To understand the mechanism by which amyloid fibrils form, we have been making real-time observations of the growth of individual fibrils, using total internal fluorescence microscopy combined with an amyloid-specific fluorescence dye, thioflavin T (ThT). At neutral pH, irradiation at 442 nm with a laser beam to excite ThT inhibited the fibril growth of β2-microglobulin (β2-m), a major component of amyloid fibrils deposited in patients with dialysis-related amyloidosis. Examination with a 22-residue K3 fragment of β2-m showed that the inhibition of fibril growth and moreover the destruction of preformed fibrils were coupled with the excitation of ThT. Several pieces of evidence suggest that the excited ThT transfers energy to ground state molecular oxygen, producing active oxygen, which causes various types of chemical modifications. The results imply a novel strategy for preventing the deposition of amyloid fibrils and for destroying preformed amyloid deposits.

Amyloid fibrils are associated with the pathogenesis of more than 20 serious diseases, including Alzheimer, Parkinson, and Huntington diseases, and dialysis-related amyloidosis (1, 2). Moreover, various proteins and peptides that are not related to diseases can also form amyloid-like fibrils, implying that the formation of fibrils is a generic property of proteins and peptides (1). In basic structure, amyloid fibrils are long and often twisted, a few nanometers in diameter, and predominantly composed of cross β-sheets (1, 3). Amyloid fibrils are formed spontaneously with a lag phase or seed-dependently without a lag time, indicating that they are formed by nucleation and extension. Further understanding of the structure, mechanism of formation, and roles in amyloidosis is one of the most important issues of protein science today.

To study amyloid fibrils, we developed a unique technique for their direct observation in which total internal reflection fluorescence microscopy (TIRFM) is combined with amyloid-specific thioflavin T (ThT) fluorescence (4–8). This technique provides important information about the morphology, growth rate, and extension direction of fibrils in real time at the single fibril level. We have applied the technique to the fibrils of β2-microglobulin (β2-m) responsible for dialysis-related amyloidosis and amyloid β associated with Alzheimer disease (4–8).

β2-m is a major component of amyloid fibrils deposited in dialysis-related amyloidosis, a common and serious complication in patients receiving hemodialysis for more than 10 years (9–11). β2-m, a typical immunoglobulin domain made of 99 amino acid residues and seven β-stands, is present as the non-polymorphic light chain of the class I major histocompatibility complex (12). 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 concentration of β2-m in blood by up to 50-fold. Although an increase in the concentration of β2-m is the most important risk factor for fibrillation, how β2-m forms amyloid fibrils under physiological conditions is unknown.

In vitro at acidic pH, the incubation of β2-m in the presence or absence of seed fibrils results in high yields of amyloid fibrils with a range of different morphologies (13–15). However, the generation of β2-m amyloid fibrils under physiological conditions at neutral pH, where fibrils deposit in patients, has been difficult. Recently, several groups have established conditions under which fibril growth is possible at neutral pH (16–22). For example, Yamamoto et al. (16, 17) found that fibrils of β2-m formed at neutral pH in the presence of trifluoroethanol or SDS. On the other hand, it has been reported that a 22-residue K3 peptide of β2-m, the sequence Ser20-Asn-Phe-Leu-Asn-Cys-Tyr-Val-Ser-Gly-Phe30-His-Pro-Ser-Ile-Glu-Val-Asp-Leu34-Lys obtained by digestion with Acromobacter protease I forms amyloid fibrils spontaneously at neutral pH (23). In addition, it has been reported that K3 peptide forms amyloid fibrils over a wide range of pH and solvent conditions (23–25). Because the K3 peptide is one of the core regions of β2-m amyloid fibrils...
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(26), it is thought to play a crucial role in the fibrillation process. We have started examining the fibrillation process under these neutral pH conditions with TIRFM combined with ThT. We also compare the formation of K3 fibrils with that of whole β2-m fibrils. When we performed real time observations of β2-m and K3 fibrils at neutral pH, fibril extension stopped during TIRFM. Furthermore, preformed K3 fibrils tended to disappear during the observation period. In this report, we focused on clarifying the effects of the laser beam on K3 fibrils. The results showed that the fibrils were decomposed by the irradiation in which ThT molecules bound to fibrils play critical roles. Our results suggest that it may be possible to selectively breakdown amyloid fibrils coupled with amyloid-specific dyes like ThT, suggesting a new strategy for destroying amyloid fibrils ultimately leading to the prevention and treatment of amyloidosis.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human β2-m was expressed with an Escherichia coli expression system and purified as described previously (27). A Met residue was always present at the N-terminal position of the recombinant β2-m. In this report, chemically synthesized K3 peptides were purchased from Peptide Institute, Inc. (Osaka, Japan), its purity being >95% according to the elution pattern from reverse phased high-pressure liquid chromatography (HPLC).

Chemical Modification of Surfaces—Polyvinylsulfonate (PVS: M, unknown) was purchased from Sigma. Polyethyleneimine (PEI: M, 60,000) was obtained from Nacalai tesque (Kyoto, Japan). The surface of quartz slides was modified by adsorbing polyelectrolytes as described before (6). The slides were first cleaned with 0.5% (v/v) Hellmanex (Hellma, Müllheim, Germany)/water and then treated with a solution of acetone for 15 min, rinsed extensively with distilled water, and finally dried in a vacuum oven at 100 °C. Positively charged surfaces were prepared by the adsorption of PEI by incubating quartz in aqueous solutions of PEI (0.1% w/v) Negatively charged surfaces were prepared by layer-by-layer deposition of polyelectrolytes. The former type was created by the adsorption of PVS onto positively charged surfaces of PEI.

Direct Observation of Amyloid Fibrils—The TIRFM system used to observe individual amyloid fibrils was developed based on an inverted microscope (IX70, Olympus, Tokyo, Japan) as described (4, 5). The ThT molecule was excited at 442 nm by a helium-cadmium laser (IK5552R-F, Kimmon, Tokyo, Japan). The laser power was 4–80 milliwatt, and the observation period was 3–5 s. The fluorescence image was filtered with a bandpass filter (D490/30, Omega Optical, Brattleboro, VT) and visualized using a digital steel camera (DP70, Olympus). In the case of β2-m, seeds of β2-m were prepared by fragmentation of amyloid fibrils with a TAITEC (Saitama, Japan) VP-30S sonicator equipped with a microtip. The seeds were added at a final concentration of 30 µg/ml to 25 µM (0.30 mg/ml) β2-m in 50 mM sodium phosphate (pH 7.0) containing 100 mM NaCl and 0.5 mM SDS. ThT solution was then added at a final concentration of 5 µM. In the case of K3, K3 (100 µM, 0.25 mg/ml) was incubated in 50 mM sodium phosphate buffer (pH 6.0) containing 100 mM NaCl. ThT was added at a final concentration of 10 µM. An aliquot (14 µl) of each sample solution was deposited on each microscopic slide, and an image of the fibrils was obtained with TIRFM. The formation of β2-m fibrils and K3 fibrils on various surfaces was also examined. After the mixing of the solutions of seeds, monomeric proteins, and ThT at the same final concentrations as above, the mixture was immediately deposited on the microscopic slide, tightly sealed with a coverslip, and incubated at 37 °C.

Effect of Laser Beam on Preformed K3 Fibrils—To examine the effect of laser irradiation, intermittent irradiation on K3 fibrils was performed. The fibrils formed on the PEI/PVS surface were irradiated by a helium-cadmium laser. The irradiation time was 3–5 s, and the laser power was 40–60 milliwatt. It is noted that the laser irradiation points were also the observation points. Then, the relation between laser power and disappearance of fibrils were examined by observing the fibrils at various laser powers. The fibrils were irradiated for 3 s every 15 min. Fluorescence images of amyloid fibrils visualized with TIRFM were quantified to obtain the time course of fibril destruction. For each TIRFM picture, we calculated the average signal intensity of background (<Ibkg>) and its standard deviation (σbkg) from several parts of the picture where fibrils are absent. Then, we counted the number of pixels whose intensity was larger than the threshold value, which was set <Ibkg> + 5σbkg. The number of pixels containing fibrils was normalized by that at time 0 under the same laser power.

ThT Assay and Light Scattering—To make K3 fibrils, K3 (100 µM) was incubated in 50 mM sodium phosphate buffer (pH 6.0) containing 100 mM NaCl in a test tube at 37 °C. The prepared K3 fibrils were diluted 4-fold with 50 mM sodium phosphate buffer (pH 6.0) containing 100 mM NaCl. ThT was added at a final concentration of 10 µM. The sample was introduced into a glass cell with a 10-mm light path, and then irradiated with a helium-cadmium laser at 442 nm or a helium-neon laser (05LHP928, MELLES GRIOT, Albuquerque, NM) at 632.8 nm continuously under agitation. The laser powers were 30–40 milliwatt and 20 milliwatt for the helium-cadmium laser and helium-neon laser, respectively. To examine the participation of oxygen, the above samples were initially purged with nitrogen or oxygen. When the dissolved oxygen of the sample decreased down to 1 mg/l under nitrogen purging or increased more than 30 mg/l under oxygen purging, the samples were started to irradiate with a laser beam under the purging of nitrogen or oxygen. The laser-irradiated K3 fibrils were monitored by fluorescence analysis with ThT and light scattering. For measurements of ThT assay, the wavelengths for excitation and emission were 445 nm and 485 nm, respectively. For measurements of light scattering, the wavelengths were both set at 350 nm. ThT fluorescence and light scattering were measured using a Hitachi F-7000 spectrophotometer (Tokyo, Japan) at 37 °C.

Detection of Singlet Oxygen—To detect singlet oxygen (1O2), singlet oxygen sensor green reagent (Molecular Probes) was used. The reagent was added at a final concentration of 10 µM to the same samples as described in the procedure of “ThT Assay and Light Scattering.” For the measurements of green fluorescence of the sensor reagent, the wavelengths for excitation and emission were 504 nm and 525 nm, respectively.
Ultrasound Measurements—Sedimentation velocity of the K3 fibrils after laser irradiation for 48 h was measured using a Beckman-Coulter Optima XL-1 analytical ultracentrifuge (Fullerton, CA) with an An-60 rotor and two-channel charcoal-filled Epon cells. The experiments were performed at 4 °C. The data were analyzed using the software Ultrascan 8.0.

High Pressure Liquid Chromatography and Mass Analysis—The laser-irradiated K3 fibrils were lyophilized and then dissolved in 6 M guanidine hydrochloride (GdnHCl). The sample was subjected to reverse-phased HPLC performed on a liquid chromatograph (GILSON, Middleton, WI) equipped with a 5C18-AR-300 (4.6 mm × 150 mm; Nacalai Tesque, Kyoto, Japan). The sample was eluted with a gradient beginning with solvent A (0.05% trifluoroacetic acid) and an increasing percentage of solvent B (0.05% trifluoroacetic acid/acetonitrile) at a flow rate of 0.5 ml/min. All peaks were collected and lyophilized. Samples dissolved in TA buffer (0.1% trifluoroacetic acid in water/acetonitrile = 2:1) were mixed with a matrix (α-cyano-4-hydroxy cinnamic acid in TA buffer) at a ratio of 1:1, and then 2 μl was deposited onto a target plate. Matrix-assisted laser desorption ionization–time of flight mass spectrometry (Bruker Daltonics) was used for identification.

Atomic Force Microscopy (AFM)—A sample solution was spotted on freshly cleaved mica. After standing for 1 min, the residual solution was blown off with compressed air. AFM images were acquired using a Digital Instruments Nanoscope IIIa scanning microscope at 25 °C (Veeco, Tokyo, Japan). Measurements were performed in an air-tapping mode.

Amino Acid Analysis—Amino acid analysis was performed on a Hitachi L-2000 amino acid analyzer after hydrolysis with constant boiling point HCl (Nacalai Tesque, Kyoto, Japan) at 110 °C for 24 h in an evacuated sealed tube. HPLC was carried out on a cation ion exchange column. The amino acids were detected by the reaction with ninhydrin.

RESULTS

Effects of Various Surfaces on β2-m and K3 Fibrils—To observe individual amyloid fibrils with TIRFM, it is necessary to investigate the best conditions for fibril growth. First, β2-m and K3 fibrils were grown in test tubes at pH 7.0 and pH 6.0, respectively, based on the conditions established in our previous studies (23, 28). TIRFM images indicated the presence of short fibrils in each sample (data not shown), confirming that amyloid-specific fluorescence from ThT enables the visualization of both β2-m and K3 fibrils.

Then, we examined the growth of β2-m and K3 fibrils on the surface of various chemically modified slides. TIRFM images taken after incubation for 12 h often showed radial extensions to both fibrils (Fig. 1). Interestingly, the growth of β2-m fibrils was independent of the properties of the surface (Fig. 1, A–C), although that of K3 fibrils varied depending on the surface (Fig. 1, D–F). The growth of K3 fibrils on the negatively charged PEI/PVS surface was explosive (Fig. 1F), while that on the positively charged PEI surface was less extensive (Fig. 1E). The results indicate that the effects of the surface differ depending on the fibrils. It should be noted that both β2-m and K3 are negatively charged at neutral pH; the net charge at pH 7.0 is −3 for β2-m and that at pH 6.0 is −1 for K3 peptide (23). To observe the fast growth of these two types of fibrils under optimal conditions, in subsequent experiments, β2-m fibrils were extended seed-dependently on the quartz surface and K3 fibrils were extended on the PEI/PVS surface. Concentrations of β2-m monomers, seeds, and ThT were 25 μM, 30 μg/ml, and 5 μM, respectively. As for K3 fibrils, concentrations of K3 monomers and ThT were 100 and 10 μM, respectively. The fibrils were observed after incubation for 12 h. The scale bar represents 10 μm.

Real-time Observations of β2-m and K3 Fibril Growth—First, we observed the seed-dependent growth of β2-m fibrils at neutral pH (Fig. 2A). The laser power was 40–60 milliwatt, and the duration of each irradiation was 3 s. Immediately after the growth was initiated, fluorescent spots of seeds were observed. However, the growth of β2-m fibrils ceased during the observation period. Because extensively elongated fibrils were observed in the absence of the laser beam (Fig. 1, A–C), we assumed that the growth was inhibited with time by the laser irradiation. In support of this assumption, β2-m fibrils grew normally under a weaker laser power of 4–6 milliwatt (data not shown). There-
fore, the growth of β2-m fibrils critically depended on the laser beam and its intensity.

Next, real-time observation of the spontaneous fibrillation of K3 was carried out at neutral pH (Fig. 2B and supplemental Movie S1). The laser power was 40–60 milliwatt, and the duration was 3 s. At time 0, the fibrils of K3 occurred concomitantly at many sites, implying that the short fibrils had already formed in the sample when K3 peptides were dissolved. Although K3 fibrils extended for a while, producing fibrils as long as several micrometers (see image at 15 min), the growth stopped at 30 min. Moreover, the extended fibrils disappeared with time (images from 45–90 min). However, K3 fibrils also grew under weaker laser irradiation (data not shown). This seizing of fibril growth and moreover the vanishing of preformed fibrils were unexpected events. We assumed that the laser beam caused the inhibition of fibril growth and decomposition of preformed fibrils.

It is noted that the laser beam was applied to the sample for duration of 3 s when we simultaneously obtained TIRFM images. The rest of the time, the sample was incubated in dark. As described below, another possibility is that the apparent fading of fibril images was caused by photobleaching of ThT retaining the fibrils intact. However, no recovery of fibril images during incubation in dark suggests that the disappearance was in fact caused by the destruction of fibrils.

Disappearance of K3 Fibrils with Irradiation—To verify the laser-induced inhibition and decomposition, we studied in detail the molecular mechanism involved using K3 peptide, which showed more dramatic effects than the whole β2-m molecule. In particular, we are interested in the apparent vanishing of K3 fibrils from the TIRFM images.

First, we examined the effect of laser irradiation on preformed K3 fibrils. The fibrils formed on the PEI/PVS surface without any irradiation. Following intermittent irradiation, the fibrils slowly disappeared with time (Fig. 3A and supplemental Movie S2). The disappearance occurred locally and discontinuously. These results suggested that K3 fibrils were destroyed by the irradiation coupled with the excitation of ThT. During the laser beam-induced destruction, fibrils vanished concomitantly at various sites in the view (Fig. 3B). Meanwhile, radially extended fibrils started to disappear in the vicinity of the growing ends of fibrils (Fig. 3C). However, the cores of clustered fibrils resisted destruction. In these single fiber fluorescence images, the diameters of fibrils cannot be determined exactly. The previous study with electron microscopy indicated that K3 fibrils formed under similar conditions at pH 6.5 are made of two types: one with a diameter of ~10 nm and the other with a diameter of ~5 nm (23). The former are likely to consist of two latter filaments associated laterally.

TIRFM images at various periods were quantified to characterize the time course of the laser beam-dependent fibril disappearance (Fig. 3D, see “Experimental Procedures”). The data points represent all of the irradiation events applied, the duration of which was 3–5 s for each. Overall fluorescence intensity estimated for TIRFM image decreased with time (i.e. with the number of exposure to the laser beam). Next, to examine the dependence on the laser power, the laser power was varied...
between 8 and 80 milliwatt. The duration of laser irradiation was 3 s every 15 min. The destruction was faster and more extensive with an increase in the laser power (Fig. 3E and supplemental Fig. S1). These results indicated that the disappearance of K3 fibrils is caused by the laser beam irradiation of ThT bound to fibrils.

Effects of Laser Irradiation on K3 Fibrils in Bulk Solution—Although TIRFM images suggested the destruction of K3 fibrils triggered by laser irradiation (Figs. 2 and 3), another possibility is that the apparent fading of images is caused by photobleaching of ThT bound to fibrils. To distinguish between these possibilities, we analyzed the structure of irradiated K3 fibrils in bulk solution. We first prepared K3 fibrils in a test tube. The fibrils were introduced into a glass cell with a 10-mm light path, and then irradiated with a laser beam at 442 nm by a helium-cadmium laser at a power of 30–40 milliwatt continuously under agitation.

As the irradiation proceeded, the intensity of the ThT fluorescence decreased (Fig. 4A). Although the intensity of the light scattering increased temporarily, it slightly decreased after about 10 h. Because of the possible fluctuation of the concentration and length of fibrils, it was difficult to reproduce exactly the intensities of light scattering and ThT fluorescence. However, the difference among the different experimental conditions as shown in Fig. 4 was reproducible, indicating that we can address the effects of laser irradiation on the basis of the relative intensities of light scattering and ThT fluorescence. The transient increase of the light scattering is likely attributable to the aggregation of K3 fibrils induced by the stirring because notable increase was observed for the samples without ThT or laser irradiation. After a long period of irradiation, the light scattering intensity was stable as the decrease in ThT intensity ceased. Although the concentration of ThT we used (10 μM) is close to its CMC (29), the similar light scattering for the sample with ThT and without laser and that without ThT and with laser (Fig. 4, A and B) suggests that the possible self-aggregation of ThT does not affect the results.

It was thought that the saturation of photobleaching of ThT also stopped the decrease in light scattering intensity. However, the results raise the possibility that the agitation during laser irradiation broke the K3 fibrils. Thus, the same experiment was carried without laser irradiation. The intensity of neither the ThT fluorescence nor light scattering decreased notably, confirming that the effects depend on the irradiation (Fig. 4A). Furthermore, when laser excitation at different wavelength (632.8 nm by a helium-neon laser) was used, the results were similar to those without laser irradiation at 442 nm by a helium-cadmium laser (Fig. 4B). Then, to examine the participation of ThT, the experiment was carried in the absence of ThT. The light scattering intensity did not decrease (Fig. 4A), indicating
that ThT is directly involved in the decrease. In other words, ThT plays a critical role in the laser irradiation-dependent damage of K3 fibrils.

To further investigate the possibility of ThT photobleaching, we added extra ThT (10 μM) to the irradiated samples after 24 h (Fig. 4C). The intensity of ThT fluorescence increased to some extent. However, it was much lower than the level before laser irradiation, confirming that the laser irradiation destructed K3 fibrils. The results also indicated that photobleaching of ThT bound to fibrils partly contribute to the apparent disappearance of ThT fluorescence. As expected, the addition of 10 μM ThT to the sample irradiated in the absence of ThT brought the high fluorescence value of ThT revealing the minimal damage in the absence of ThT.

We then assumed that active oxygen generated upon the excitation of ThT plays a role (30, 31). To examine the involvement of oxygen in the destruction of K3 fibrils, the samples were purged with nitrogen or oxygen under laser irradiation (Fig. 4B). With the nitrogen purged, the intensity of light scattering after 24 h did not decrease significantly. But with the oxygen purged, the intensity decreased at a much greater rate. These results suggested that oxygen is involved in the destruction of K3 fibrils. ThT intensity decreased significantly independent of the purging of nitrogen or oxygen, indicating that substantial fractions of ThT molecules were decomposed by the laser beam, separate from the ThT-coupled decomposition of amyloid fibrils. It is likely that specific binding of ThT to amyloid fibrils accelerates the decomposition of ThT itself: amyloid fibrils catalyze the laser-dependent decomposition of ThT, consuming bulk ThT molecules.

To examine the generation of active oxygen upon the excitation of amyloid bound ThT, we used a singlet oxygen (1O2) detection reagent (Fig. 4D). In the presence of singlet oxygen, the sensor reagent exhibits green fluorescence at around 525 nm when exited at 504 nm. The laser-irradiated sample in the presence of ThT showed high fluorescence. Both ThT and fibrils were required for the high fluorescence. Moreover, the fluorescence intensity increased with oxygen purged, whereas it was slightly suppressed with nitrogen purged. These results indicated that singlet oxygen is generated specifically by the laser irradiation of amyloid-bound ThT.

Analysis of Morphology and Chemical Structure of Laser-irradiated K3 Fibrils—We also examined the effects of laser irradiation on the morphology of K3 fibrils monitored by AFM. The AFM images revealed the fibrils to be relatively long before the irradiation (Fig. 5A) and shortened after the irradiation for 48 h (Fig. 5B). To clarify the size distribution of the irradiated K3 fibrils, we performed an analysis of sedimentation velocity. The sedimentation coefficient exhibited several peaks, indicating the existence of various fibrils with different molecular weights (Fig. 5, C and D). At 226,000 × g, the peaks at about 0.5 S and 0.8 S correspond to the K3 monomer (2,498 Da) and dimer, respectively (Fig. 5D). It is noted that the K3 peptide has a free thiol at Cys23, and the disulfide-linked dimer was produced during the experiments (23). On the other hand, because of the supramolecular structure, non-irradiated K3 fibrils readily precipitated even at 900 × g, and we could not analyze their sedimentation velocity (data not shown). These results also confirmed that K3 fibrils were broken down or decomposed by the laser irradiation into shorter fibrils. Moreover, products similar in molecular weight to the K3 monomer existed in the sample of the irradiated fibrils, suggesting that the irradiation brought about chemical modifications damaging the ability of K3 peptides to polymerize.

Then, to investigate whether laser-irradiation causes chemical modifications of K3 fibrils, we carried out analyses using HPLC (Fig. 5E) and mass spectroscopy (Fig. 5, F and G). To analyze the chemical damage to the monomer, irradiated K3 fibrils were dissolved in 6 M GdnHCl. In the HPLC analysis, the sample of dissolved fibrils showed multiple broad peaks (Fig. 5E). The peaks with retention times of 28 min and 31 min are those of the K3 monomer and dimer, respectively. As described above, the disulfide-linked dimer was produced during the experiments. When compared with the elution profile of the equivalent amount of fresh K3 solution, the monomeric peak of GdnHCl-treated K3 fibrils was significantly decreased in intensity, instead producing a broad tailing peak. These results indi-
cated the existence of products of various molecular weights, other than the K3 monomer and dimer. Moreover, in comparison with K3 fibrils without irradiation (Fig. 5G), the mass analysis of irradiated K3 fibrils revealed several decomposed peaks (e.g. \( m/z = 665 \) and \( 2,225 \)) as well as a peak of the monomer (\( m/z = 2,496 \)) (Fig. 5F). Interestingly, these peaks also had a smaller molecular weight than the monomer, suggesting the cleavage of peptide bonds by the laser.

To address the chemical modification further, we performed amino acid analysis (Fig. 6, A and B). K3 peptide of 22 amino acid residues contains 2 Asp, 2 Asn, 3 Ser, 1 Glu, 1 Pro, 1 Gly, 1 Cys, 2 Val, 1 Ile, 3 Leu, 1 Tyr, 2 Phe, 1 Lys, and 1 His residues. It is known that Tyr, Met, Cys, Trp, and His are amenable to oxidation (30, 31). In this experiment, the contents of Pro, Cys, and Tyr residues were not quantitatively determined. Consistent with these, notable decrease upon laser irradiation was observed for His; after 3 h of irradiation, the intensity decreased with these, notable decrease upon laser irradiation was confirmed that singlet oxygen is generated by the laser irradiation of amyloid bound ThT. Thus, the excited energy of ThT is transferred to ground-state oxygen (triplet oxygen), producing singlet oxygen and moreover oxygen peroxide and oxygen radicals. These lead to an inhibition of growth and ultimately the destruction of K3 fibrils.

DISCUSSION

Mechanism of the Light-induced Damage of K3 Fibrils—In this report, we showed that the growth of K3 and \( \beta_2 \)-m fibrils is inhibited by irradiation with a laser beam at 442 nm. Furthermore, preformed K3 fibrils were broken down into shorter fibrils and monomers and underwent chemical modifications. The reactions require ThT, indicating that the laser beam-excited ThT plays critical roles. The effects of purging samples with nitrogen or oxygen suggested the participation of active oxygen, in particular, singlet oxygen and oxygen radicals. We confirmed that singlet oxygen is generated by the laser irradiation of amyloid bound ThT. Thus, the excited energy of ThT is transferred to ground-state oxygen (triplet oxygen), producing singlet oxygen and moreover oxygen peroxide and oxygen radicals. These lead to an inhibition of growth and ultimately the destruction of K3 fibrils.

Although the exact mechanism involved is still unclear, we suggest a basic model for the laser beam-dependent decomposition of amyloid fibrils (Fig. 7). First, as described below, ThT binds specifically to K3 fibrils, and its optical properties change. Irradiation at 442 nm excites ThT from a ground state. Then, the excited ThT transfers its energy to ground-state molecular oxygen (triplet oxygen), producing singlet oxygen. Labile singlet oxygen attacks directly Tyr, Cys, and His side chains in K3 fibrils. Additionally, excited triplet oxygen generates hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) and various types of free radicals causing chemical modifications. It is also possible that excited ThT directly attacks K3 fibrils. These lead to the destruction of K3 fibril.

We consider the mechanism of damage to K3 fibrils to be similar to that caused by photodynamic therapy (PDT). PDT, a promising treatment for cancer, involves the use of photo-
chemical reactions mediated through the interaction of light with photosensitizing agents (33, 34). Through photochemical reactions, various types of active oxygen including singlet oxygen, hydrogen peroxide, and free radicals are generated causing cell death. PDT can select a photo-oxidized target, including deoxyribonucleic acid or protein, depending on the photosensitizing agent, which binds a specific target. Similarly, we showed that ThT, which specifically binds to amyloid fibrils, causes the inhibition of fibril growth and moreover the destruction of fibrils.

Although the reaction takes advantage of the amyloid-specific fluorescence dye ThT, the underlying mechanism of the binding of ThT to amyloid fibrils and consequent dramatic change in fluorescence quantum yield remains unclear (35–38). ThT is made of benzothiazole and aminobenzene rings connected by a rotating single bond, and in the ground state, has a nonplanar conformation. It has been suggested that an excimer formation in cavities which can accommodate two ThT ions is responsible for the characteristic fluorescence (37, 38). On the other hand, a quantum-chemical calculation suggests that, when internal rotation of the dye molecule is blocked because of steric hindrance upon binding to amyloid fibrils, the internal charge-transfer process in the excited singlet state leading to quenching is suppressed, thus producing a high quantum yield of fluorescence (35). Another quantum-chemical calculation suggests that, in the planer excited state, a population of fluorescent ThT molecules with π-conjugated bonds of benzothiazole and aminobenzene rings is produced (36). To achieve the efficient decomposition of fibrils by laser irradiation, understanding the mode of binding of ThT to fibrils is important.

The difference in the effects of laser irradiation on β2-m and K3 fibrils may be caused by the difference in the stability and morphology of fibrils. β2-m fibrils are made of several protofilaments tightly associated laterally, while K3 fibrils represent a single protofilament or the bundles of a couple of protofilaments, which may be different from that of mature β2-m fibrils (23). β2-m fibrils were more stable than K3 fibrils against denaturation induced by pH change (26) or unfolding induced by GdnHCl. In accordance with these observations, β2-m fibrils were more stable against the laser beam than K3 fibrils. Although the inhibition of fibril growth and decomposition of preformed fibrils were observed for K3 fibrils, only the former was observed for β2-m fibrils. The rigid architecture of mature β2-m fibrils may prevent decomposition by the laser, suggesting that the effects directly depend on the stability and rigidity of fibrils.

In contrast, the promoting effects of oxidative stress on fibrillation have been implicated in Alzheimer disease and Parkinson disease (39). Lipid peroxidation products formed via lipid alkoxy radicals accelerated the fibrillation of amyloid β and α-synuclein. Thus, although oxidative stress can trigger the formation of fibrils under certain conditions, fine tuning of their effects (e.g. extensive photooxidation more than inducing fibril growth) may enable the specific destruction of amyloid fibrils. In fact, we observed the destruction of ThT-bound amyloid β fibrils triggered by an extensive laser beam irradiation of ThT, suggesting the generality of the approach reported here. Interestingly, acceleration of fibrillation of amyloid β in the presence of ThT by a relatively weak laser irradiation was also observed. The detailed analysis of the adverse effects depending on the laser power is underway and will be published elsewhere.

Effects of Surfaces on β2-m and K3 Fibril Growth—Finally, we address the effects of various surfaces on fibril growth because we employed special conditions accelerating the fibril growth. The effects of the surface differed between β2-m and K3 (Fig. 1). We did not observe a clear dependence of β2-m fibril formation on the charge of the surface (Fig. 1, B and C). This independence may be caused by our special conditions including 0.5 mM SDS. On the other hand, K3 fibrils did not grow well on the positively charged PEI surface (Fig. 1E), but exhibited explosive growth on the negatively charged PEI/PVS surface (Fig. 1F).

Previously, we reported that negatively charged slides enhance the fibril growth of negatively charged amyloid β (1–40) and positively charged slides inhibit it (6). Zhu et al. (40) similarly reported that amyloidogenic immunoglobulin light chain variable domain SMA with a negative net charge formed fibrils on a negatively charged mica surface, but not on positively charged or hydrophobic surfaces. In this context, it has been reported that collagen plays a crucial role in the deposition of β2-m amyloid under physiological conditions (20). The authors proposed that positively charged surfaces along the collagen fiber directly enhance the fibrillation of negatively charged β2-m.

Although electrostatic interaction is important to the formation of β2-m fibrils, other interactions may also be involved. The net charge of the β2-m monomer and K3 peptide is −3 at pH 7.0 and −1 at pH 6.0, respectively. The molecular weights are 11,854 and 2,498, respectively. Thus, it is likely that the fibril growth of K3 is more affected by electrostatic interactions than that of β2-m.

CONCLUSION

In conclusion, we revealed that K3 fibrils were decomposed by the laser beam in the presence of amyloid-bound ThT molecules. Although we only observed inhibition of fibril growth for β2-m, it is likely that ThT-induced decomposition of β2-m fibrils occurs under appropriate conditions. Because amyloid fibrils have a unique supramolecular structure, it may be possible to create very specific and powerful PDT reagents for them. The present results suggest the light-induced decomposition of amyloid fibrils coupled with an amyloid-specific dye to be useful in the treatment or prevention of dialysis-related amyloidosis, for which no effective method has yet been established.

Acknowledgments—We thank Miyo Sakai (Institute for Protein Research) for performing ultracentrifuge analysis and Dr. Tetsuchika Wazawa (Tohoku University) for support with the TIRFM system.

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