Conformation of Amyloid Fibrils of β2-Microglobulin Probed by Tryptophan Mutagenesis*

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β2-Microglobulin (β2-m), a protein responsible for dialysis-related amyloidosis, adopts an immunoglobulin domain fold in its native state. Although β2-m has Trp residues at positions 60 and 95, both are located near the surface of the domain. Hence, β2-m does not have a conserved Trp common to other immunoglobulin domains, which is buried in close proximity to the disulfide bond. To study the structure of amyloid fibrils in relation to their native fold, we prepared a series of Trp mutants. Trp60 and Trp95 were both replaced with Phe, and a single Trp was introduced at various positions. Among various mutants, W39-β2-m, in which a Trp was introduced at the position corresponding to the conserved Trp, exhibited a remarkable quenching of fluorescence in the native state, as observed for other immunoglobulin domains. An x-ray structural analysis revealed that W39-β2-m assumes the native fold with Trp95 located in the vicinity of the disulfide bond. Comparison of the fluorescence spectra of various mutants for the native and fibrillar forms indicated that, while the Trp residues introduced in the middle of the β2-m sequence tend to be buried in the fibrils, those located in the C-terminal region are more exposed. In addition, the fluorescence spectra of fibrils prepared at pH 2.5 and 7.0 revealed a large difference in the fluorescence intensity for W60-β2-m, implying a major structural difference between them.

Amyloidosis accompanies the deposition of normally soluble proteins into insoluble amyloid fibrils (1–3). Among various amyloidogenic proteins, β2-microglobulin (β2-m) is a target of extensive study because of its clinical importance and a suitable size for examining the relation between protein folding and amyloid fibril formation (4–11). β2-m, a typical immunoglobulin domain made of seven β-strands and one intramolecular disulfide bond (12, 13), is present as the non-polymorphic light chain of the class I major histocompatibility complex (14) (Fig. 1). As a part of its normal catabolic cycle, β2-m is dissociated from the complex and is transported in serum to the kidneys where the majority (95%) of it is degraded (15). Renal failure disrupts the clearance of β2-m from the serum and moreover β2-m does not pass through the dialysis membrane, resulting in an increase in the β2-m concentration by up to 50-fold in the blood circulation (15). When a high blood level is retained for more than 10 years, β2-m then self-associates to form amyloid fibrils, causing dialysis-related amyloidosis (15, 16).

The results so far obtained with various approaches suggest a picture of β2-m amyloid fibrils with an increased amount of β-structure in comparison with the native structure, including the transformation of native β-turns, but with disordered N- and C-terminal regions (6–9, 17). However, details remain unknown. To address the structure of amyloid fibrils, we have taken advantage of the unique properties of Trp fluorescence. One important feature of immunoglobulin domains is the presence of a buried Trp residue located close to the conserved disulfide bonds (18), leading to a strong quenching of Trp fluorescence (19, 20). We noticed that, although β2-m has two Trp residues (i.e. Trp60 and Trp95), neither of these corresponds to the buried Trp common to other immunoglobulin domains. In the x-ray crystallographic structure of wild-type β2-m, while Trp60 located on the β-turn connecting β-strands D and E is exposed to solvent, Trp95 at the end of β-strand G is partially buried (Fig. 1). We designed and expressed a series of single Trp mutants in which Trp residues of wild-type β2-m have been deleted and a new Trp residue was introduced at various positions. Examination of the fluorescence spectra of mutants in the native and amyloid states revealed a notable conformational change upon the formation of amyloid fibrils.

EXPERIMENTAL PROCEDURES

β2-m Amyloid Fibril Formation—The expression and purification of human recombinant β2-m and the single Trp mutants were achieved as described previously (21). Acidic pH fibrils were prepared by a repeated seed-dependent extension with human recombinant β2-m expressed in Escherichia coli (21), or yeast Pichia pastoris (22). Neutral pH fibrils were prepared at pH 7.0 by a repeated seeding starting with the acidic pH fibrils.
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The fibril concentrations were measured with a Micro BCA™ Protein Assay Reagent kit (Pierce).

The amyloid extension reactions of mutant β2-ms were carried out using the wild-type neutral pH seeds and wild-type acidic pH seeds at pH 7.0 and 2.5, respectively. The extension reaction mixture at pH 2.5 contained 25 μM monomeric β2-m and 5 μg/ml acidic seeds. The extension reaction mixture at pH 7.0 contained 25 μM monomeric β2-m, 30 μg/ml neutral pH seeds, and 0.5 mM SDS (23). A low concentration of SDS, below its critical micelle concentration, is considered to stabilize the seeds and fibrils, to induce conformational change of monomers, and most importantly, to induce the oligomerization of β2-m. The reaction was monitored by the fluorometric assay with thioflavin T (ThT) at pH 8.5 as described previously (21–23).

AFM images were obtained using a Nano Scope IIIa (Digital Instruments) as described previously (24). The total internal reflection fluorescence microscopy system to observe ThT-bound amyloid fibrils was developed based on the use of an inverted microscope (IX70; Olympus, Tokyo, Japan) as described previously (25).

Trp Fluorescence Measurements—Trp fluorescence spectra were measured with a Hitachi F-4500 spectrofluorometer at 25 °C and a β2-m concentration of 0.05 mg/ml with excitation at 295 nm and monitored at 300–450 nm. Urea- or Gdn-HCl-dependent unfolding transitions of β2-m in the monomeric or amyloid state were measured using the change of Trp fluorescence. Before the measurements, the samples were incubated for 24 h at 25 °C. To compare the spectra of various single Trp β2-m mutants in the native or fibrillar state, the spectra of the unfolded states in 5.5 M Gdn-HCl or 10 M urea are assumed to be the same independent of mutations. We did not normalize the spectra of the unfolded states under different conditions. The spectra of wild-type β2-m with two Trp residues in 5.5 M Gdn-HCl or 10 M urea are assumed to have an intensity 2-fold those of single Trp mutants. We consider that this normalization provides a more accurate comparison of the spectra of various mutants particularly for amyloid fibrils.

A Two-state Unfolding Transition Analysis of the Native State—For the unfolding of the native state at pH 8.0, we further analyzed the results assuming a two-state transition between the native and denatured states (21). To obtain the fraction of unfolded species ($f_U$), the fluorescence spectra from 310 nm to 450 nm were fitted to Equation 1,

$$ F = \alpha f_U F_U + (1 - f_U) F_N $$

where $F$ is the spectrum for a sample with each concentration of denaturant, $F_N$ and $F_U$ that for without denaturant and the highest concentration of denaturant, respectively, $f_U$ is the fractional population of unfolded species, and $\alpha$ is a variable term for fluorescence intensity (21). Exceptionally, for the monomeric states of W39- and W33- β2-m, which did not show a change in maximal wavelength but exhibited a significant change in intensity upon unfolding (see

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(23). Throughout the experiments, we incubated the solutions for the formation of fibrils without agitation. The standard buffers used were 50 mM sodium citrate buffer (pH 2.5) containing 100 mM NaCl and 50 mM sodium phosphate buffer (pH 7.0) containing 100 mM NaCl. β2-m fibrils were originally purified from patients suffering from dialysis-related amyloidosis. Seeds (i.e. fragmented fibrils) were prepared by sonication of 200-μl aliquots of a fibril stock solution using a Microson sonicator (Misonix, Farmingdale, NY) as described previously (23).
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Comparison of conformation and stability of the wild-type and single Trp mutants (W60, W95, and W39) in the monomeric state at pH 8.0 (A) and in the amyloid fibrils at pH 2.5 (B) and pH 7.0 (C). A and B, concentration of Gdn-HCl increases from 0 M (red) to 5.5 M (purple) in steps of 0.5 M, as guided by an arrow. C, concentration of urea increases from 0 M (red) to 10.0 M (purple) in steps of 1.0 M, as guided by an arrow.

**FIGURE 2. Unfolding transitions of the native state and amyloid fibrils of various β2-m mutants.** Unfolding transitions were monitored by Trp fluorescence spectrum of wild-type and single Trp β2-m mutants (W60, W95, and W39) in the monomeric state at pH 8.0 (A) and in the amyloid fibrils at pH 2.5 (B) and pH 7.0 (C). A and B, concentration of Gdn-HCl increases from 0 M (red) to 5.5 M (purple) in steps of 0.5 M, as guided by an arrow. C, concentration of urea increases from 0 M (red) to 10.0 M (purple) in steps of 1.0 M, as guided by an arrow.

**TABLE 1** Comparison of conformation and stability of the wild-type and single Trp β2-ms

| Protein | ΔGU[20°C] | m' | R | Q | λ20 | λ34 | λ39 | Cm | Q' | λ20 | λ34 | λ39 |
|---------|-----------|-----|---|---|-----|-----|-----|-----|----|-----|-----|-----|
| Wild type | 22.4 | 10.2 | 2.2 | 19.5 | 81 | 353 | 350 | 4.1 | 200 | 342 | 347 | 6.4 | 200 | 339 | 349 |
| W60 | 25.5 | 11.1 | 2.3 | 9.3 | 106 | 331 | 348 | 3.6 | 35 | 342 | 347 | 6.5 | 124 | 339 | 351 |
| W95 | 18.2 | 12.0 | 1.5 | 14.6 | 107 | 349 | 350 | 4.7 | 27 | 337 | 350 | 4.9 | 72 | 338 | 349 |
| W39 | 19.5 | 8.8 | 1.8 | 10.5 | 78 | 325 | 349 | 4.2 | 8 | 348 | 344 | 4.6 | 17 | 331 | 350 |

- ΔGU[20°C] = (GΔGU[20°C])Δ and a measure for cooperativity of unfolding (m') as described by Equation 3.
- MIDPOINT denaturant concentration of unfolding.
- Distance between the Cα of the Trp residue and the center of the Cys25–Cys80 disulfide bond, which was calculated from the coordinate of wild-type β2-m with Trp residues defined to be 200%.
- Relative fluorescence spectral area of Trp among various mutants in the same conformational states where those of the wild-type β2-m with two Trp residues were defined to be 200%.
- Maximal wavelength of Trp fluorescence in the native state (λ20), denatured state (λ39), or amyloid fibrils (λ39).
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monitored by comparing the R factor to the free R factor (32). The geometry of the model was monitored using the program PROCHECK (33), the Ramachandran plot showing that 99% of the residues lie in the allowed region.

RESULTS

CD Spectra of Single Trp Mutants—CD spectra of single Trp β2-ms were measured at pH 8.0 (see supplemental Fig. S1). The far-UV CD spectra of all the mutants were similar to those of wild-type β2-m (23). On the other hand, the near-UV CD spectra were differed, indicating that Trp residues contribute to the near-UV CD and that the environment of these residues differs depending on the mutant. Thus, in overall structure, the single Trp mutants were similar to the wild type.

Trp Fluorescence Spectra of Single Trp Mutants—We characterized the single Trp mutants using Trp fluorescence (Fig. 2, Table 1, see also supplemental Fig. S2). The fluorescence spectrum of wild-type β2-m in the native state at pH 8.0 showed a maximum (λmax) at 337 nm (Fig. 2A). The addition of high concentrations of Gdn-HCl resulted in a red shift of the λmax to 350 nm, accompanied by a decrease in the intensity of fluorescence. W60-β2-m showed a λmax at 353 nm even in the absence of Gdn-HCl and an increase in the Gdn-HCl concentration resulted in only a slight shift of the λmax to a shorter wavelength accompanied by a slight decrease in fluorescence (Fig. 2A). The results are consistent with the complete exposure of Trp60 in the crystallographic structure (12). On the other hand, the fluorescence spectrum of W95-β2-m showed a similar change to that of the wild type, indicating that, although Trp95 is partly buried, it is not quenched by the disulfide bond (Fig. 2A). In fact, the fluorescence spectrum of wild-type β2-m was reproduced by adding the spectra of W60- and W95-β2-ms (Fig. 3A, dashed line). These results clearly demonstrate that Trp60 predominantly contributes to the fluorescence of wild-type β2-m.

W39-β2-m is a triple mutant in which Trp39 and Trp95 were replaced by Phe, and Leu39, the position of the conserved Trp residue common to many immunoglobulin domains, was replaced by Trp. The fluorescence spectrum of W39-β2-m exhibited complete quenching in the absence of Gdn-HCl (Fig. 2A). These observations are consistent with an immunoglobulin domain having a Trp residue at the corresponding position (19, 20), in which the fluorescence of the conserved Trp is quenched by the disulfide bond. A cooperative change in fluorescence intensity was observed with an increase in the concentration of Gdn-HCl, suggesting that W39-β2-m assumes the native fold in the absence of Gdn-HCl (see below).

Other mutants studied were W33-, W49-, and W78-β2-ms (Fig. 3A, see supplemental Fig. S2). With an increase in the Gdn-HCl concentration, the fluorescence of W33-β2-m showed no shift in λmax and the intensity decreased slightly, indicating that Trp33 is exposed. The Trp fluorescence of W49-β2-m showed a slight shift in the λmax toward a longer wavelength and a slight increase in intensity, indicating that Trp49 is partly buried and slightly quenched. The Gdn-HCl-induced spectral change of W78-β2-m was similar to that of wild-type β2-m, although the λmax of the native state was shorter than that of the wild type. The result indicates that Trp78 of W78-β2-m is more buried than Trp95 of the wild type.

Altogether, the fluorescence spectra of the single Trp mutants are consistent with the location of mutated residues in the x-ray native structure (Fig. 1), arguing that these mutants provide excellent probes with which to monitor the conformational change upon the formation of fibrils.

The Gdn-HCl-induced unfolding transitions were constructed on the basis of the spectral change (Fig. 4). In the cases...
of W49- and W60-/H9252 2-ms, we could not obtain a transition curve because the location of the Trp residues on the surface resulted in no significant spectral change upon unfolding. For other mutants, it has been revealed that the stability decreased to various extents in comparison with that of the wild type. In particular, the destabilization was significant for W39-/H9252 2-m. It is conceivable that the replacement of Leu39 located in the tightly packed interior of β2-m by bulky Trp induced significant steric hindrance. Thus, it is important to confirm the native fold of W39-/H9252 2-m.

X-ray Crystallographic Structures—We determined the crystallographic structures of wild-type and W39-/H9252 2-ms (Fig. 5) using the molecular replacement method on the basis of the monomeric crystal structure prepared at pH 5.7 (12). Both crystals belong to space group C2 and the unit cell dimensions are $a = 77.4$ Å, $b = 28.8$ Å, $c = 54.8$ Å, and $β = 121.7^\circ$ for the wild-type and $a = 77.4$ Å, $b = 28.8$ Å, $c = 57.2$ Å, and $β = 123.6^\circ$ for W39-/H9252 2-m. A summary of the refinement statistics is given in Table 2. The coordinates and structure factor amplitudes have been deposited in the Protein Data Bank (ID code 2D4F for the wild-type and 2D4D for W39-/H9252 2-m). Our structure of wild-type β2-m at pH 5.6 without a bulge in the βD strand is basically the same as that previously reported (12).

Despite the triple mutations (W60F/W95F/L39W), the crystal structure of W39-/H9252 2-m was very similar to that of wild-type.
The RMS deviations between them are 0.51 Å using main chain atoms, N, Ca, C, and O. Slight differences exist at residues Arg45-His51 on the CD loop and Lys58-Trp60 on the DE loop. The difference in the CD loop is caused by a difference in crystal packing interactions with symmetry-related β2-m molecules. For the DE loop, the mutation of Trp to Phe should yield a void, possibly producing the deviation. The structure of W39-β2-m shows tight contact between Trp39 and the disulfide bond, with the aromatic ring plane facing the disulfide bond in parallel as observed for other immunoglobulin domains (Fig. 5D) (18). The results reveal the native fold of W39-β2-m and furthermore the proximity of Trp39 to the disulfide bond, proposing the structural basis for the complete quenching of Trp fluorescence. Moreover, a clear calorimetric peak typical of the unfolding of globular proteins was observed for W39-β2, although decreases in stability were also evident (see supplemental Fig. S3). Additionally, W49- and W60-β2-ms, whose conformational stability could not be evaluated based on Trp spectral change, also exhibited calorimetric peaks, supporting the native fold of these mutants.

Formation of Amyloid Fibrils—The formation of amyloid fibrils of single Trp mutants was studied using seed-dependent fibril growth at pH 2.5 and 7.0, and monitored by measuring ThT fluorescence (Fig. 6). At each pH, seed fibrils prepared with wild-type β2-m were used, ensuring the formation of fibrils with wild-type structures even when various amyloid conformations can coexist (23, 34). At pH 2.5, most of the mutants except W33- and W49-β2-ms completed the extension reaction in a couple of hours, similar to the standard kinetics of fibril growth with wild-type β2-m (21, 24). W33- and W49-β2-ms showed a lag-phase. Because we used the wild-type seeds, it is possible that the lag-phase represents the difficulty of accommodating these bulky mutations at Ser33 and Val49, producing a slightly different type of fibrils. The final intensity of ThT depended on the mutant, suggesting that the exact structure of the amyloid fibrils depends on the site of mutation, as previously discussed (21).

At pH 7.0, wild-type β2-m exhibits seed-dependent fibril extension in the presence of maturated seeds and 0.5 mM SDS, although several hours are required, and the final level of ThT fluorescence is less than that at pH 2.5 (23). At this stage of our experiments, 0.5 mM SDS was essential (23, 35). W60-, W95- and W78-β2-ms showed similar extension kinetics to wild-type β2-m. On the other hand, the formation of fibrils by W33-, W39- and W49-β2-ms was suppressed and enough amounts of fibrils for the subsequent experiments were not obtained for W33-β2-m. As was the lag-phase at pH 2.5, the significant suppressions may represent the difficulty of accommodating the bulky Trp mutations at Ser33, Leu39, and Val49 (Fig. 6B).

Fibrils of W39-β2-m as well as those of wild-type β2-m were confirmed to have formed at pH 2.5 and 7.0 by AFM (Fig. 7, A–D). Fibrils of W39-β2-m were also visualized with total internal reflection fluorescence microscopy taking advantage of

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**TABLE 2**

| Crystal | Wild type | W39 |
|---------|------------|-----|
| Space group | C2 | C2 |
| Unit cell |
| $a$ | 77.6 | 77.4 |
| $b$ | 28.9 | 28.8 |
| $c$ | 54.8 | 57.2 |
| $\beta$ | 121.7 | 123.6 |
| Resolution | 38.0-1.7 (1.79-1.70) | 47.67-2.1 (2.21-2.20) |
| Number of observed reflections | 39670 (4129) | 22387 (3270) |
| Number of unique reflections | 11279 (1454) | 6343 (927) |
| Completeness (%) | 97.3 (87.1) | 100 (100) |
| $R$ factor (%) | 8.1 (1.8) | 8.2 (2.4) |
| $R$ free (%) | 20.8 | 21.6 |
| Number of protein atoms | 918 | 857 |
| Number of water molecules | 72 | 48 |
| Average overall $B$ factor (Å$^2$) | 18.42 | 26.44 |

**RMS deviation ideal stereochemistry**

| Bond length (Å) | 0.013 | 0.010 |
| Bond angle (degree) | 1.45 | 1.30 |

* Values in parentheses are for the highest resolution shell.
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ThT fluorescence (Fig. 7, E and F). When measured by AFM, those that formed at pH 2.5 had a morphology typical of β2-m amyloid fibrils with a diameter of about 5–8 nm and a length of more than 1 μm as reported previously (36). Although the AFM of the fibrils prepared at pH 7.0 was more difficult as previously reported (24), we could detect some fibrils of wild-type β2-m and W39-β2-m. We previously confirmed that the fibrils of various β2-m mutants formed at pH 2.5 by electron microscopy (21). Therefore, in the present article, although we did not measure the AFM micrographs of fibrils of other Trp mutants, we believe that other mutants, except W33-β2-m at pH 7.0, also formed amyloid fibrils with an overall morphology similar to that of wild-type β2-m. However, for fibrils with Trp residue newly introduced, their exact structures might be slightly different from that of wild-type fibrils by the steric hindrance of Trp residue and other effects introduced by mutations.

Fluorescence Spectra of Amyloid Fibrils at pH 2.5—The amyloid fibrils of wild-type β2-m at pH 2.5 showed a λmax at 342 nm (Fig. 2B and Table 1). Importantly, this λmax is slightly longer than that of the monomeric native conformation at pH 7.0. As the Gdn-HCl concentration increased, the λmax shifted to 347 nm accompanied by a marked decrease in intensity. The fluorescence intensity of the fibrils relative to the unfolded form in 5.5 mM Gdn-HCl is much larger than that of the native structure at pH 7.0, implying an increased distance between the Trp residues and the disulfide bond.

The Gdn-HCl-dependent spectral change of W60-β2-m in the fibrils was similar to that of wild-type β2-m, although the relative change of fluorescence was larger than that of the wild type (Fig. 2B). In contrast, W95-β2-m in fibrils showed a moderately quenched spectrum with a λmax at 342 nm and, upon unfolding, the fluorescence intensity increased accompanied by a red shift to 348 nm (Fig. 2B). Thus, the change in fluorescence upon the unfolding of the wild-type fibrils at pH 2.5 is mostly attributed to Trp60, the opposite to the situation in the native state. The fluorescence spectrum of wild-type β2-m in the fibrils was reproduced exactly by summing those of W60- and W95-β2-ms (Fig. 3B, dashed line).

Regarding other mutants at pH 2.5, the fluorescence spectrum of W33-β2-m in fibrils was similar to that of W95-β2-m, suggesting that Trp residues of these mutants are buried and quenched to a similar extent (Fig. 3B, Table 1, and supplemental Fig. S2). In a marked contrast, the fluorescence of either W39-, W49-, or W78-β2-ms in the fibrils was strongly quenched and it was difficult to accurately estimate λmax, suggesting that they are buried and are probably located close to the disulfide bond (Fig. 3B).

Fluorescence Spectra of Amyloid Fibrils at pH 7.0—The wild-type β2-m fibrils prepared at pH 7.0 showed a maximum at 339 nm (Fig. 2C and Table 1). Again, this maximum wavelength is slightly longer than that in the monomeric native state, suggesting more exposed Trp residues on average. Because the presence of 0.5 mM SDS caused precipitation of Gdn-HCl, we used urea as a denaturant. As the urea concentration increased, the fluorescence increased with a shift of λmax to 349 nm. It is noted that the change in intensity is the opposite to that of the fibrils at pH 2.5.

The Gdn-HCl-induced change in fluorescence of W60-β2-m fibrils at pH 7.0 was similar to that of the wild type (Fig. 2C). On the other hand, the increase in the fluorescence of the W95-β2-m fibrils was moderate, indicating that Trp60 dominantly contributes to the observed increase in fluorescence of the wild-type β2-m at pH 7.0. The fluorescence spectrum of the wild type was the sum of the spectra of W60- and W95-β2-ms (Fig. 3C, dashed line). Thus, the large difference in the wild-type fibrils at pH 2.5 (Fig. 3B) and 7.0 (Fig. 3C) represents that, while Trp60 in the fibrils at pH 2.5 is slightly buried but not quenched, that at pH 7.0 is buried more and significantly quenched.

For other single Trp mutants, W39-β2-m showed a maximum at 331 nm (Fig. 2C, Table 1, supplemental Fig. S2). This wavelength was the shortest among the fibrils of the single Trp mutants, suggesting that Trp39 is most deeply buried in the fibrils at pH 7.0. The fluorescence spectra of the W49- and W78-β2-m fibrils were similar to the spectrum of the W60-β2-m fibrils (Fig. 2C). However, the quenching of the W39-, W49- and W78-β2-m fibrils was less extensive at pH 7.0 than at pH 2.5.

Unfolding Transitions of Amyloid Fibrils—To further address the mutational effects, the unfolding transition curves of the
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fibrils against the concentration of Gdn-HCl at pH 2.5 and urea at pH 7.0 were constructed (Fig. 4, B and C and Table 1). Considering the complication of reversibility of fibril unfolding (37), the curves obtained after similar incubation periods give apparent transitions with which to compare the overall stability of fibrils. The fibrils exhibited cooperative unfolding transitions at both pH 2.5 and 7.0. The variation in the transition curves was not correlated with that in the native state at pH 7.0 (Fig. 4A). Intriguingly, the most unstable fibrils at pH 7.0 (W39- and W49-β2-ms) showed decreased amyloidogenicity at pH 7.0 (Figs. 4C and 6B).

**DISCUSSION**

**Fluorescence Spectra in the Native State**—Trp fluorescence is one of the most popular probes with which to study the conformation of proteins and peptides. By observing the λmax, one can estimate whether Trp is in a polar environment exposed to water or in a non-polar environment buried in the polypeptide chain. Although the intensity of fluorescence increases with a decrease in polarity, various quenchers, most importantly the disulfide bond located close by, decreases the intensity (38). However, interpreting the spectra of fluorescence is not always straightforward since the presence of multiple Trp residues averages the respective contributions. Moreover, Trp is not so frequent among 20 amino acid residues, restricting the site of the conformational probe. In the present study, to take advantage of Trp fluorescence while excluding its disadvantages, a variety of single Trp mutants of β2-ms were prepared.

The x-ray crystallographic analysis demonstrated that even W39-β2-m, the most destabilized mutant, assumes the native structure (Fig. 5). The introduced Trp is located close to the disulfide bond as observed for other immunoglobulin domains (18), explaining the remarkable quenching of fluorescence. For other single Trp mutants, the variation in the spectra of fluorescence is exactly consistent with the location of substituted residues in the native structure (Fig. 1). The results argue that a series of single Trp mutants are indeed promising tools with which to probe the protein conformation.

**Fluorescence Spectra in the Amyloid Fibrils**—Most importantly, at both pH 2.5 and 7.0, the fluorescence spectrum of wild-type β2-m in the fibrils was reproduced exactly by summing those of W60- and W95-β2-ms (Fig. 3, B and C, dashed lines). This shows that W60- and W95-β2-ms form the amyloid fibrils similar to those of wild-type β2-m, validating the present approach to address the structure of amyloid fibrils. We consider that the fibrils of other mutants are also similar to those of wild-type β2-m in morphology and overall structure, even if not the same (21). However, presence of a lag-phase for W33- and W49-β2-ms at pH 2.5 and significant suppression for W33-, W39-, and W49-β2-ms at pH 7.0 suggests that the wild-type seeds do not easily accommodate these bulky mutations, leading to the formation of amyloid fibrils with altered local structures (Fig. 6). This interpretation is consistent with the observation that Ser33, Leu39, and Val49 are involved in the core of amyloid fibrils at pH 2.5 (17, 21) and that most unstable fibrils at pH 7.0 are those of W39- and W49-β2-ms. In other words, adequate packing of these residues is important for the fibril formation. Thus, Trp mutagenesis is also useful for probing the internal packing of amyloid fibrils.

The fluorescence spectra of amyloid fibrils were markedly different from those of the native conformation, providing important insights into the conformational differences between the two (Fig. 3). In the amyloid state at pH 2.5, Trp fluorescence of all the mutants except W60-β2-m was highly quenched, indicating these residues are located near quenchers, most importantly the disulfide bond. Interestingly, Trp39 was not completely quenched in the fibrils, suggesting a disruption of the tight contact between Trp39 and the disulfide bond in the native structure. Except W95-β2-m, the λmax values are smaller in the fibrils than in the native state, suggesting that the Trp residues are more buried in the fibrils. Taken together, although Trp60 exposed on the native structure is buried upon the formation of a fibril, it is located relatively far from the disulfide bond. Trp95 is relatively buried in the native structure, while it is more exposed to the solvent in the fibrils, consistent with the idea that N- and C-terminal regions are more exposed in the fibrils (6–9, 17).

Importantly, the present results suggest the structural basis of the polymorphism of β2-m amyloid fibrils (23, 39) and, furthermore, fibrils of other proteins (40–42). The fluorescence spectra of the amyloid forms at pH 7.0 are similar to those at pH 2.5, except for W60-β2-m (Figs. 2 and 3). While the fluorescence of W60-β2-m fibrils at pH 2.5 was significantly intense, it was quenched at pH 7.0 as for other mutants, suggesting the distance between Trp60 and the disulfide bond to be closer at pH 7.0. It is conceivable that the two types of fibrils can be distinguished in terms of the geometry between the DE loop on which Trp60 resides and the disulfide bond.

Finally, although the architecture of amyloid fibrils is still controversial, the most common model is that of several protofibrils associated laterally and that a single protofibril consists of a large number of monomers associated in tandem by cross-β sheet interactions (1–3). Consequently, when one considers burial and quenching using the Trp fluorescence, it is inevitable to consider the effects from other molecules in the same protofibril, and furthermore, from molecules in other protofibrils. The order of effects is likely to be: first the intramolecular interactions, second the intermolecular interactions in the protofilament, and then the intermolecular interactions between protofibrils. Therefore, although further studies distinguishing the different interactions and moreover additional evidence validating the present observations are essential, we believe that a series of residue-specific information obtained here will be useful to construct the overall images of amyloid fibrils as summarized below.

**CONCLUSIONS**

For most of the single Trp mutants, we observed signal changes indicating a significant conformational change between the native structure and amyloid fibrils. This suggests that the formation of amyloid fibrils of β2-m accompanies a significant conformational change to the entire molecule. Notable suppression of fibril formation at pH 7.0 observed for W33-, W39-, and W49-β2-ms suggests that the corresponding residues (*i.e.*, Ser33, Leu39, and Val49) are tightly packed in the wild-type fibrils, requiring local
conformational change for accommodating bulky Trp residues. The fibrils prepared at pH 2.5 and pH 7.0 exhibited a marked change in Trp fluorescence suggesting the structural basis of the polymorphism of β2-m amyloid fibrils (23, 39). Thus, mutagenesis of Trp would be a promising tool with which to probe the mechanism by which amyloid fibrils form.

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REFERENCES

1. Rochet, J. C., and Lansbury, P. T., Jr. (2000) Curr. Opin. Struct. Biol. 10, 60–68
2. Dobson, C. M. (2003) Nature 426, 884–890
3. Uversky, V. N., and Fink, A. L. (2004) Biochim. Biophys. Acta 1698, 131–153
4. Naiki, H., Hashimoto, N., Suzuki, S., Kimura, H., Nakakuki, K., and Gejyo, F. (1997) Amyloid 4, 223–232
5. Kad, N. M., Myers, S. L., Smith, D. P., Smith, D. A., Radford, S. E., and Thomson, N. H. (2003) J. Mol. Biol. 330, 785–797
6. McParland, V. J., Kalverda, A. P., Homans, S. W., and Radford, S. E. (2002) Nat. Struct. Biol. 9, 326–331
7. Jones, S., Smith, D. P., and Radford, S. E. (2003) J. Mol. Biol. 330, 935–941
8. Esposito, G., Michelutti, R., Verdone, G., Viglino, P., Hernandez, H., Robinson, C. V., Amoresano, A., Dal Piaz, F., Monti, M., Pucci, P., Mangione, P., Stoppani, M., Merlini, G., Ferri, G., and Bellotti, V. (2000) Protein Sci. 9, 831–845
9. Corazza, A., Pettirossi, F., Viglino, P., Verdone, G., Garcia, J., Demy, P., Giorgetti, S., Mangione, P., Raimondi, S., Stoppani, M., Bellotti, V., and Esposito, G. (2004) J. Biol. Chem. 279, 9176–9189
10. Chiti, F., Mangione, P., Andreola, A., Giorgetti, S., Stefani, M., Dobson, C. M., Bellotti, V., and Taddei, N. (2001) J. Mol. Biol. 307, 379–391
11. Ivanova, M. I., Sawaya, M. R., Gingery, M., Attinger, A., and Eisenberg, D. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 10584–10589
12. Trinh, C. H., Smith, D. P., Kalverda, A. P., Phillips, E. V., and Radford, S. E. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 9771–9776
13. Verdone, G., Corazza, A., Viglino, P., Pettirossi, F., Giorgetti, S., Mangione, P., Andreola, A., Stoppani, M., Bellotti, V., and Esposito, G. (2002) Protein Sci. 11, 487–499
14. Bjorkman, P. J., Saper, M. A., Samraoui, B., Bennett, W. S., Strominger, J. L., and Wiley, D. C. (1987) Nature 329, 506–512
15. Floege, J., and Ketteler, M. (2001) Kidney Int. 59, 164–171
16. Gejyo, F., Yamada, T., Odani, S., Nakagawa, Y., Arakawa, M., Kunitomo, T., Kataoka, H., Suzuki, M., Hirawaza, Y., Shirahama, T., Cohen, A. S., and Schmid, K. (1985) Biochem. Biophys. Res. Commun. 129, 701–706
17. Hoshino, M., Kato, H., Hagiwara, Y., Hasegawa, K., Naiki, H., and Goto, Y. (2002) Nature Struct. Biol. 9, 332–336
18. Amzel, L. M., and Poljak, R. L. (1979) Annu. Rev. Biochem. 48, 961–997
19. Tsunenaga, M., Goto, Y., Kawata, Y., and Hamaguchi, K. (1987) Biochemistry 26, 6044–6051
20. Goto, Y., Ichimura, N., and Hamaguchi, K. (1988) Biochemistry 27, 1670–1677
21. Chiba, T., Hagiwara, Y., Higurashi, T., Hasegawa, K., Naiki, H., and Goto, Y. (2003) J. Biol. Chem. 278, 47016–47024
22. Kozukh, G. V., Hagiwara, Y., Kawakami, T., Hasegawa, K., Naiki, H., and Goto, Y. (2002) J. Biol. Chem. 277, 1310–1315
23. Kihara, M., Chatani, E., Sakai, M., Hasegawa, K., Naiki, H., and Goto, Y. (2005) J. Biol. Chem. 280, 12012–12018
24. Ohhashi, Y., Kihara, M., Naiki, H., and Goto, Y. (2005) J. Biol. Chem. 280, 32843–32848
25. Ban, T., Hoshino, M., Takahashi, S., Hamada, H., Hasegawa, K., Naiki, H., and Goto, Y. (2004) J. Mol. Biol. 344, 757–767
26. McPherson, A. (1992) J. Cryst. Growth 122, 161–167
27. Leslie, A. G. W. (1992) Acta Crystallogr. Sect. D 55, 1696–1702
28. CCP4 Collaborative Computational Project, Number 4. (1994) Acta Crystallogr. Sect. D 50, 760–763
29. Vagin, A., and Teplyakov, A. (1997) J. Appl. Crystallogr. 30, 1022–1025
30. Jones, T. A., Zou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1991) Acta Crystallogr. Sect. A 47, 110–119
31. Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) Acta Crystallogr. Sect. D 53, 240–255
32. Brunger, A. T. (1992) Nature 355, 472–475
33. Laskowski, R. A., Rullmann, J. A., MacArthur, M. W., Kaptein, R., and Thornton, J. M. (1996) J. Biomol. NMR 8, 477–486
34. Yamaguchi, K., Takahashi, S., Kawai, T., Naiki, H., and Goto, Y. (2005) J. Mol. Biol. 352, 952–960
35. Yamamoto, S., Hasegawa, K., Yamaguchi, K., Tsutsumi, S., Kardos, J., Goto, Y., Gejyo, F., and Naiki, H. (2004) Biochemistry 43, 11075–11082
36. Katou, H., Kanno, T., Hasegawa, K., Naiki, H., and Goto, Y. (2002) Protein Sci. 11, 2218–2229
37. Narimoto, T., Sakurai, K., Okamoto, A., Chatani, E., Hoshino, M., Hasegawa, K., Naiki, H., and Goto, Y. (2004) FEBS Lett. 576, 313–319
38. Cowgill, R. W. (1970) Biochim. Biophys. Acta 207, 556–559
39. Chatani, E., Kato, M., Kawai, T., Naiki, H., and Goto, Y. (2004) J. Mol. Biol. 342, 941–951
40. Dzwolak, W., Smirnovas, V., Jansen, R., and Winter, R. (2004) Protein Sci. 13, 1927–1932
41. Jones, E. M., and Suredwicz, W. K. (2005) Cell 121, 63–72
42. Tanaka, M., Chien, P., Yonekura, K., and Weissman, J. S. (2005) Cell 121, 49–62
43. Koradi, R., Billeter, M., and Wuthrich, K. (1996) J. Mol. Graph. 14, 51–55