Expansion of Polyglutamine Induces the Formation of Quasi-aggregate in the Early Stage of Protein Fibrillization*

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We examined the effects of the expansion of glutamine repeats on the early stage of protein fibrillization. Small-angle x-ray scattering (SAXS) and electron microscopic studies revealed that the elongation of polyglutamine from 35 to 50 repeats in protein induced a large assembly of the protein upon incubation at 37 °C and that its formation was completed in ~3 h. A bead modeling procedure based on SAXS spectra indicated that the largely assembled species of the protein, quasi-aggregate, is composed of 80 to ~90 monomers and a bowl-like structure with long and short axes of 400 and 190 Å, respectively. Contrary to fibril, the quasi-aggregate did not show a peak at S = 0.21 Å⁻¹ corresponding to the 4.8 Å spacing of β-sheet-like sheets in SAXS spectra, and reacted with a monoclonal antibody specific to expanded polyglutamine. These results imply that β-sheets of expanded polyglutamines in the quasi-aggregate are not orderly aligned and are partially exposed, in contrast to regularly oriented and buried β-pleated sheets in fibril. The formation of non-fibrillar quasi-aggregate in the early phase of fibril formation would be one of the major characteristics of the protein containing an expanded polyglutamine.

Amyloid is a fibrillar deposit observed in various proteins associated with human neurodegenerative diseases including Alzheimer’s diseases, Creutzfeldt-Jakob diseases, and polyglutamine diseases. Amyloid has a “cross-β structure,” with β-strands perpendicular to, and backbone hydrogen bonds parallel to, the fibril axis (1, 2). Recent in vitro studies have revealed that an intermediate termed protofibril is transiently formed prior to the formation of the amyloid fibrils of amyloid-β peptide and α-synuclein, the depositions of which in brain are hallmarks of Alzheimer’s disease and Parkinson’s disease, respectively (3–7). The protofibril as well as oligomeric states of amyloid-β peptide has been reported to induce cellular dysfunction and neuron death (8–10). Recently, Bucicentini et al. (11) showed that non-disease-related proteins might generally form globular intermediates prior to the formation of amyloid fibrils and that the intermediates induce cellular toxicity. Thus, studies on the earliest phase of protein fibrillization, in particular the oligomerization and nucleation processes, are of great importance for identifying therapeutic targets as well as for understanding the molecular mechanism of fibril formation. In addition, structural characterization of such globular precursors of amyloid fibril would be important to reveal origins of the cellular toxicity of the amyloid-forming proteins.

Amyloid fibrils are also observed in the aggregates of proteins bearing an expanded polyglutamine (12, 13). Expansion of polyglutamine to more than ~35 repeats in certain proteins induces the formation of intranuclear inclusions that are characteristic of polyglutamine diseases such as Huntington’s disease and hereditary spinocerebellar ataxia (14). Although the protein aggregate including amyloid fibril might be closely involved in the cellular dysfunction that causes polyglutamine diseases (15), little is known about the effects of the expansion of glutamine repeats on the aggregation mechanism of the causative proteins. Although Porrier et al. (16) recently reported spheroids and protofibrils as precursors in the fibrillization of the N-terminal fragment of mutant huntingtin containing 44 glutamine repeats, an early phase of the fibrillization process of the protein has not been fully characterized. In particular, structural and physico-chemical properties of such precursors have remained unclear.

We have recently designed sperm whale myoglobin (Mb) mutants in which a varying length of glutamine repeats was inserted (17). Although the expansion of glutamine repeat in general renders the protein insoluble in water, we successfully established preparation of the mutant Mbs containing 50 (Mb-Gln₅₀) or 35 (Mb-Gln₃₅) glutamine repeats on a large scale, which is required for their structural analysis. 50 glutamine repeats inserted into protein are pathological, whereas 35 glutamine repeats lie between pathological and non-pathological status in polyglutamine diseases. In our previous study (17), Mb-Gln₅₀ and Mb-Gln₃₅, but not a mutant Mb bearing shorter, 12 glutamine repeats (Mb-Gln₁₂), reacted with a monoclonal 1C2 antibody specific for an expanded polyglutamine (18), suggesting that the expanded polyglutamines in Mb-Gln₅₀ and Mb-Gln₃₅ form a characteristic structure similar to that of the native proteins that cause polyglutamine diseases. In addition, the tendency to form amyloid fibrils was highly dependent on the length of glutamine repeats inserted into Mb. Mb-Gln₅₀ and Mb-Gln₃₅ formed amyloid fibrils under physiological conditions rapidly and slowly, respectively, whereas Mb-Gln₁₂ and Mb wild-type (Mb-WT) did not form any amyloid fibrils under the

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‡ The abbreviations used are: Mb, myoglobin; WT, wild type; SAXS, small-angle x-ray scattering; MOPS, 4-morpholinopropanesulfonic acid; SVD, singular value deconvolution; DAMMIN, dummy atom minimization.
same conditions (17). We suggest, by these observations, that Mb-Gln50 and Mb-Gln35 would be appropriate to investigate the effects of the expansion of glutamine repeats on the fibrilization process of polyglutamine-bearing proteins. To elucidate the formation mechanism of fibril would be crucial for better comprehension of the molecular basis for polyglutamine diseases.

In the present study, we applied small-angle x-ray scattering (SAXS) to examine and compare the early stage of fibril formation of the mutant Mbs, because SAXS is a powerful technique for quantitatively defining the MB of smaller particles with accurate time dependence, compared with light scattering. We revealed that the expansion of polyglutamine from 35 to 50 repeats in a mutant Mb induced a large assembly of the protein in the early phase of fibrilization. The formation of a largely assembled species of Mb-Gln50 was confirmed by electron microscopy. We further examined structural properties of β-sheets of expanded polyglutamines in the Mb-Gln50 assembled species and determined its low-resolution structure based on SAXS spectra.

**Experimental Procedures**

**Protein Expression and Purification**—Recombinant wild-type and mutant Mbs were expressed in TB1 *Escherichia coli* coli and purified as reported previously (17). The concentration of ferric Mbs was determined by the extinction coefficient of 157 μM⁻¹ cm⁻¹ at the Soret band in the UV-visible spectra. The sample solutions of Mbs in 10 mM MOPS buffer at pH 7.0 were centrifuged at 25,000 × g for 30 min, before incubation at 37 °C, to remove any pre-existing precipitates.

**Congo Red Binding Assay**—We incubated 100 μl of 1.86 mg/ml Mb-Gln50 and Mb-Gln35 and 4.49 mg/ml Mb-Gln35 and Mb-WT solutions at 37 °C. We also incubated the supernatant fraction (1.86 mg/ml) of a Mb-Gln35 solution, which was pre-incubated at 37 °C for 6 h at 37 °C. The precipitate, respectively (21), which was pre-incubated for 6 h, was resuspended with 100 μl of 10 mM MOPS buffer (pH 7.0) and seeded into fresh 1.86 mg/ml Mb-Gln50 and 4.49 mg/ml Mb-Gln35 solutions (10% v/v). At appropriate time points, 5 μl of the protein solutions was added to 10 μl Congo red in 10 mM MOPS buffer (pH 7.0) and incubated at room temperature for 30 min. UV-visible spectra of these samples were measured with a Shimadzu 2400-PC spectrophotometer. Congo red bound to fibril was determined using the following equation: Congo red (μmol/l) = A610,295/25,295 – A610,46,306 (8).

**Small-angle X-ray Scattering (SAXS)**—The measurement of SAXS was performed by synchrotron radiation of RIKEN structural biology beamline I (BL45XU) at Spring-8 in Harima, Japan (19). The sample-to-detector distance was 30 cm, which was calibrated using meridional diffraction of dried chicken collagen. The temperature of the sample was maintained at 37 °C by an incubator, and the sample cell and stage were also set to 37 °C. Using an x-ray image intensifier and cooled CCD detector (XR-II CCD) (20), each scattering profile was collected for 5 s. Data points were normalized to the intensity of the incident beam, and the buffer was subtracted. The radius of gyration ($R_g$) was determined by the Guinier approximation: $I(S) = I(0) \exp(-4πR_g^2 S^2)$, where $S$ and I(0) are the momentum transfer and intensity at the zero scattering angle, respectively, with fitting ranges of $S^2$ (Å⁻²) from $5 \times 10^{-2}$ to $40 \times 10^{-2}$. $S$ is defined as $S = 2\sin(θ)/λ$, where $θ$ and $λ$ are the scattering angle and the x-ray wavelength, respectively (21). The range used for $R_g$ determination satisfied the condition $2πR_g < 1.3$. The distance distribution function, $P(r)$, was calculated by GNOM, which uses an indirect Fourier transform method (22). The maximum dimension, $D_{max}$, was determined from the first zero cross-point of the $P(r)$ function (21). The cross-sectional radius of gyration, $R_g$, was determined by fitting the data points, using the equation $I(S)/IS = I(0) \exp(-πR_g^2 S^2)$ over $S^2$ ranges (Å⁻²) from $2 \times 10^{-6}$ to $10 \times 10^{-6}$ and from $8 \times 10^{-6}$ to $20 \times 10^{-6}$ for oligomer and quasi-aggregates, respectively. The spectral analysis by singular value deconvolution (SVD) was used by SPECFIT (version 3.0, Spectrum Software Associates, Marlborough, MA), using the $S$ versus $S^2$ versus $I(S)$ (Kratyk) plot of the data.

**Low-resolution Modeling**—Low-resolution particle shapes were restored from SAXS intensity profiles using a bead modeling procedure of DAMMIN (23). In the dummy atom minimization (DAMMIN), a protein molecule was approximated by densely packed small spheres (dummy atoms). Minimization was performed by the simulated annealing method (22), starting from the dummy atoms placed at random coordinates within the search sphere, sphere of diameter $D_{max}$. The detailed procedure of DAMMIN has been described previously (24). The stability of the model was checked by repeating the minimization 10 times in different runs. The three-dimensional models were displayed using the program VMD.

**Electron Microscopy and Immunoblotting**—The negative staining for the 1.86 mg/ml of sample of Mb-Gln35 and the 4.49 mg/ml of samples of Mb-Gln35 and Mb-WT was performed as reported previously (17), and the images were recorded on a LEI 912AB electron microscope (LEO, Cambridge, UK) normally at a magnification of 63,000 × g. For immunoelectron microscopy of Mb, the copper grid was treated with a polyclonal Mb (1:250–500, Chemicon) or IC2 antibody (1:50–100, Chemicon), followed by a 5-nm gold-conjugated rabbit or mouse second antibody, respectively (25). The sample was examined by transmission electron microscopy (HR-TEM, Amersham Biosciences). Each 1.86 mg/ml of sample of Mb-Gln50 with various incubations (0, 3, 10, 24, and 72 h) at 37 °C was also processed to immunoblotting by the Mb (1:5000) and IC2 (1:2000) antibodies.

**X-ray Diffraction Experiment**—The sample-to-detector distance was 30 cm, which was calibrated using meridional diffraction of dried chicken collagen. Mb-Gln50 and Mb-Gln35 fibrils grown from 5 mg/ml of solution in 10 mM MOPS buffer at pH 7.0 were collected by brief centrifugation and mounted onto the last guard slit with a width of 0.6 mm. The sample-to-detector distance was 30 cm. The sample holder was vacuumized to remove the scattering of air, and the fibrils were exposed to x-ray radiation operating at ~90 mA. Data were collected at room temperature for 30 s on a modified RIGAKU R-Axis IV™ imaging plate detector.

**Size Exclusion Chromatography**—A 1.86 mg/ml of solution of Mb-Gln50 and 4.49 mg/ml of solutions of Mb-Gln35 and Mb-Gln12 were incubated at 37 °C, and 40 μl of each sample at 3, 10, and 24 h was centrifuged at 25,000 × g for 20 min. The supernatant was analyzed by size exclusion chromatography using the Superdex-200 in the SMART system (Amersham Biosciences). The running buffer was 10 mM MOPS containing 150 mM NaCl at pH 7.0, and the flow rate was 40 μl/min.

**Results**

**Formation of Quasi-aggregate by Expanded Polyglutamine**—We examined the fibril formation of mutant Mbs by a Congo red binding assay. We incubated separate solutions (1.86 mg/ml) of Mb-Gln50 and Mb-Gln35 at 37 °C and determined the amount of Congo red bound to fibril, using UV-visible spectroscopy (8). The time-dependent profiles showed that the fibril formation of Mb-Gln50 was initiated in ~50 h, whereas Mb-Gln35 did not clearly exhibit fibrillation within 200 h (Fig. 1A). We then increased the concentration of a Mb-Gln35 solution from 1.86 mg/ml to 4.49 mg/ml and examined the fibril formation by the Congo red binding assay. The 4.49 mg/ml of solution of Mb-Gln35 formed fibrils slowly with a lag time of ~150 h (Fig. 1A). Contrary to Mb-Gln50 and Mb-Gln35, 4.49 mg/ml of solutions of Mb-Gln12 and Mb-WT did not show the binding to Congo red upon incubation >200 h (Fig. 1A), indicating no fibril formation as reported previously (17). We also verified that the lag time of fibrillation did not change when we used mutant Mbs of different sample preparations.

Because Mb-Gln50 and Mb-Gln35 formed fibrils under the physiological condition, we examined the early phase of fibrillation of the mutant Mbs by SAXS. We incubated separate solutions (1.86 mg/ml) of the mutant Mbs at 37 °C and measured their SAXS spectra with various incubation times. The Guinier plot of Mb-Gln35 showed an increase in the intensity at $S^2 = -1 \times 10^{-3}$ Å⁻² upon incubation at 37 °C, suggesting formation of an assembled species of the mutant Mb (data not shown). We plotted the scattering profiles of Mb-Gln50 as $S$ versus $S^2$ versus $I(S)$ (Kratyk plot) to clarify the protein assembly. As shown in Fig. 1B, an intensity of the peak at $S = 1.4 \times 10^{-3}$ Å⁻¹ was clearly increased with incubation times. The peak showed a maximum scattering intensity in ~3 h of incubation, and the intensity was constant until 27 h (Fig. 1C). Because a

[2] Y. Nishikawa and T. Fujisawa, unpublished results.
Fig. 1. Expansion of the glutamine repeat induced the formation of quasi-aggregate. A, fibril formation was monitored by Congo red binding to fibril, using UV-visible spectroscopy. 1.86 mg/ml of Mb-Gln_{35} (●), Mb-Gln_{12} supernatant (1.86 mg/ml) after incubation at 37°C for 6 h (◼), 1.86 mg/ml of Mb-Gln_{35} with 10% (v/v) Mb-Gln_{35} quasi-aggregate (▲), 1.86 mg/ml of Mb-Gln_{35} (○), 4.49 mg/ml of Mb-Gln_{35} (□), 4.49 mg/ml of Mb-Gln_{35} with 10% (v/v) Mb-Gln_{35} quasi-aggregate (△), 4.49 mg/ml of Mb-Gln_{35} (◇), and 4.49 mg/ml of Mb-WT (○) are shown. The level of variability of each data point by different sample preparations was within 25%. B–E, time-dependent S versus S^2 × I(S) (Kratky) profiles of SAXS spectra for 1.86 mg/ml of Mb-Gln_{35} (B) and 4.49 mg/ml of Mb-Gln_{35} (D). The scattering intensities at S = 1.4 × 10^{-3} Å^{-1} (○) and S = 1.2 × 10^{-2} Å^{-1} (◼) in D were plotted against incubation time (hour) in C and E, respectively.

peak at the extremely small-angle region (S = 1.4 × 10^{-3} Å^{-1}) corresponds to a large-sized particle, our result indicated that a largely assembled Mb-Gln_{35} was formed in the early time of the lag phase (~50 h) of fibrillization. Hereafter, we referred such a largely assembled species of protein, which was formed in the early stage of the Mb-Gln_{35} fibrillization, as a quasi-aggregate. In contrast to Mb-Gln_{35}, SAXS spectra of a 1.86 mg/ml of solution of Mb-Gln_{35} did not change upon incubation at 37°C for more than 20 h, suggesting an absence of such an assembled species of protein as observed for Mb-Gln_{35} (data not shown). Thus, we measured SAXS spectra of a 4.49 mg/ml of solution of Mb-Gln_{35} (Fig. 1D), which facilitated the fibril formation as shown in Fig. 1A. However, the peak at S = 1.0 × 10^{-3} Å^{-1} did not increase with incubation times (Fig. 1A), which confirmed the absence of an assembled species of protein in the early stage of the fibrillization for Mb-Gln_{35}. In addition, we did not detect any peak at the extremely small-angle region in SAXS spectra for Mb-Gln_{12} and Mb-WT upon incubation at 37°C over 50 h, indicating absence of any largely assembled species of protein (data not shown). The reliability of time-dependent SAXS spectra was verified by repeating measurements with mutant Mbs of different sample preparations and with different incubation times.

To gain more physico-chemical insights into the formation of the quasi-aggregate of Mb-Gln_{35}, we measured time-dependent SAXS spectra at various temperatures (25, 29, 33, and 37°C) for Mb-Gln_{35}. The formation of quasi-aggregates was greatly affected by temperature, and representative scattering profiles at 25°C and 33°C are shown in Fig. 2, A and B. The scattering intensity at S = 1.4 × 10^{-3} Å^{-1} was plotted against incubation times, and the formation rate of the quasi-aggregate was calculated. We found that these plots were best fitted by a single exponential (Fig. 2C), indicating that the formation of the quasi-aggregate follows the first-order kinetics. We also show an Arrhenius plot of the formation rate of the Mb-Gln_{35} quasi-aggregate in Fig. 2D. The activation energy for the formation of the quasi-aggregate was calculated from the slope of the Arrhenius plot (22.8 ± 1.8 kcal/mol). Furthermore, we examined the effects of the Mb-Gln_{35} quasi-aggregate on fibrillization of Mb-Gln_{35} or Mb-Gln_{35} by the Congo red binding assay. Elimination of the quasi-aggregate, which accumulated upon incubation at 37°C for 6 h, in the Mb-Gln_{35} sample prolonged a lag time of fibrillization from ~50 to ~70 h (Fig. 1A). On the other hand, addition of the Mb-Gln_{35} quasi-aggregate into a fresh Mb-Gln_{35} or Mb-Gln_{35} solution as a seed eliminated or shortened the lag time, respectively (Fig. 1A).

We ascertained the formation of quasi-aggregate by electron microscopy. We carried out the negative staining for mutant Mbs, which had been incubated at 37°C for 3 h, and observed them by electron microscopy. A typical electron micrograph is shown in Fig. 3A. We clearly detected particles with a length of ~20–45 nm in the Mb-Gln_{35} sample, whereas such particles were not observed for Mb-Gln_{12}, Mb-Gln_{35}, and Mb-WT samples (data not shown). This electron micrograph suggested that the observed particles for Mb-Gln_{35} correspond to the quasi-aggregate, which was indicated in the SAXS experiment for Mb-Gln_{35} (Fig. 1B), because the formation of quasi-aggregate was completed in 3 h (Fig. 1B). We further performed an immunoelectron microscopic study for the quasi-aggregate with a polyclonal Mb and a monoclonal 1C2 antibody (18). Although some of the quasi-aggregates initiate to assemble each other by further incubation during the immunostaining procedure, the quasi-aggregate reacted with both of the Mb and 1C2 antibodies (Fig. 3, B and C). We also investigated the reactivity of these antibodies to the Mb-Gln_{35} fibrils that were formed by incubation at 37°C for 7 days. The Mb antibody
reacted with the fibrils along the fibers (Fig. 3D), whereas the 1C2 antibody recognized only ends and branch sites of the fibrils (Fig. 3E). We further investigated this observation by the following immunoblotting experiment. Each sample of Mb-Gln50, with various incubations (0, 3, 10, 24, and 72 h) were separated by SDS-PAGE and processed for immunoblotting by the Mb and 1C2 antibodies. We focused on the gel top in the immunoblot, because the quasi-aggregate and/or fibril can be detected at the gel top. We found a clear difference in the immunoreactivity to the gel top in 72 h between the Mb and 1C2 antibodies. Although the Mb antibody stained the gel top in 72 h, the reactivity of the 1C2 antibody to the gel top was obviously decreased in 72 h (Fig. 3F).

Furthermore, we followed the morphological changes of Mb-Gln50 upon incubation at 37 °C for 1 to −7 days, using electron microscopy. The quasi-aggregates of Mb-Gln50 were associated with each other to form largely assembled clusters, but fibrils were not observed at 1 day (data not shown), whereas fibrils were clearly detected at 3 days. We display typical electron micrographs of Mb-Gln50 at 3 or 5 days in Fig. 3, G–I. In the electron micrographs, we frequently observed the fibrils that were connected to and associated with clusters of the quasi-aggregates (arrow, Fig. 3, G–I).

Models of Mb-Gln50 Monomer, Oligomer, and Quasi-aggregates—To gain more structural information on the quasi-aggregate, we attempted to perform SVD analysis for the time-dependent SAXS spectra. Because the SVD analysis required more data points in the first h of protein fibrilization, we measured SAXS of Mb-Gln50 (1.86 mg/ml), again in shorter incubation intervals from 1 to 60 min at 37 °C (Fig. 4A). The resultant 11 scattering profiles were analyzed using SPECFIT. The SVD analysis showed two species, and the scattering profiles were best fitted by single exponentials. This analysis together with the presence of an isoscattering point at $S = 4.4 \times 10^{-3}$ Å⁻¹ in the SAXS spectra (Fig. 4A) indicated that this reaction follows the first-order kinetics. Therefore, we deconvoluted the time-dependent SAXS profiles of Mb-Gln50 into two spectra, which displayed the scattering profiles of the initial (0 min) and final (60 min) states of Mb-Gln50. In the first-order kinetic scheme, the scattering profile of the final state (60 min) corresponded to that of the quasi-aggregate. We calculated Kratky plots of the two states of Mb-Gln50 and show them in Fig. 4B. From the scattering profiles, we calculated $R_g$ values of the initial and final states of Mb-Gln50 (68 and 146 Å, respectively). We realized that the initial state (0 min) was not a monomer but an oligomer, because the $R_g$ of 68 Å was larger than that of the Mb-Gln50 monomer (27 Å) (25). We found, by SAXS and dynamic light scattering analyses, that the Mb-Gln50 monomer is rarely isolated as a complete monomeric state and that some oligomerized species are formed during the purification. From the SAXS spectra, we also calculated cross-sectional radii of gyration, $R_g$, of the Mb-Gln50 oligomer (30 Å) and quasi-aggregate (92 Å). In addition, $D_{\text{max}}$ values of the oligomer (213 Å) and quasi-aggregate (463 Å) were determined by the pair distance distribution functions using GNOM. We found that these values were not changed by different data sets, confirming the reliability of the scattering profiles of the Mb-Gln50 oligomer and quasi-aggregate. We summarized the structural parameters of the Mb-Gln50 monomer, oligomer, and quasi-aggregate in Table I.

On the basis of the SAXS spectra, bead models of the Mb-Gln50 oligomer and quasi-aggregate were constructed by a fitting procedure, using DAMMIN. The calculated model structures are illustrated in Fig. 4C. The converged model of the oligomer showed an elongated structure that has long and short axes of 210 and 50 Å, respectively. The model structure of the quasi-aggregate showed a bowl-like shape with some torsion, having long and short axes of 400 and 190 Å, respectively.

![Fig. 2. Temperature-dependent formation of Mb-Gln50 quasi-aggregate. Representative $S$ versus $S^2 \times I(S)$ (Kratky) plots at 25 °C (A) and 33 °C (B) are shown. C, the plots of the scattering intensity at $S = 1.4 \times 10^{-3}$ Å⁻¹ against the incubation time at 25 °C (○), 29 °C (■), 33 °C (▲), and 37 °C (▲) were best fitted by a single exponential, which allowed determination of the formation rate of the quasi-aggregate at various temperatures. D, an Arrhenius plot (ln($K_{\text{app}}$) versus $K^{-1}$) for the formation rate of the quasi-aggregate.](image-url)
which approximately corresponded to those observed by electron microscopy in Fig. 3A. The model structure of the Mb-Gln$_{50}$ monomer was also constructed from the SAXS spectrum by the bead modeling shape-determination method. We calculated the particle volumes of the Mb-Gln$_{50}$ monomer, oligomer, and quasi-aggregate and listed them in Table I. We obtained the relative ratios between monomer, oligomer, and quasi-aggregate and revealed that the oligomer and quasi-aggregate are composed of 4 to −5 and 80 to −90 monomers, respectively.

\textbf{β}-sheet Structure of Polyglutamine in Quasi-aggregate and Fibril—We found, by the SAXS experiment, that the expansion of polyglutamine from 35 repeats to 50 caused the different mechanism in the early stage of protein fibrillization. Next, we explored the effects of the expansion of polyglutamine on the end-state fibril structure by x-ray diffraction analysis for the Mb-Gln$_{50}$ and Mb-Gln$_{35}$ fibril. The x-ray diffraction patterns of both dried fibrils showed a strong ring at a spacing of $\sim 4.8$ Å and a weak diffraction ring at the 8 to $\sim 9$ Å region (Fig. 5, A and B). To inspect the spacing values, we plotted the circular averaged intensity against $S$ (Å$^{-1}$) (Fig. 5C). This analysis clearly showed diffraction rings at spacings of 4.79 Å$^{-1}$ and 8.33 Å, and 4.83 Å$^{-1}$ and 8.42 Å for the Mb-Gln$_{50}$ and Mb-Gln$_{35}$ fibril, respectively. Although both spacing values of the diffraction rings for the Mb-Gln$_{50}$ fibril were slightly smaller than those for the Mb-Gln$_{35}$ fibril, the spacing values did not change substantially by the expansion of glutamine repeats.

Because the Mb-Gln$_{50}$ fibril diffracted at the 4.8-Å spacing very strongly, we expected that a peak at $S = 0.21$ Å$^{-1}$ corresponding to the 4.8-Å spacing in β-sheet pleated sheets would be observed in the high-S region of SAXS spectra. The peak at $S = 0.21$ Å$^{-1}$ was indeed unambiguously detected for the Mb-Gln$_{50}$ sample after incubation at 37 °C for 120 h (bold arrow in Fig. 5D), when formation of the Mb-Gln$_{50}$ fibril was almost completed (Fig. 1A). We examined whether this peak is detected during formation of the quasi-aggregate. SAXS spectra of Mb-Gln$_{50}$ were measured after incubation at 37 °C for 3 to $\sim 12$ h (Fig. 5D). As a result, we could not observe the peak at $S = 0.21$ Å$^{-1}$ in 3 h when formation of the quasi-aggregate was almost completed (Fig. 1B). Similarly, the incubation for 12 h was still insufficient for detection of the peak at $S = 0.21$ Å$^{-1}$, although formation of the quasi-aggregate is definitely completed in 12 h (Fig. 1B).

\textbf{Gel Filtration Experiment—}We investigated whether we can isolate the quasi-aggregate of Mb-Gln$_{50}$ as a soluble state. A 1.86 mg/ml of solution of Mb-Gln$_{50}$ was incubated at 37 °C for 3, 10, and 24 h, and the supernatant fraction was separated by size exclusion chromatography. Because the quasi-aggregate is composed of 80 to $\sim 90$ monomers of 26,000 (Table I), the molecular weight of the quasi-aggregate was estimated to be 2,000,000–2,500,000, suggesting that the quasi-aggregate should be eluted at the void volume. However, we could not detect a peak at the void volume (data not shown), indicating that the quasi-aggregate does not exist in the supernatant but in the pellet fraction. We also verified absence of a peak at the void volume position for respective samples of Mb-Gln$_{35}$ and Mb-Gln$_{12}$. The presence of the Mb-Gln$_{50}$ quasi-aggregate in the pellet fraction was confirmed by an immunoblotting experiment. When we dissolved each pellet of the Mb-Gln$_{50}$ sample (3, 10, and 24 h) with SDS-sample buffer and it was processed for immunoblotting by Mb antibody, we detected a distinct band at the gel top and another band for monomeric Mb-Gln$_{50}$.
Effect of Polyglutamine on Protein Fibrillization

**TABLE I**

| Mb-Gln50 | $R_g$ (Å) | $R_m$ (Å) | $D_{max}$ (Å) | Volume of bead model (Å$^3$) | Relative ratio of volume |
|----------|-----------|-----------|--------------|----------------------------|-------------------------|
| Monomer  | 27 ± 1    | 11 ± 1    | 95 ± 5       | 5.82 ± 0.30 × 10^4          | 1                       |
| Oligomer | 68 ± 7    | 30 ± 2    | 213 ± 13     | 2.56 ± 0.34 × 10^6          | 4.4 ± 0.6               |
| Quasi-aggregate | 146 ± 3 | 92 ± 4 | 463 ± 13     | 5.07 ± 0.76 × 10^6          | 87 ± 13                 |

Fig. 5. **β-sheet structures in fibril and quasi-aggregate.** X-ray diffraction patterns from Mb-Gln50 (A) and Mb-Gln35 (B) fibrils are shown. The white and black arrows show reflections at ~4.8 and ~8.4 Å, respectively. C, the intensity of the diffraction was plotted against $S$ (Å$^{-1}$) to clarify the positions of the diffraction rings of the Mb-Gln50 (solid line) and Mb-Gln35 (dotted line) fibrils. D, time-dependent Kratky profiles in the high-$S$ region were shown during the formation of Mb-Gln50 fibrils. A bold arrow indicates a peak at $S = 0.21$ Å$^{-1}$, which corresponds to the 4.8-Å spacing of β-pleated sheets.

Contrary to the similar fibril structures of the mutant Mbs, we revealed a major difference in the early stage of the fibrillation process between Mb-Gln50 and Mb-Gln35. We demonstrated, by SAXS spectra and electron micrographs, that a largely assembled, non-fibrillar species of protein, quasi-aggregate, was formed in the early phase of fibrillation of Mb-Gln50. The formation of quasi-aggregate was almost completed within 3 h of incubation at 37 °C, and the amount of quasi-aggregate was constant until 27 h (Fig. 1B). Because the lag time in the fibril formation of Mb-Gln50 was ~50 h (Fig. 1A), the quasi-aggregate was formed in the early stage of the lag phase, namely the nucleation process of fibrillation, and the amount of quasi-aggregate was constant until at least half-time of the lag phase. On the basis of SAXS spectra of the quasi-aggregate, we constructed the bead model and determined the shape and dimensions of the quasi-aggregate (Fig. 4C). The SVD analysis revealed that only two species (oligomer and quasi-aggregate) exist during the formation of the quasi-aggregate, and that the formation of the quasi-aggregate follows the first-order kinetics (Fig. 4, A and B), which was consistent with the presence of an isoscattering point in the Kratky profiles (Fig. 4A). These results indicated that any stable intermediates are not formed in the formation process of quasi-aggregate from oligomers. Because the formation rate of quasi-aggregate was unambiguously affected by temperature (Fig. 2), we suggest that the formation of quasi-aggregate would be induced by hydrophobic interactions (28). The partial solubility of quasi-aggregate in SDS (data not shown) would be in agreement with the major contribution of the hydrophobic interactions to the formation of quasi-aggregate, because the interactions between expanded polyglutamines are so strong that a quasi-aggregate formed by such interactions could not be readily dissociated into monomers by the treatment with SDS.

**DISCUSSION**

**Effect of Expansion of Glutamine Repeats on the Fibrillation Process**—The x-ray diffraction analysis revealed that both diffraction spacings in the Mb-Gln50 fibrils (4.79 and 8.33 Å) are slightly smaller than but quite similar to those in the Mb-Gln35 fibrils (4.83 and 8.42 Å) (Fig. 5, A and B). The diffraction rings at ~4.8 and ~8.4 Å correspond to the hydrogen-bond distances between intra- and inter-β-sheets, respectively (26). Because the inserted polyglutamine forms the β-sheet structure in the mutant Mbs (17), we suggest that the β-pleated sheets in fibril were constituted by the inserted glutamine repeats. Although intra- and inter-β-sheets in the Mb-Gln50 fibril were slightly more compact, presumably because of the longer glutamine repeats than those in the Mb-Gln35 fibril, we can conclude that the expansion of glutamine repeats did not have substantial effects on the β-sheet structure in fibril. Interestingly, Perutz et al. (27) recently reported the same spacing values (~4.8 and ~8.4 Å) in the diffraction ring of fibrils for N-terminal huntingtin, which contains similar glutamine repeats (Gln51) to Mb-Gln50, suggesting that a similar length of polyglutamine, even in the different context of protein, forms a similar β-pleated sheet structure in fibril.
were connected to and associated with clusters of quasi-aggregates at 3 or 5 days (arrow, Fig. 3, G–I), which might support some involvement of the quasi-aggregate in fibril formation. It is noted here that the dimension of the quasi-aggregate (20–40 nm) was somewhat larger than the diameter of fibrils (10–20 nm), implying that a fibril is not formed by the simple tandem association of the quasi-aggregate. If fibrils were formed by the assembly of quasi-aggregates, conformational rearrangement in the quasi-aggregate would be essential, as discussed in fibrillization of Ure2p (32), or some protein molecules would dissociate from the quasi-aggregates during the fibril maturation (27). Alternatively, the quasi-aggregate might play a role on the scaffold where monomers or oligomers interact and finally form a mature fibril, because we could not observe the tandem assembly of quasi-aggregates during the fibril formation by electron microscopy.

Contrary to the formation of quasi-aggregate for Mb-Gln50, we could not observe such a largely assembled species of protein during the fibrillization of Mb-Gln35 by SAXS and electron microscopy. A major difference in the physical property between Mb-Gln35 and Mb-Gln50 is that Mb-Gln35 is not unfolded as greatly as Mb-Gln50 (17). The lesser extent of unfolding of Mb-Gln35 would prevent the exposure of hydrophobic residues on protein surface and the formation of quasi-aggregate in the early stage of fibrillization. Because the addition of the Mb-Gln50 quasi-aggregate into a fresh Mb-Gln50 solution accelerated the fibril formation of Mb-Gln50 (Fig. 1A), we suggest that the absence of quasi-aggregate in the early phase is responsible for the slower formation rate of the Mb-Gln50 fibril. Because Mb-Gln50 did not form a quasi-aggregate, the mutant Mb would form a mature fibril in a different way from the quasi-aggregate-based fibrillization suggested for Mb-Gln50.

**Structural Features of Quasi-aggregate Formed in the Early Stage of Fibrillization**—We examined structural features of the β-sheet formed by the inserted polyglutamine in the quasi-aggregate of Mb-Gln50. Although the Mb-Gln50 fibril, which was prepared by the incubation for 120 h at 37 °C, clearly showed the peak at $S = 0.21$ Å$^{-1}$ in the Kratky plot, corresponding to the 4.8-Å spacing of orderly aligned β-sheeted sheets, such a peak was not observed in the Mb-Gln50 sample in 3 to 12 h incubation when formation of the quasi-aggregates is completed (Fig. 5D). This result suggested the absence of orderly aligned β-sheeted sheets in the quasi-aggregate. We also addressed the structural property of β-sheet in the quasi-aggregate by the Congo red binding assay. The formation of quasi-aggregate was completed in 3 h, and the amount of quasi-aggregate was constant until 27 h. The level of bound Congo red to Mb-Gln50 in 3–27 h was quite lower than that in ~120 h (Fig. 1A), when fibrils were clearly observed by electron microscopy. This result suggested that the quasi-aggregate has a low affinity for Congo red, contrary to the high affinity of fibril for Congo red. Because Congo red binds to regularly oriented β-sheeted sheets in fibril (33), we interpreted that the low affinity of the quasi-aggregate for Congo red might correspond to unordered β-sheets in the quasi-aggregate. This interpretation seemed to be reconciled with the observation that the 1C2 antibody was not reactive to the fibril but to the quasi-aggregate of Mb-Gln50 in the immunoelectron microscopic and immunoblotting experiments (Fig. 3, B–F). The Mb-Gln50 fibril was not labeled by the 1C2 antibody along with the fibers, and the reaction of the 1C2 antibody with the gel top in the immunoblot was decreased in 72 h. This decreased reactivity was correlated with the increase in the amount of fibril at the gel top, because fibrils were not observed at 1 day but clearly detected at 3 days by electron microscopy (Fig. 3G). The low reactivity of the 1C2 antibody to fibril suggested that β-sheets of expanded polyglutamines in fibril are regularly oriented, interact each other extensively, and form a core structure in the fibril such that the 1C2 antibody is not accessible to the buried, expanded polyglutamines. On the other hand, the 1C2 antibody was reactive to the quasi-aggregate as evidenced by the electron micrograph (Fig. 3C) and the immunoblot (3, 10 and 24 h in Fig. 3F). These results implied that β-sheets of expanded polyglutamines in the quasi-aggregate do not yet form regularly aligned β-sheeted sheets and do not interact each other so extensively as to inhibit the accessibility of the 1C2 antibody. On the basis of these results, we propose that the β-sheets of expanded polyglutamines in the quasi-aggregate of Mb-Gln50 would not be orderly aligned and are partially exposed, which are quite different from the regularly oriented and buried β-sheeted sheets in fibril.

Previous studies reported that some fibril precursors such as protofibril or an oligomerized form of amyloid-β exhibited cellular toxicity in vitro and in vivo (8–11). In our study, the presence of the Mb-Gln50 quasi-aggregates in the pellet fraction unfortunately prevented us from isolating the quasi-aggregate as soluble state and evaluating its cellular toxicity. This observation was in contrast to isolation of the amyloid-β protofibril as a soluble form by gel filtration chromatography (5). Because the protofibril of amyloid-β constitutes 30 to ~50 monomers (4,500) (34), its molecular weight is estimated to be 135,000 to ~225,000, which is much smaller than that of the Mb-Gln50 quasi-aggregate (2,000,000 to ~2,500,000). The higher molecular weight together with the extreme insolubility of expanded polyglutamines would render the Mb-Gln50 quasi-aggregate insoluble in water. Although we could not investigate cellular toxicity of the quasi-aggregate, the formation of quasi-aggregate only for the expanded polyglutamine-bearing protein suggested that the quasi-aggregate might be implicated with the cellular dysfunction that is caused by the expansion of glutamine repeats.

In summary, we revealed that the expansion of polyglutamine to 50 repeats in protein induced the formation of a largely assembled non-fibrillar species of the protein, a quasi-aggregate, in the early stage of protein fibrillization. A bead modeling procedure based on SAXS spectra showed that the quasi-aggregate is a bowl-like structure with long and short axes of 400 and 190 Å, respectively. We suggested, by spectroscopic and electron microscopic studies, that properties of the β-sheets formed by expanded polyglutamines in the quasi-aggregate are quite different from those of regularly oriented and buried β-sheeted sheets in fibril.
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REFERENCES
1. Sunde, M., and Blake, C. (1997) Adv. Protein Chem. 50, 123–159
2. Jimenez, J. L., Guijarro, J. I., Orlova, E., Zurdo, J., Dobson, C. M., Sunde, M., and Saibil, H. R. (1999) EMBO J. 18, 815–821
3. Harper, J. D., Lieber, C. M., and Lansbury, P. T. (1997) Chem. Biol. 4, 119–125
4. Harper, J. D., Lieber, C. M., and Lansbury, P. T., Jr. (1997) Chem. Biol. 4, 951–959
5. Walsh, D. M., Lomakin, A., Benedek, G. B., Condron, M. M., and Teplow, D. B. (1997) J. Biol. Chem. 272, 22364–22372
6. Harper, J. D., Wong, S. S., Lieber, C. M., and Lansbury, P. T., Jr. (1999) Biochemistry 38, 8972–8980
7. Rochet, J. C., and Lansbury, P. T., Jr. (2000) Curr. Opin. Struct. Biol. 10, 69–68
8. Hartley, D. M., Walsh, D. M., Ye, C. P., Diehl, T., Vásquez, S., Vassilev, P. M., Teplow, D. B., and Selkoe, D. J. (1999) J. Neurosci. 19, 8876–8884
9. Walsh, D. M., Hartley, D. M., Kosunomo, Y., Fezouni, Y., Condron, M. M., Lomakin, A., Benedek, G. B., Selkoe, D. J., and Teplow, D. B. (1999) J. Biol. Chem. 274, 25945–25952
10. Walsh, D. M., Klivinib, I., Fadeeva, J. V., Cullen, W. K., Anwyl, R., Wolfe, M. S., Rowan, M. J., and Selkoe, D. J. (2002) Nature 416, 535–539
11. Bucciantini, M., Giannoni, E., Chiti, F., Baroni, F., Formigli, L., Zurdo, J., Taddei, N., Ramponi, G., Dobson, C. M., and Stefani, M. (2002) Nature 416, 507–511
12. Scherzinger, E., Lurz, R., Turmaine, M., Mangiarini, L., Hollembach, B., Hasenbank, R., Bates, G. P., Davies, S. W., Lehrach, H., and Wanker, E. E. (1997) Cell 86, 549–558
13. McGowan, D. P., van Reon-Mom, W., Holloway, H., Bates, G. P., Mangiarini, L., Cooper, G. J., Faull, R. L., and Snel, R. G. (2000) Neuroscience 100, 671–680
14. Cummings, C. J., and Zoghbi, H. Y. (2000) Annu. Rev. Genomics Hum. Genet. 1, 281–328
15. Zoghbi, H. Y., and Orr, H. T. (2000) Annu. Rev. Neurosci. 23, 217–247
16. Porier, M. A., Li, H., Masosko, J., Cai, S., Amzel, M., and Ross, C. A. (2002) J. Biol. Chem. 277, 41032–41037
17. Tanaka, M., Morishima, I., Akagi, T., Hashikawa, T., and Nukina, N. (2001) J. Biol. Chem. 276, 45479–45475
18. Trottier, Y., Lutz, Y., Stevanin, G., Imbert, G., Devys, D., Cancel, G., Saudou, F., Weber, C., David, G., Tora, L., et al. (1995) Nature 378, 403–436
19. Fujisawa, T., Inoue, K., Oku, T., Iwamoto, H., Uruga, T., Kunnamasa, T., Inoko, Y., Yagi, N., Yamamoto, M., and Ueki, T. (2000) J. Appl. Crystallogr. 33, 797–800
20. Amezey, Y., Ino, K., Yagi, N., Asano, Y., Wakabayashi, K., Ueki, T., and Endo, T. (1995) Rev. Sci. Instrum. 66, 2290–2294
21. Guinier, A., and Fournet, G. (1955) Small-angle Scattering of X-rays, Wiley, New York
22. Svergun, D. I., Semenyuk, A. V., and Feigin, L. A. (1988) Acta Crystallogr. A 44, 244–250
23. Svergun, D. I. (1999) Biophys. J. 76, 2879–2886
24. Fujisawa, T., Kostyukova, A., and Maeda, Y. (2001) FEBS Lett. 498, 67–71
25. Tanaka, M., Machida, Y., Nishikawa, Y., Akagi, T., Morishima, I., Hashikawa, T., Fujisawa, T., and Nukina, N. (2002) Biochemistry 41, 10277–10286
26. Sunde, M., Serpell, L. C., Bartlam, M., Fraser, P. E., Popps, M. B., and Blake, C. C. (1997) J. Mol. Biol. 273, 729–739
27. Perutz, M. F., Pope, B. J., Owen, D., Waneker, E. E., and Scherzinger, E. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 5596–5600
28. Uversky, V. N., and Fink, A. L. (2001) J. Biol. Chem. 276, 10737–10744
29. Heiser, V., Scherzinger, E., Boeddrich, A., Nordhoff, E., Lurz, R., Schugardt, N., Lehrach, H., and Waneker, E. E. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 6739–6744
30. Jarrett, J. T., and Lansbury, P. T., Jr. (1993) Cell 74, 1055–1058
31. Lansbury, P. T., Jr. (1995) Neuron 15, 1151–1154
32. Rousset, L., Thomson, N. H., Radford, S. E., and Melki, R. (2002) EMBO J. 21, 2903–2911
33. Klunk, W. E., Jacob, R. F., and Mason, R. P. (1999) Methods in Enzymology, Vol. 309, pp. 285–305, Academic Press, San Diego
34. Yong, W., Lomakin, A., Kirkitadze, M. D., Teplow, D. B., Chen, S. H., and Benedek, G. B. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 150–154