a lesser extent of the random-coil structure than Wil (Fig. 4A).

Since thermodynamic stability is known to be closely related to the fibrillation of immunoglobulin light chains [10,11,25–27], we investigated the thermodynamic stability and amyloid fibrillation of 3HmutWil at pH 7.5. It has been reported that Wil is thermodynamically less stable and consequently forms fibrils with a shorter lag time than Jto at pH 7.5 [10]. We further confirmed that Wil was thermodynamically less stable than Jto at pH 7.5 by urea denaturation experiments (Table 1) and found that the ThT fluorescence intensity of Wil increased earlier and was 4-fold higher than that of Jto by fibrillation experiments (Fig. 4B). TEM analysis revealed the existence of fibrils formed by Wil, while no fibrils formed by Jto were detected (Fig. 4D and E). On the other hand, there was little difference between the stabilities of 3HmutWil and Wil (Table 1). 3HmutWil showed a small increase in ThT fluorescence within 30 h, similar to Wil. These results suggest that the three histidine residues had little effect on the stability and consequently the lag time of fibrillation of Wil at pH 7.5. However, the ThT fluorescence of 3HmutWil was much lower than that of Wil after 72 h (Fig. 4B). It is widely established that amyloid fibrillation is initiated by the formation of nuclei, followed by further associations of either monomers or oligomers with the nuclei [28]. Therefore, the present findings suggest that the three histidine residues are likely to affect other steps of fibrillation at pH 7.5, rather than nucleation.

We also prepared three single histidine Wil mutants (H34Q-Wil, H53Q-Wil and H93S-Wil) to examine the roles of the individual histidine residues in the fibrillation of Wil at pH 7.5. Similar to the case for 3HmutWil, each single mutant exhibited little effect on the stability of Wil at pH 7.5 (Table 1). On the other hand, there were clear differences in the fibrillation among the three Wil mutants. Although the ThT fluorescence of H34Q-Wil and H53Q-Wil increased as much as that of Wil, H93S-Wil showed a marked decrease in ThT fluorescence compared with that of Wil (Fig. 4C). H93S-Wil seemed to form fewer fibrillar aggregates than H34Q-Wil and H53Q-Wil by TEM analysis (Fig. 4G–I). These results suggest that His93 is more important for the fibrillation of Wil than His34 and His53 at pH 7.5.

In this study, we observed critical effects of the three histidine residues on the fibrillation of the Vλ6 protein Wil at both pH 2 and pH 7.5. At pH 2, mutations of the three histidine residues caused disruption of the N-terminal region of the residual structure and delayed the amyloid fibrillation of Wil. These effects can be attributed to the three histidine residues, which would be positively charged and promote intramolecular and/or intermolecular repulsion at pH 2. In the case of human islet amyloid polypeptide (IAPP), which contains a single histidine residue at position 18, the fibrillation rate was found to be dramatically faster at a higher pH (8.8 ± 0.3) than at a lower pH (4.0 ± 0.3), indicating that the protonation of His18 at the lower pH delayed the fibrillation of IAPP [29]. These results suggest that positive charges of histidine residues would affect the amyloid fibrillation of many proteins at acidic pH. In the case of Wil, mutations of the three histidine residues also caused disruption of the residual structure at pH 2. In addition to the previous findings that hydrophobic residues and disulfide bonds are important for the maintenance of a residual structure [19,23,30–32], the present data suggest that charged histidine residues may be closely related to the residual structure of Wil. On the other hand, we found that the histidine residues, especially His93, also affected the amyloid fibrillation of Wil without being positively charged at pH 7.5. Mutations of the three histidine residues had little effect on the stability and lag time of fibrillation of Wil but drastically decreased the ThT fluorescence of Wil incubated at pH 7.5, suggesting that a fibrillation process other than nucleation was affected by the mutations. Histidine residues are also reported to be important for binding to metals [33–35] and ganglioside micelles [36] in amyloid β-peptide (Aβ) and monomer–monomer interactions in the amyloidogenic Vκ1 protein AL-09 [37,38]. In the present study, we have demonstrated the importance of the three histidine residues for fibrillation of the Vλ6 protein Wil not only at pH 2 but also at pH 7.5. Our findings provide new insights into the mechanism of amyloid fibrillation of the Vλ6 protein Wil.

Table 1
Thermodynamic parameters characterizing the urea denaturation of rVλ6 proteins.

rVλ6 proteins	$C_{1/2}$ (M)[a]	m (kcal mol^{-1} M^{-1})[b]	ΔG (H_2O) (kcal/mol)[c]	$\Delta\Delta G$ (H_2O) (kcal/mol)[d]
Wil	1.49 ± 0.05	1.7 ± 0.2	2.5 ± 0.2	0
Jto	2.27 ± 0.02	1.6 ± 0.0	3.6 ± 0.1	+1.1
3HmutWil	1.73 ± 0.03	1.7 ± 0.0	2.8 ± 0.0	+0.3
H34Q-Wil	1.49 ± 0.01	1.8 ± 0.1	2.6 ± 0.1	+0.1
H53Q-Wil	1.75 ± 0.03	1.5 ± 0.1	2.7 ± 0.1	+0.2
H93S-Wil	1.49 ± 0.04	1.8 ± 0.1	2.7 ± 0.0	+0.1

[a] The denaturant concentration at the midpoint for the denaturation of rVλ6 proteins.
[b] The free energy change per mole of denaturant.
[c] ΔG in the absence of denaturant.
[d] $\Delta G_{V\lambda6}$ protein(H_2O) − ΔG_{Wil}(H_2O).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.11.108.

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