

Title	A Comprehensive Model for Packing and Hydration for Amyloid Fibrils of β 2-Microglobulin
Author(s)	Lee, Young-Ho; Chatani, Eri; Sasahara, Kenji et al.
Citation	Journal of Biological Chemistry. 2009, 284(4), p. 2169-2175
Version Type	VoR
URL	https://hdl.handle.net/11094/71287
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A Comprehensive Model for Packing and Hydration for Amyloid Fibrils of β_2 -Microglobulin^{*[S]}

Received for publication, September 8, 2008, and in revised form, November 10, 2008 Published, JBC Papers in Press, November 18, 2008, DOI 10.1074/jbc.M806939200

Young-Ho Lee[‡], Eri Chatani[‡], Kenji Sasahara[‡], Hironobu Naiki[§], and Yuji Goto^{‡1}

From the [‡]Institute for Protein Research, Osaka University and CREST, Japan Science and Technology Agency, 3-2 Yamadaoka, Suita, Osaka 565-0871 and [§]Faculty of Medical Sciences, University of Fukui, and CREST, Japan Science and Technology Agency, Fukui 910-1193, Japan

Volume can provide informative structural descriptions of macromolecules such as proteins in solution because a final volumetric outcome accompanies the exquisite equipose of packing effects between residues, and residues and waters inside and outside proteins. Here we performed systematic investigations on the volumetric nature of the amyloidogenic conformations of β_2 -microglobulin (β_2 -m) and its amyloidogenic core peptide, K3, using a high precision densitometer. The transition from the acid-denatured β_2 -m to the mature amyloid fibrils was accompanied by a positive change in the partial specific volume, which was larger than that observed for the transition from the acid-denatured β_2 -m to the native structure. The data imply that the mature amyloid fibrils are more voluminous than the native structure because of a sparse packing density of side chains. In contrast, the formation of the mature amyloid-like fibrils of the K3 from the random coil was followed by a considerable decrease in the partial specific volume, suggesting a highly compact core structure. Interestingly, the immature amyloid-like fibrils of β_2 -m exhibited a volume intermediate between those of the mature fibrils of β_2 -m and K3, because of the core structure at their center and the relatively noncompact region around the core with much hydration. These volumetric differences would result from the nature of main-chain-dominated fibrillogenesis. We suggest comprehensive models for these three types of fibrils illustrating packing and hydrational states.

A polypeptide chain folds spontaneously to the most stable form, the native structure. However, it has been recognized that a misfolding-induced ordered aggregate, known as an amyloid, is an alternative fold that might be in the global minimum of free energy (1). Intriguingly, various nonamyloidogenic polypeptides can also assemble into fibrillar structures, implying that fibrillation is a generic property of polypeptides (2). This raises the possibility that although the native structure depends on interactions among side chains of amino acid resi-

dues, *i.e.* a side-chain-dominated structure, the amyloidogenic structure does not depend on interactions of side chains, *i.e.* a main-chain-dominated structure. It is therefore conceivable that a main-chain-dominated fibrillar structure is misfolded without optimal packing of side chains, and a side-chain-dominated native structure has evolved pursuing a unique fold with optimal packing of residues (3–6). This defective packing results in a lot of cavities, thereby rendering multiple fibrillar conformations, the so-called polymorphism (4). By contrast, fibrils of amyloidogenic core peptides were suggested to possess tight packing (7–12) that could constitute the core structure of the fibrils made of whole protein accommodating this peptide.

Although the past decade has seen progress in our biophysical understanding of amyloid fibrils using various approaches (3, 4, 6–16), much remains to elucidate about the conformational and thermodynamic features of fibrous proteins. In this respect, the volumetric technique for fibrillogenesis is a promising alternative, because volume is a key thermodynamic quantity and can sensitively probe changes of molecular hydration (17), intrinsic packing (18), and dynamics (19) upon conformational transitions and molecular binding events such as protein (un)folding and formation of aggregates in solution (20).

Indeed, although there have been a few studies of the volume of fibrils (3, 21–25), there is no consensus over the volumetric properties of fibrillation. Our previous study (3) showed that mature amyloid fibrils of β_2 -microglobulin (β_2 -m)² (99 residues) are in a voluminous state with loose packing. Similarly, Foguel *et al.* (21) reported that amyloidogenesis of transthyretin (TTR, 127 residues) and α -synuclein (140 residues) correlated with less packed structures (larger volume changes). Akasaka *et al.* (22) also found that a disulfide-deficient variant of hen egg white lysozyme (HEWL, 129 residues) formed flexible amyloid-like fibrils with an increase in volume. Conversely, Winter and co-workers (23, 24) observed that the transition from poly-D-lysine (~200 residues) and insulin (51 residues) monomers to mature fibrils was in both cases accompanied by reductions in volume (*i.e.* structural compaction). It is also intriguing that the mature and the early fibrils of a short fragment of TTR-(105–115) (11 residues) take denser and looser packing than the mature fibrils of full-length TTR, respectively (21, 25). These disparities of volumetric changes on fibrillation

* This work was supported by the Japanese Ministry of Education, Culture, Sports, Science, and Technology (to Y. G.) and by the Japan Society for Promotion of Science postdoctoral fellowship (to Y.-H. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at <http://www.jbc.org>) contains additional Experimental Procedures, Figs. S1 and S2, and additional references.

¹ To whom correspondence should be addressed. E-mail: ygoto@protein.osaka-u.ac.jp.

² The abbreviations used are: β_2 -m, β_2 -microglobulin; ASA, accessible surface area; AUC, analytical ultracentrifugation; AFM, atomic force microscopy; Gdn-HCl, guanidine hydrochloride; HD_{ex}, hydrogen deuterium exchange; PMV, partial molar volume; SV, sedimentation velocity; ThT, thioflavin T; HEWL, hen egg white lysozyme; TTR, transthyretin.

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could reflect the intrinsic feature of polymorphism of amyloid(-like) fibrils.

Most importantly, to obtain a reliable volume value of a solute of interest, one should certify that solutes are dispersive enough without precipitation (17) and confirm the constituent components with their populations. These prerequisites become more serious in the case of a polymerization reaction such as fibrillation. Fibrillar or amorphous aggregates are apt to precipitate because of high molecular weight, and unreacted monomers are often left.

In view of the notions for the polymorphism and core structures of fibrils, the model of the amyloidogenicity of $\beta 2$ -m is excellent because volumetric properties of the various fibrillar structures formed by the identical $\beta 2$ -m and its fragment can be examined systematically. Typically, rigid and long amyloid fibrils referred to as “mature fibrils” are assembled by acid-denatured $\beta 2$ -m (3–5, 16, 26). At high concentrations (>200 mM) of sodium chloride at acidic pH, $\beta 2$ -m can assemble spontaneously into flexible thin amyloid-like fibrils, often called “immature fibrils” (26, 27). In addition, K3 peptide, an amyloidogenic core fragment corresponding to Ser-20 to Lys-41 of $\beta 2$ -m, can form amyloid-like fibrils similar to mature fibrils at neutral pH (14) and thin filamentous fibrils at acidic pH in the absence and presence of 20% (v/v) 2,2,2-trifluoroethanol, respectively (28).

Keeping the prerequisites for preparing sample solution for densitometry described above in mind and to gain insight into volumetric properties of amyloidogenesis, we have performed direct measurements of changes in volume accompanying the fibrillation of $\beta 2$ -m and K3, using a high precision densitometry. To our surprise, the extent of the partial volumetric changes upon the formation of the mature fibrils and immature fibrils of $\beta 2$ -m, and the mature fibrils of K3, differed. Here we report the partial volume and states of these three fibrillar conformations in light of the main-chain-dominated fibrillation and the core structure of fibrils with polymorphism.

EXPERIMENTAL PROCEDURES

Preparation and Characterization of the Samples—Recombinant human $\beta 2$ -m was expressed and purified using the *Escherichia coli* expression system as described (15). K3 was obtained by digestion of $\beta 2$ -m with lysyl endopeptidase (*Achromobacter protease I*) as reported (16). For CD and measurements of density, $\beta 2$ -m was dissolved in 6 mM HCl (pH 2.3) and 38 mM NaCl or 50 mM citrate buffer (pH 2.5) in the presence and absence of 5.7 M Gdn-HCl and, in turn, K3 in solution of 6 mM HCl and 38 mM NaCl. Mature $\beta 2$ -m fibrils of 1.2 mg·ml⁻¹ were elongated in a 6 mM HCl solution (pH 2.3) containing 38 mM NaCl in the presence of 5 μ g·ml⁻¹ of seeds, ultrasonicated fibrils, at 37 °C for ~ 14 h. The short mature fibrils were prepared by ultrasonating the fresh mature fibrils 100 times on ice using a Microson sonicator (Misonix, Farmingdale, NY) at intensity level 2 and with 20 1-s pulses. The fibrils were confirmed to be fragmented with AFM (data not shown). Immature fibrils of $\beta 2$ -m were spontaneously polymerized as reported (26) in a reaction mixture containing 0.6 mg·ml⁻¹ of recombinant $\beta 2$ -m, 50 mM citrate buffer (pH 2.5), and 200 mM NaCl. The reaction solution was incubated at 37 °C for ~ 90 h. Mature fibrils of K3 were seed-dependently polymerized by K3 monomer in the solution

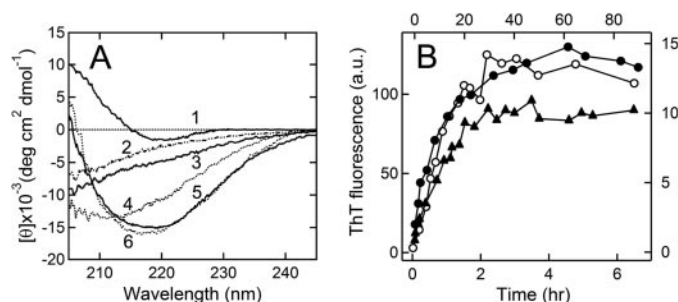


FIGURE 1. Conformational characterization and formation of fibrils. *A*, far-UV CD spectra for the different structures of $\beta 2$ -m and K3 peptide were obtained. 1, native $\beta 2$ -m; 2, K3 peptide; 3, acid-denatured $\beta 2$ -m; 4, immature fibrils of $\beta 2$ -m; 5, mature fibrils of $\beta 2$ -m; and 6, mature fibrils of K3. *B*, formation of the three types of fibrils was monitored by ThT fluorescence. The intensity of ThT for the mature $\beta 2$ -m fibrils (○) and both the immature $\beta 2$ -m fibrils (▲) and the mature K3 fibrils (●) was plotted against incubation time on the lower and upper axes, respectively. The left and right axes are corresponding to the intensity of ThT of the mature $\beta 2$ -m fibrils and the other fibrils, respectively.

containing 6 mM HCl, 7 mM NaCl, and 0.00091% 2,2,2-trifluoroethanol. Details of the preparation of K3 fibrils are presented in the supplemental material. All the polymerization reactions in this study were traced by fluorometric analysis using ThT and monitored at 485 nm with excitation at 445 nm with a Hitachi fluorescence spectrophotometer, F4500, at 25 °C as reported (16, 26). Far-UV CD spectra of all samples were measured at 25 °C with a Jasco J-600 spectropolarimeter, using a cell with a light-path of 1 mm. AFM images were obtained using a Nano Scope IIIa (Digital Instruments/Veeco) at 25 °C.

Sedimentation Velocity (SV) with Analytical Ultracentrifugation (AUC)—SV measurements were performed on the mature and immature fibrils of $\beta 2$ -m and the mature fibrils of K3 at 5 °C using a Beckman-Coulter Optima XL-1 analytical ultracentrifuge (Fullerton, CA) equipped with an An-60 rotor and two- or six-channel charcoal-filled Epon cells. After pre-centrifugation at $700 \times g$ for 5 min, the rotor speed was increased to $17,400$ – $31,000 \times g$. The sedimentation curves were recorded using absorbance data at 280 nm at intervals of 10–20 min. All conditions for SV were identical to those for polymerization reactions except temperature. Each measurement was conducted with a radial increment of 0.003 cm in a continuous scanning mode.

High Precision Density Measurements—All measurements of density were made using a vibrating tube density meter (DMA5000, Anton Paar, Austria) with a precision of 1×10^{-6} g·ml⁻¹, and the polypeptide concentration was between 0.25 and 4 mg·ml⁻¹. To prevent condensation in measuring density at 5 °C, a U-shaped densitometric cell was thoroughly dried with the use of a dry air pump, and ambient relative air humidity and temperature were kept under 30% and 25 °C, respectively. The adjustments with water and air were performed following every single set of measurements, and almost no deviation of water density before and after a series of density measurements was confirmed.

RESULTS

Characterization of Various Conformational States—The far-UV CD spectrum of native $\beta 2$ -m at pH 7.0 (Fig. 1A) is con-

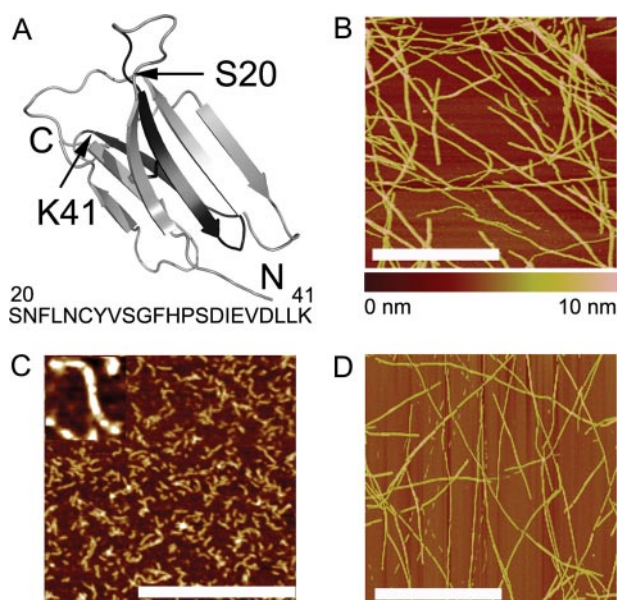


FIGURE 2. **Distinct conformational states of β 2-m.** A, structure of native β 2-m and amino acid sequence of K3 peptide. The region corresponding to the K3 is shown in dark gray. The three-dimensional structure was drawn by PyMOL (38) with the crystal structure (Protein Data Bank entry 2d4f) (15). AFM images of mature fibrils of β 2-m (B), immature fibrils of β 2-m (C), and mature fibrils of K3 (D) are shown. C, inset, curved immature fibrils are magnified. The scale bars represent 1 μ m.

sistent with that of our earlier study that exhibited a 7-stranded β -sheet as shown by x-ray crystallography (Fig. 2A) (15, 26). In contrast, β 2-m is largely unfolded at pH 2.3 as indicated by far-UV CD (Fig. 1A).

We obtained a clear solution of the mature fibrils of β 2-m by performing seed-dependent elongation in 6 mM HCl (pH 2.3) and 38 mM NaCl. The intensity of the ThT fluorescence increased with the incubation period without a lag time (Fig. 1B). The far-UV CD spectra of these fibrils possessed a minimum at 220 nm and were compatible with those of mature fibrils studied previously (Fig. 1A) (16, 26). In AFM images, mature fibrils showed a morphology similar to that of the typical amyloid fibrils formed at acidic pH and 100 mM NaCl, *i.e.* several micrometers in length with a diameter of \sim 5 nm (Fig. 2B) (3).

We ensured the satisfactory dispersion of fibrils for measurements of density and confirmed the presence of residual monomers using SV at $17,400 \times g$ and 5°C for several hours with AUC equipped with a UV-visible absorption optical system. We obtained a series of sedimentation curves by monitoring absorbance at 280 nm, suggesting the absence of precipitates, *i.e.* uniformly dispersed solutes (Fig. 3A). If much larger molecules not suitable for densitometric measurements are present, one cannot obtain a series of well defined sedimentation curves. For instance, amyloid β -(1–40) fibrils formed at pH 7.2 rapidly sedimented during pre-centrifugation at $700 \times g$ within 5 min, as shown in supplemental Fig. S1A. On the basis of SV, a distribution of sedimentation coefficient ($s_{20,w}$) values for mature β 2-m fibrils was obtained (supplemental Fig. S1B). The $s_{20,w}$ which reflects a molecular weight, showed a broad distribution from 100 S to 350 S. The variance in $s_{20,w}$ values may come from the difference in length of fibrils. The base lines of UV spectra of

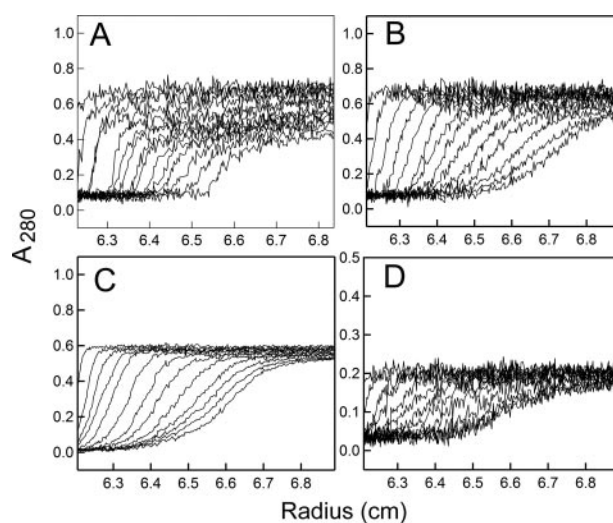


FIGURE 3. **Sedimentation velocity analyses of the fibrils of β 2-m and K3.** The sedimentation curves of mature fibrils of β 2-m (A), short mature fibrils of β 2-m (B), immature fibrils of β 2-m (C), and mature fibrils of K3 (D) were obtained at 5°C by monitoring the absorbance at 280 nm.

the sedimentation curves were slightly above zero, indicating that not all monomers transformed into fibrils. The residual monomers accounted for up to about 10% of the total intensity of absorbance.

To examine the effects on volume of the increase in accessible surface area (ASA) of the terminal parts of the fibrils, we further investigated the partial volume of short mature fibrils of β 2-m prepared by ultrasonically long mature fibrils. SV measurements for the short mature fibrils were conducted at $17,400 \times g$ and 5°C for several hours. We obtained a sedimentation pattern indicating the fibrils to be well dispersed (Fig. 3B). The $s_{20,w}$ values of short fibrils exhibited a narrow distribution from 50 S to 90 S (supplemental Fig. S1B). The values were smaller than those of long fibrils, implying that the fibrils were shortened by sonication. About 10% of residual monomers remained unreacted based on the absorbance at 280 nm (Fig. 3B).

The immature fibrils of β 2-m were spontaneously polymerized in solution containing 200 mM NaCl at pH 2.5 and 37°C . The formation of fibrils was monitored by the changes in intensity of ThT, and immature fibrils were elongated without a lag time as in our previous study (Fig. 1B) (26). The far-UV CD spectrum was consistent with that of the earlier study (Fig. 1A) (26). The AFM image revealed fibrils that were curved and short with a small diameter (\sim 2 nm) (Fig. 2C). The immature fibrils prepared here were shorter than the previously reported immature fibrils (26) because of the low concentration of salt (200 mM NaCl) compared with the previous study (400 mM NaCl) (1). However, this difference could not affect volumetric properties as in the case of long and short mature fibrils of β 2-m (see under "Discussion"). Based on the results of the SV experiments with AUC at $31,000 \times g$ and 5°C for several hours, immature fibrils were in a sufficiently dispersed state (Fig. 3C). The $s_{20,w}$ values showed a narrow distribution from 10 S to 30 S (supplemental Fig. S1B). The smaller $s_{20,w}$ values than those of mature fibrils would be due to the short length and single-stranded property (Fig. 2C). The base lines of UV spectra were

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nearly zero, indicating that almost all monomers were transformed into fibrils (Fig. 3C).

The far-UV CD spectrum of K3 peptides at 6 mM HCl (pH 2.3) and 38 mM NaCl showed a substantially unfolded structure (Fig. 1A). The mature fibrils were seed-dependently polymerized at 6 mM HCl (pH 2.3) and 7 mM NaCl. The fibril formation monitored by ThT intensity exhibited a similar profile to that of immature fibrils but was slightly higher (Fig. 1B). The CD spectrum of K3 fibrils was similar with that of mature β 2-m fibrils (Fig. 1A). The AFM image of K3 fibrils revealed long thin fibrillar structures with a diameter of ~ 3 nm (Fig. 2D). SV experiments were also undertaken at $17,400 \times g$ and 5°C for several hours. We found that mature K3 fibrils were dispersive enough, and about 10% K3 monomers were left as judged from absorbance baselines (Fig. 3D). The distribution of $s_{20,w}$ values of K3 fibrils had a broad range from 10 S to 120 S (supplemental Fig. S1B). Considering the long length of K3 fibrils (Fig. 2D), the small $s_{20,w}$ values compared with those of β 2-m fibrils could result from the low molecular weight of K3 monomers and the single- or double-stranded property.

Density and Volumetric Measurements—Density values of individual samples measured were transformed into the apparent specific volume (ν_{app}) using Equation 1. To obtain the partial specific volume at infinite dilution, ν° , a series of ν_{app} values were plotted against concentration and then linearly extrapolated to the zero concentration according to Equation 2 (Fig. 4),

$$\nu_{\text{app}} = 1/c - (\rho - c)/\rho_0c \quad (\text{Eq. 1})$$

$$\nu^\circ = \lim_{c \rightarrow 0} \nu_{\text{app}} \quad (\text{Eq. 2})$$

where ρ and ρ_0 are the densities of the solution and solvent, respectively; and c is the specific concentration of the protein in grams/ml of solution. The measured densities and calculated ν_{app} of all the samples are shown in Fig. 4, and the mean ν° values of the three series of density measurements for all the samples are presented Table 1.

The ν° values of several native proteins, *i.e.* ferredoxin-NADP⁺ reductase (FNR), ferredoxin, HEWL, and β 2-m, varied depending on the kinds of proteins and the temperature. We first obtained a ν° of HEWL comparable with that of an earlier study under the same conditions (29). The ν° of FNR was larger than that of ferredoxin at pH 6.0 and 20°C . Native β 2-m at pH 7.0 and 20°C had a small ν° compared with ferredoxin and FNR. The ν° of native β 2-m dropped as the temperature was lowered. For the densitometry of all fibrils prepared, we conducted measurements at low temperature, 5°C , to avoid destabilization and lateral aggregation of fibrils. Hence, to compare differences in the volume of diverse conformational states at the same temperature, the densities of native and denatured β 2-m as well as K3 peptide were also measured at 5°C . The ν° values obtained were in the following order: mature fibrils > native monomer > acid-denatured monomer \approx K3 monomer > immature fibrils \gg mature fibrils of K3.

The formation of the main-chain-dominated structure of fibrils from monomers showed various changes in volume. Mature fibrils of β 2-m showed the largest ν° of the polypeptides explored here. The ν° values of immature and mature K3 fibrils

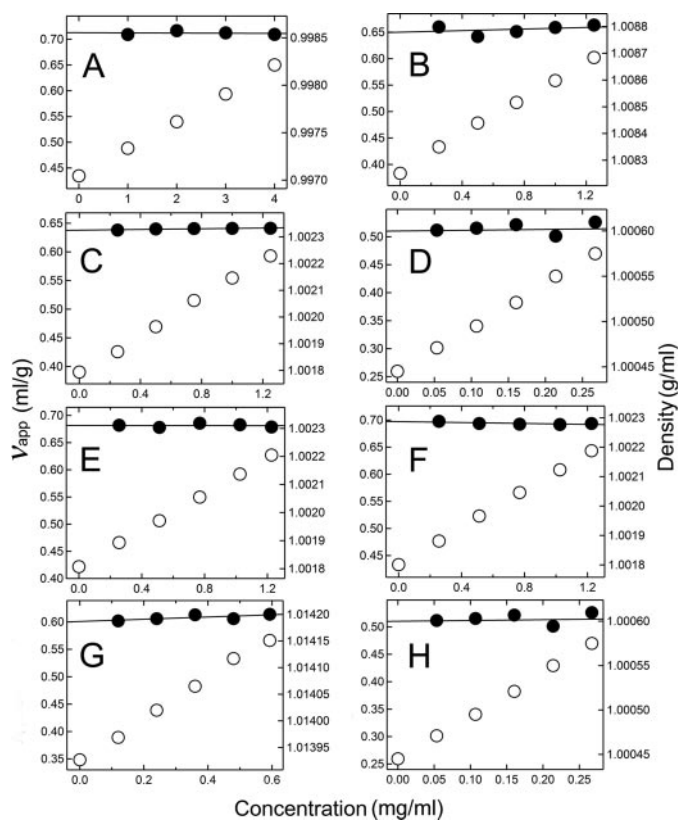


FIGURE 4. Density and apparent specific volume (ν_{app}) values for various conformational states of polypeptides. A–H, measured densities (\circ) were plotted against concentration for HEWL (A), native β 2-m (B), acid-denatured β 2-m (C), K3 peptide (D), long mature fibrils of β 2-m (E), short mature fibrils of β 2-m (F), immature fibrils of β 2-m (G), and mature fibrils of K3 peptide (H). The density measurements were conducted at 5°C except for HEWL, which was measured at 25°C . Individual density values of polypeptides were converted to ν_{app} (\bullet) using Equation 1 and plotted as a function of concentration. To obtain partial specific volume at infinite dilution (ν°), ν_{app} values were linearly extrapolated to the zero concentration on the basis of Equation 2. One series of density and ν_{app} values of the three are shown. The left and right axes represent ν_{app} and density, respectively.

were lower than a side-chain-dominated native β 2-m. The ν° value of mature K3 fibrils was the lowest of the three fibrous conformations. As largely extended β 2-m folded to its a native structure, the $\Delta\nu^\circ$ increased as much as $0.016 \pm 0.009 \text{ ml}\cdot\text{g}^{-1}$. This $\Delta\nu^\circ$ corresponds to the change in partial molar volume (PMV) of $190 \pm 106 \text{ ml}\cdot\text{mol}^{-1}$. The transition from the unfolded β 2-m to mature fibrils was accompanied by an augmentation of volume, $\Delta\nu^\circ = 0.046 \pm 0.008 \text{ ml}\cdot\text{g}^{-1}$ and $\Delta\text{PMV} = 499 \pm 108 \text{ ml}\cdot\text{mol}^{-1}$. Interestingly, the formation of immature fibrils from the unfolded β 2-m caused decreases in volume, $\Delta\nu^\circ = -0.044 \pm 0.008 \text{ ml}\cdot\text{g}^{-1}$ and $\Delta\text{PMV} = -523 \pm 96 \text{ ml}\cdot\text{mol}^{-1}$. The transition of K3 from a monomer to fibrils was followed by a much larger reduction in volume, $\Delta\nu^\circ = -0.137 \pm 0.006 \text{ ml}\cdot\text{g}^{-1}$ and $\Delta\text{PMV} = -343 \pm 16 \text{ ml}\cdot\text{mol}^{-1}$. It should be noted that ν° values of mature fibrils of β 2-m and K3 were corrected on the basis of those of the unreacted monomers (10%).

DISCUSSION

Determinants of Changes in Volume—The partial specific volume of a solute at infinite dilution, ν° , can be presented as the sum of four terms (Equation 3) (18, 19, 30, 31).

TABLE 1
Summary of partial specific volume (ν°) for polypeptides of various conformations

Polypeptide	ν° $\text{ml}\cdot\text{g}^{-1}$	Conditions
Native HEWL	0.711 ± 0.002	25 °C, water from this study
Native HEWL	0.712 ± 0.001	25 °C, water from Gekko and Noguchi (29)
Native FNR	0.709 ± 0.001	20 °C, 25 mM sodium phosphate (pH 6.0) and 50 mM NaClO ₄
Native ferredoxin	0.686 ± 0.003	20 °C, 25 mM sodium phosphate (pH 6.0) and 50 mM NaClO ₄
Native β 2-m	0.668 ± 0.004	20 °C, 50 mM sodium phosphate (pH 7.0) and 38 mM NaCl
Native β 2-m	0.652 ± 0.004	5 °C, 50 mM sodium phosphate (pH 7.0) and 38 mM NaCl
Denatured β 2-m	0.640 ± 0.001	5 °C, 50 mM sodium citrate (pH 2.5)
Denatured β 2-m	0.620 ± 0.002	5 °C, 50 mM sodium citrate (pH 2.5) and 5.7 M Gdn-HCl
Denatured β 2-m	0.636 ± 0.008	5 °C, 6 mM HCl (pH 2.3) and 38 mM NaCl
K3 peptide of β 2-m	0.635 ± 0.006	5 °C, 6 mM HCl (pH 2.3) and 38 mM NaCl
Mature β 2-m fibrils (long)	0.682 ± 0.009	5 °C, 6 mM HCl (pH 2.3) and 38 mM NaCl
Mature β 2-m fibrils (short)	0.697 ± 0.013	5 °C, 6 mM HCl (pH 2.3) and 38 mM NaCl
Mature K3 fibrils	0.498 ± 0.002	5 °C, 6 mM HCl (pH 2.3) and 7 mM NaCl
Immature β 2-m fibrils	0.596 ± 0.008	5 °C, 50 mM sodium citrate (pH 2.5) and 200 mM NaCl

$$\nu^\circ = \nu_{\text{geo}} + \nu_{\text{cav}} + \Delta\nu_{\text{hyd}} + \nu_{\text{therm}} \quad (\text{Eq. 3})$$

where ν_{geo} represents the geometric volume of the constituent atoms such as the van der Waals and excluded volumes, and ν_{cav} reflects the cavity (void) volumes in the interior of the molecule because of imperfect atomic packing. Thus increases in ν_{geo} or ν_{cav} serve as elevating the volume. $\Delta\nu_{\text{hyd}}$ indicates the changes in volume resulting from hydration of the molecule and can be divided into three terms, the hydration of apolar ($^{\text{apol}}\nu_{\text{hyd}}$), polar ($^{\text{pol}}\nu_{\text{hyd}}$), and charged ($^{\text{cha}}\nu_{\text{hyd}}$) atomic groups. $^{\text{pol}}\nu_{\text{hyd}}$ and $^{\text{cha}}\nu_{\text{hyd}}$ contribute negatively to $\Delta\nu_{\text{hyd}}$ because of hydrogen bonding or electrostriction (18, 19, 30). Although there is the report of the positive contribution of $^{\text{apol}}\nu_{\text{hyd}}$ to $\Delta\nu_{\text{hyd}}$, the sign of $^{\text{apol}}\nu_{\text{hyd}}$ is still controversial (32). ν_{therm} refers to thermal volume that represents the void space around the solute molecules that arises from the mutual thermal motions of the solute and solvent molecules (18, 19, 30), and contributes positively to changes in volume.

It should be noted that increasing the ASA is closely related to increasing ν_{geo} , ν_{hyd} , and ν_{therm} . It is generally accepted that denaturation of a protein causes the increase in ASA with the decrease in volume (31), although relative contributions of four terms in Equation 3 to volume changes is difficult to access. An eventual positive or negative change in volume in a system of interest is a result of the delicate balance between these four terms.

From the Acid-denatured β 2-m to Native β 2-m—To investigate the effect of a possible residual structure in the acid-denatured β 2-m, we compared the ν° values of the different denatured species. We first obtained a ν° of $0.636 \pm 0.008 \text{ ml}\cdot\text{g}^{-1}$ for the β 2-m solution containing 6 mM HCl at pH 2.3 and 38 mM NaCl, which is similar to the ν° of $0.640 \pm 0.001 \text{ ml}\cdot\text{g}^{-1}$ for the acid-denatured β 2-m in 50 mM citrate buffer at pH 2.5 (Table 1). Adding 5.7 M Gdn-HCl to 50 mM citrate buffer at pH 2.5, the ν° values of acid-induced unfolded β 2-m further diminished to $0.620 \text{ ml}\cdot\text{g}^{-1}$ (Table 1). This might imply the presence of residual structures in the acid-denatured β 2-m as indicated by the ability to bind 1-anilino-8-naphthalenesulfonate (27) and by the fast relaxation rates of ¹⁵N with NMR spectroscopy (33). We suggest that the decrease in volume upon the addition of Gdn-HCl might result from disruption of the non-native cavities in the residual structure and from the increases in hydra-

tion, although there are possible changes in volume depending on the concentration of Gdn-HCl.

The structural transition of β 2-m from the HCl-denatured (pH 2.3) to native (pH 7.0) species was accompanied by the increase in volume. This elevation is most likely because of the formation of internal cavities and a decrease in hydration despite reductions in excluded and thermal volumes. It is difficult, however, to distinguish between the contribution to changes in volume arising from the different pH values.

The ν° of the native β 2-m at pH 7.0 and 5 °C was rather low ($0.652 \text{ ml}\cdot\text{g}^{-1}$) as compared with ν° at pH 7.0 and 20 °C and ν° of other native proteins (Table 1) (18, 30). This can be explained by the positive value for the thermal expansibility of a protein, because thermal expansibility is defined by $(1/\nu^\circ)(\delta\nu^\circ/\delta T)_p$, where T is temperature, and p is pressure (18).

From the Acid-denatured β 2-m to Mature Amyloid Fibrils—The formation of mature fibrils from monomers led to the increase in volume (Fig. 5A). This would be a balance between the increase in volume caused by the formation of cavities and the reduction in hydration and the decrease in volume because of reductions in thermal and excluded volumes. The magnitude of the change in volume was larger for the misfolding reaction (*i.e.* fibril formation) than folding reaction, by $0.025 \text{ ml}\cdot\text{g}^{-1}$ (Fig. 5A and Table 1). The different extent of volumetric change between folding and misfolding implies that the difference in volume between amyloid fibrils and native species was caused by the formation of loosely packed amyloid fibrils.

This interpretation can be supported by our previous studies. A calorimetric study on the transitions of the amyloid fibrils and native β 2-m to the unfolded β 2-m suggested a similar extent of surface burial (similar ΔC_p) in both conformational transitions and a lower level of internal packing (lower ΔH) of amyloid fibrils than native monomers (5). Moreover, a high hydrostatic pressure study on amyloid fibrils of β 2-m also indicated a lower level of internal packing of fibrils (3).

Interestingly, the ν° of mature β 2-m fibrils fragmented by sonication was indistinguishable from that of the original long fibrils (Table 1). This signifies that the increased ASA of terminal parts of fibrils on the shortening of mature fibrils was too small to affect the volumetric properties of mature fibrils, or was a result canceled out between four terms in Equation 3.

Volumetric Variation of Amyloidogenesis

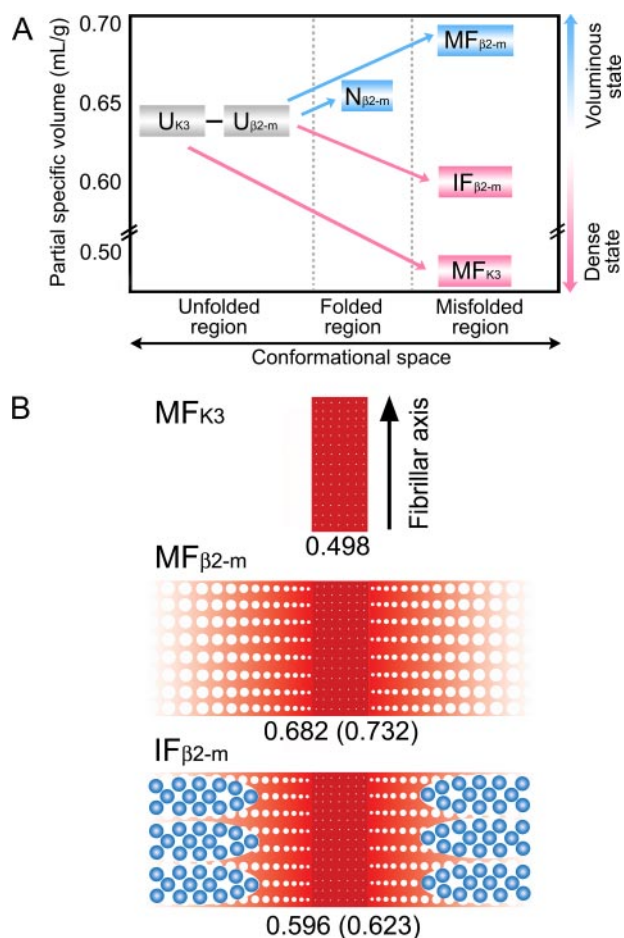


FIGURE 5. Volumes of various conformational states and schematic models for distinct fibrillar conformations. *A*, partial specific volume (ν°) of each conformation is shown as a function of conformational space, and boundaries of conformational regions are denoted with dotted lines. $U_{\beta 2-m}$ exhibits acid-denatured $\beta 2-m$, and $N_{\beta 2-m}$ indicates native $\beta 2-m$. $MF_{\beta 2-m}$ exhibits mature amyloid fibrils of $\beta 2-m$. Immature fibrils of $\beta 2-m$ are denoted as $IF_{\beta 2-m}$. An amyloidogenic fragment of $\beta 2-m$, K3 peptide, is shown as U_{K3} . MF_{K3} indicates the mature fibrils of K3. The tilted arrows indicate directions of reactions for conformational transitions. Magenta arrows and rectangles represent decreases in volume following structural transition, and blue arrows and rectangles indicate increases in volume. The short horizontal black line indicates small differences in ν° values between both structural states. *B*, proposed structures for the three types of fibrils are shown as side views along the fibrillar axis. The conformations of MF_{K3} , $MF_{\beta 2-m}$, and $IF_{\beta 2-m}$ are depicted. The white spheres of various sizes indicate internal cavities, and blue circles indicate water molecules. Red rectangles at the center show a compact core structure, and those outside the core structures indicate exhibit packing-defective regions. The values of ν° in units of $\text{ml}\cdot\text{g}^{-1}$ are denoted below the corresponding models, and the ν° values for outside of the core structure are given in parentheses.

From K3 Peptide to Mature Amyloid-like Fibrils—The transition from the unfolded K3 monomer to the fibrils dropped significantly the ν° (Fig. 5A and Table 1) in contrast to the transition of monomers to fibrils of $\beta 2-m$. The negative volumetric change would be caused by the formation of remarkably tight packing inside the fibril. This coincides with our previous solid-state NMR study that suggested that the conformation of the K3 molecule in fibrils retains tight packing with adjacent K3 molecules (34). Moreover, the highly persistent protection profile of K3 fibrils, except the two residues of the N terminus revealed by hydrogen-deuterium exchange (HD_{ex}) experiments, suggests that most of residues of K3 constitute the core structure of the K3 fibrils (14).

Several other reports also pointed out that a tightly packed cross- β -structure, *i.e.* core structure, in the fibrils was made of short polypeptides (7–12, 25). It may also be conceivable that a water molecule is trapped in the cavity during the formation of fibrils, which cause a reduction in volume (35, 36). Taken together, mature K3 fibrils assume a highly packed conformation representative of a core structure of amyloid fibrils, although the effect of associations of fibrils on changes in volume is not considered here.

From the Acid-denatured $\beta 2-m$ to Immature Amyloid-like Fibrils—The changes in volume caused by the transition from monomers to immature fibrils were explored. Intriguingly, the ν° of immature fibrils was lower than that of unfolded $\beta 2-m$ (Fig. 5A and Table 1). This decrease would result from the formation of a compact core structure in the center of immature fibrils rendering the outside of the core structure loosely/moderately packed and highly hydrated simultaneously. To investigate solvent accessibility, we further conducted Gdn-HCl-induced unfolding experiments for mature and immature fibrils (details are provided in supplemental Fig. S1). We found that mature fibrils were more stable than immature fibrils (supplemental Fig. S2), implying that immature fibrils are more prone to contact with the solvent than mature fibrils.

In addition, our recent work of HD_{ex} for mature and immature fibrils reinforces this interpretation. Both fibrils formed at acidic pH showed distinct protection patterns (13, 14). Almost 80% of the entire sequence of $\beta 2-m$ was protected from HD_{ex} in the mature fibrils involving the region corresponding to the core structure of K3 fibrils, whereas only 30% of the entire sequence was protected in the immature fibrils accommodating many residues of the core structure of K3 fibrils. Although the core structure of mature K3 fibrils was not exactly consistent with that of the mature and immature fibrils, the concept of a core structure at the center of mature and immature fibrils could still be valid.

Simple Models for Various Fibrous Structures—As indicated in Fig. 5A, an unstructured polypeptide folds to the native conformation with an increase in volume. However, misfolding of a random coil-like structure toward main-chain-dominated fibrous conformations can be followed by an increase or decrease in volume. To better understand various fibrils possessing distinct volumes and morphologies, we suggest concise models for three types of fibrous structures examined here with simple quantitative evaluation of ν° (supplemental equation S1).

Mature fibrils of K3 would be comprised of a core structure with compact packing as evidenced by the ν° of $0.498 \text{ ml}\cdot\text{g}^{-1}$ (Fig. 5B). The tight packing of K3 fibrils is a special case achieved only when all of the side chains can form ideal stabilizing interactions with their counterparts without any steric hindrance.

Mature fibrils of $\beta 2-m$ would accommodate a highly packed core structure that causes a decrease in volume, and the rest of the core structure mainly consists of solvent-inaccessible areas with extended hydrogen bonds and loose packing (Fig. 5B) that causes an increase in volume. Indeed, the estimated ν° of the exterior region of the core structure (ν°_{OUT}) of mature $\beta 2-m$ fibrils was $0.732 \text{ ml}\cdot\text{g}^{-1}$ (supplemental equation S1) and larger

than ν° of native β 2-m (0.652 ml g^{-1}) (Table 1), indicating a highly bulky state. Therefore, outside of the center can produce cavities, thereby rendering mature fibrils compressible and multiple structures called “polymorphism.”

Likewise, the calculated ν°_{OUT} of the immature fibrils was 0.623 ml g^{-1} , which is comparable with the unfolded state (Fig. 5B and Table 1). This value may be explained by much hydration and a small sparse area outside the core region with fewer hydrogen bonds (Fig. 5B). These volumetric features might endow immature fibrils with a thin curved conformation. It should be noted that the less volume changes of fibrillogenesis of the short TTR-(105–115) than that of the full-length TTR implying the core structure would support our model (21, 25).

In conclusion, taking the present and our previous results together, we suggested three concise and straightforward models illustrating the conformational states of the three fibrils at acidic pH. Although an evolutionally attained native structure is folded pursuing the optimal packing and burial of side chains (37), a main-chain-dominated structure of amyloid fibrils is formed with a lot of void spaces because of loose packing, which can subsequently trigger polymorphism of amyloid fibrils. This tunable volume change might signify that loosely packed parts of amyloid fibrils serve as its survival for responses of changes in ambient conditions such as robust folding pathways, in turn, and might mean an alternative evolutionary selection as a rudimentary structure.

Finally, the comparisons between the partial volumes of the β 2-m and K3 fibrils formed at acidic and neutral pH values would provide more detailed insights into structural models of amyloid fibrils, although we need further efforts of preparing well dispersed fibrils at neutral pH for ensuring the reliable and precise measurements of density.

Acknowledgments—We thank Azusa Okamoto and Miyo Sakai for help with the Gdn-HCl-induced unfolding experiments and AUC measurements of the fibrils, respectively. We thank Shun-ichi Kidokoro (Nagaoka University of Technology) for technical support of densitometry.

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A Comprehensive Model for Packing and Hydration for Amyloid Fibrils of β_2 -Microglobulin

Young-Ho Lee, Eri Chatani, Kenji Sasahara, Hironobu Naiki and Yuji Goto

J. Biol. Chem. 2009, 284:2169-2175.

doi: 10.1074/jbc.M806939200 originally published online November 18, 2008

Access the most updated version of this article at doi: [10.1074/jbc.M806939200](https://doi.org/10.1074/jbc.M806939200)

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