Amyloid formation of native folded protein induced by peptide-based graft copolymer

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Abstract **We report here that a native folded *holo*-myoglobin, when incubated with a synthetic amyloidogenic peptide in aqueous solutions, forms fibrils. These fibrils took a cross-β form (inter-strand spacing: 4.65 Å and inter-sheet spacing: 10.65 Å) and bound the amyloidophilic dye Congo red as did the authentic amyloid fibrils. In contrast such fibril formation of myoglobin did not occur in the absence of the peptide. These results suggest the possibility that inter-molecular interaction of native protein with the amyloidogenic peptide trigger the amyloid formation even for the non-pathogenic native protein like myoglobin, which itself exists as a globular form, under certain conditions.**

Key words: Amyloid fibril; Artificial peptide; Myoglobin; Conformation; Self-assembly; Graft copolymer; Molecular interaction

1. Introduction

Amyloid fibril formation of peptides and proteins that cause neurodegenerative diseases such as Alzheimer's and Creutzfeldt–Jakob's are currently the focus of biochemical and biophysical research [1–5]. In order to understand the pathogenesis of and therapeutics for these diseases, it is important to elucidate the molecular-level structures and formation mechanisms of these peptides and proteins. In previous studies, many proteins were identified in amyloid diseases, and these aggregates were found to have a common core structure (a straight, long, cross-β structure), even though the proteins involved had no sequential or structural similarities [6–8]. Recently, Dobson's group found that non-pathogenic proteins, such as those of the SH3 domain [9], acylphosphatase [10] and *apo*-myoglobin [11], also form amyloid fibrils in vitro under particular conditions, and suggested that the formation of amyloid fibrils is a genetic property of the proteins. In fact, our previous results strongly supported this idea, namely, a simple artificial protein containing a homopeptide-graft chain was found to form amyloid-like fibrils under certain conditions [12]. In this letter we describe the first example of amyloid-like fibril formation in native folded *holo*-myoglobin by mixing myoglobin with the artificial peptide in aqueous solution. This result demonstrates the possibility that amyloid fibril formation of the non-pathogenic native protein is caused by interaction with the artificial peptide, which itself can form β-sheet fibrils, under certain conditions. Our findings also support the idea that amyloid formation is a fundamentally common property of peptides and proteins.

2. Materials and methods

2.1. Materials

The poly(γ-methyl-L-glutamate) grafted polyallylamine (**1**) was prepared as previously described [12]. Myoglobin from horse heart muscle was purchased from Sigma Chemical and used without further purification. The myoglobin containing nitrobenzofurazan at the amino groups (myoglobin–NBD) was obtained as follows. The myoglobin (50 mg) was dissolved in 5 ml of water (pH 9.5), and 30 μl of 4-fluoro-7-nitrobenzofurazan (NBD–F, 0.54 mg) ethanol solution was added into the solution. The molar ratio of NBD–F to amino groups of myoglobin was 0.05. After incubation for 1 min at 45°C, this solution was rapidly quenched to 0°C and dialyzed against 1 l of water, using a Spectra/Pore molecular porous membrane tube (Spectrum Medical Industries, MWCO 3500). After dialysis, the solution was lyophilized to obtain myoglobin–NBD: yield 45 mg. The peptide **1** containing Rhodamine B at the amino groups of the PAA unit (4.9%) (**1**–RhB) was prepared by reacting the peptide **1** with RhB isothiocyanate in water–TFE (1:1 v/v) at pH 11.5, and purified by dialyzing against 1 l of water–ethanol (6:4 v/v): yield 37 mg.

A sample solution of a **1**/myoglobin mixture was prepared by diluting TFE stock solution of peptide **1** with purified water containing myoglobin (final concentrations of **1** and myoglobin: [1] = [myoglobin] = 1.1×10^{-4} amino acid unit M, TFE content 20%, pH 4.0). To form fibril structures, the sample solution was incubated for 5 months at room temperature. The pH of the sample solution was adjusted with 0.1 M HCl.

2.2. Measurements

Far-UV CD spectra were recorded on a J-720 WI spectropolarimeter (JASCO) under a nitrogen atmosphere. Experiments were performed in a quartz cell with a 5-mm-path length over the range of 190–250 nm at ambient temperature. Transmission-FTIR spectra were measured by a Perkin-Elmer Spectrum 2000, using a mercury-cadmi-

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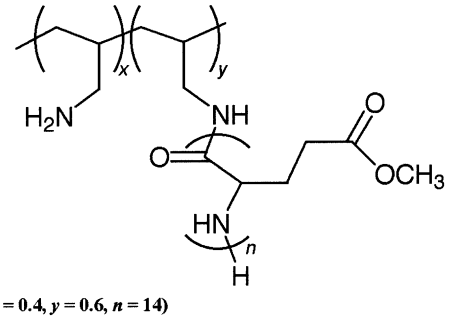
um-tellurium (MCT) detector (resolution, 2 cm^{-1}; number of scans, 1024). The sample and the detector chamber were purged with dried nitrogen before and during measurement. Fluorescence spectra of the mixed solution of myoglobin–NBD and **1**–RhB were measured on a RF-5300 PC fluorescence spectrophotometer (Shimadzu, Japan) at a wavelength of 465 nm for NBD excitation. Experiments were performed at room temperature in a quartz cell with a 10-mm-path length. In order to investigate the fluorescent resonance energy transfer from NBD to RhB, fluorescence spectra were obtained by subtracting the spectra of **1**–RhB from that of myoglobin-NBD/**1**–RhB in the mixed solution, since only the RhB gave a small amount of fluorescence when the solution was excited at 465 nm. The AFM images were collected at ambient temperature on a Nanoscope IIIa (Digital Instruments) operating in a tapping mode using a super sharp silicon tip (tip radius: 3 nm). An aliquot of the sample (myoglobin or **1**/myoglobin mixture) in water–TFE (8:2 v/v, pH 4.0) was placed on freshly cleaved mica. After adsorption for 3 min, the excess solution was removed by absorption onto filter paper. A 10 μm×10 μm scanner was used for imaging. The scanning speed was at a line frequency of 1 Hz, and the original images were sampled at the resolution of 512×512 points. The X-ray diffraction experiment was carried out using a Rigaku rotating-anode X-ray generator, R-AXIS IV (Japan). The solution of the **1**/myoglobin mixture was lyophilized after incubation for 5 months, and the obtained powder was put into a capillary of 1.0-mm diameter. The X-ray source was operating at 40 kV and 30 mA.

2.3. Congo red binding study

A Congo red (Wako Pure Chemical Industries) stock solution (150 μM) was prepared by dissolving the dye in water-TFE (8:2 v/v) containing NaCl. The fibrillar **1**/myoglobin mixture was prepared by incubating the sample solution for 5 months at room temperature as described above. Binding studies were carried out by diluting the **1**/myoglobin solution (after 5 months incubation) with the Congo red solution. The final concentration of Congo red was 3 μM in water–TFE (8:2 v/v) at pH 4.0 and containing 100 mM NaCl. The resulting solution was incubated at room temperature for 3 h. The ultraviolet spectra were then recorded for the sample mixture by using a V-530 spectrometer (JASCO). The Olympus optical microscope (BX50-34-FLAD-1) was used for birefringence measurement.

3. Results and discussion

We used a simple artificial peptide with an α-helical homo-polypeptide, poly(γ-methyl-L-glutamate)-grafted polyallylamine (**1**) (Fig. 1), as a trigger molecule for amyloid formation, and we chose a myoglobin as a non-pathogenic folded protein. It was previously shown that peptide **1** forms amyloid-like fibrils through the association of globular species with a structural transition from α-helix to β-sheet, when the amino groups of allylamine units of **1** were protonated (i.e. pH <8) in water–2,2,2-trifluoroethanol (TFE) mixed solution [12]. On the other hand, myoglobin has been well



1 (*x* = 0.4, *y* = 0.6, *n* = 14)

Fig. 1. Chemical structure of synthetic amyloidogenic peptide **1**. The molar ratio of free amino groups (x) of the polyallylamine to peptide-conjugated amino groups (y) was 0.4:0.6, and the number average degree of polymerization (n) of the PMLG graft-chain was 14.

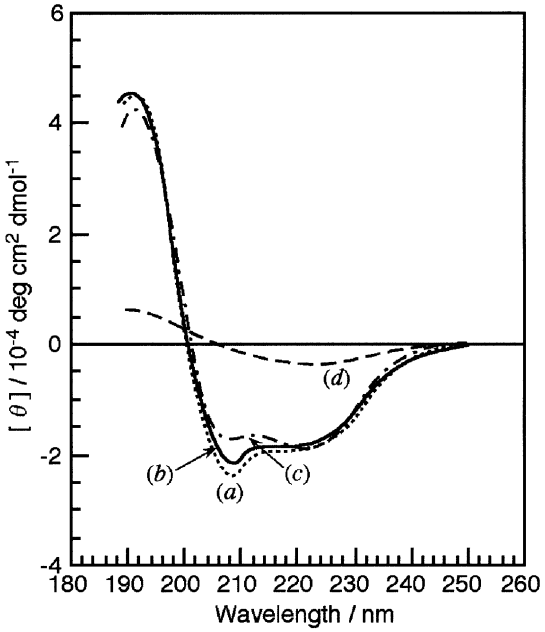


Fig. 2. Far-UV CD spectral changes of myoglobin ([myoglobin] = 1.1×10^{-4} unit M; amino acid unit) (*a* and *c*) and **1**/myoglobin mixture ([**1**] = [myoglobin] = 1.1×10^{-4} unit M; amino acid unit) (*b* and *d*) in water–TFE (8:2 v/v) at pH 4.0. The peptide and the protein were incubated for 0 h (*a* and *b*) and 5 months (*c* and *d*) at room temperature. The pH of the solution was adjusted with 0.1 M HCl.

known as a water-soluble globular protein that contains a heme prosthetic group, which stabilizes its structure. The details of the conformational properties of myoglobin (e.g. effects of pH and solvent) have been studied [13]. The effects of peptide **1** on the conformation of myoglobin were first examined by mixing each component in water–TFE (8:2 v/v) at pH 4.0. In this condition, the pure myoglobin exists in an α-helical native state (the pure peptide **1** forms β-sheet structure as described above). Fig. 2 shows the far-ultraviolet circular dichroism (far-UV CD) spectral change of myoglobin (1.1×10^{-4} amino acid unit M) in the absence and presence of peptide **1** in water–TFE (8:2 v/v) at pH 4.0. The far-UV CD spectra of myoglobin for both solutions showed negative maxima at 222 and 208 nm, respectively, shortly after preparation of the sample solutions, indicating the presence of a right-handed α-helix structure (ca. 65% content) (Fig. 2a,b). The vertical scale of the spectra in Fig. 2 was calculated using the total peptide concentration. This conformation of myoglobin without peptide **1** was also kept after incubation for 5 months in this solution at room temperature, although the folded structure (α-helical form) was partially converted to the unfolded (random form) structure (Fig. 2c). On the other hand, in the presence of peptide **1** (1.1×10^{-4} glutamate unit M) after incubation for 5 months, the far-UV CD measurement revealed a drastic change in the spectrum from the α-helical to the β-like pattern, in which a single negative maximum was observed at 221 nm and the ellipticity was extensively reduced (Fig. 2b,d). The transmission FTIR spectrum of **1**/myoglobin (after incubation for 5 months) was consistent with the results of far-UV CD measurements. The **1**/myoglobin mixture and myoglobin were adsorbed onto a CaF_2 plate after incubation for 5 months in water–TFE (8:2 v/v, pH 4.0). In the amide I and II regions [14], characteristic absorptions

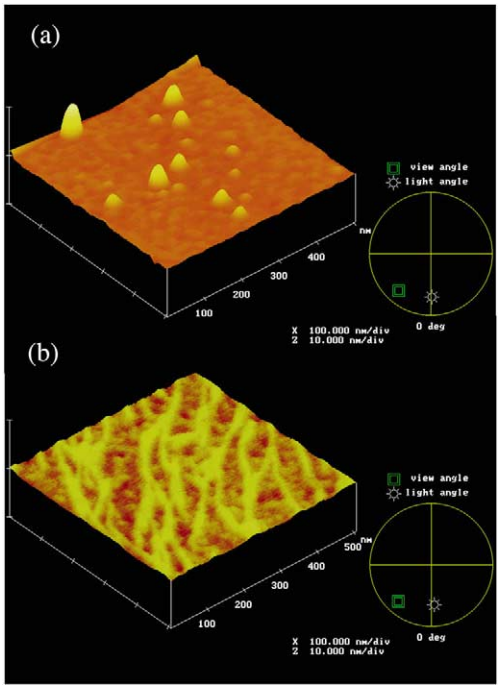


Fig. 3. Three-dimensional TM–AFM height images (500 nm×500 nm) of myoglobin ([myoglobin] = 1.1×10^{-4} unit M; amino acid unit) (*a*) and **1**/myoglobin mixture ([**1**] = [myoglobin] = 1.1×10^{-4} unit M; amino acid unit) (*b*) after incubation for 5 months in water–TFE (8:2 v/v) at pH 4.0. z-scale, 0–10 nm.

with the anti-parallel β-sheet structure were mainly observed at 1695, 1625 and 1512 cm^{-1} in the case of the **1**/myoglobin mixture, whereas pure myoglobin took mainly the α-helix structure (peak maxima at 1656 and 1545 cm^{-1}). These results strongly suggest that the myoglobin interacted with peptide **1** and that, as a result, the conformation of native myoglobin was changed from α-helix to β-sheet.

To examine the morphological properties of **1**/myoglobin mixture with its α-to-β conformational transition, atomic force microscopy (AFM) was measured after incubation for 5 months in water–TFE (8:2 v/v) at pH 4.0. Fig. 3 shows the AFM height images (500×500 nm^2) for myoglobin and for the **1**/myoglobin mixture. AFM is a useful technique in the field of structural biology to evaluate the three-dimensional structural features of protein and their assemblies. It is, however, well known that the convolution of the scanning tip leads to an overestimation of the sample's width [15]. All sample dimensions were therefore estimated from the height of the sample in cross section. An AFM image of pure myoglobin revealed the presence of only globular species (average height 2.5 nm) (Fig. 3a). This value was in fair agreement with the molecular size of native folded myoglobin. In contrast, an amyloid-like fibrillar structure with an average height of 1.1 nm was observed only with the **1**/myoglobin mixture, in which conformation of the myoglobin was changed from α-helix to β-sheet (Fig. 3b). It seems that a fibril formation based on a structural transition into β-sheet structure, as is observed in a **1**/myoglobin system, is an intrinsic process for amyloid deposition. In the case of amyloid β-peptides (Aβ), a nucleation-dependent polymerization model has been proposed to explain the mechanisms of Aβ fibril formation in vitro [3,16,17]. Nucleus formation requires an association step of Aβ monomers that are thermodynamically unfavorable. In our case, it is supposed that myoglobin and peptide **1** form aggregates owing to the hydrophobic interaction, and the resultant aggregates may act as a 'nucleus' for amyloid formation. In fact, such aggregate formations between myoglobin and peptide **1** was strongly supported by the results of fluorescence resonance energy transfer (FRET) experiments. The efficiency of FRET depends on the inverse sixth power of the distance between donor and acceptor [18]. This allows FRET measurements to be used with high sensitivity to verify the association of fluorescent-labeled myoglobin and peptide **1**. For the FRET study, we synthesized nitrobenzofurazan (NBD)-labeled myoglobin and Rhodamine B (RhB)-labeled peptide **1** to use as donors and acceptors, respectively. Fluorescence spectra of the mixture of myoglobin–NBD and **1**–RhB ([myoglobin–NBD] = [**1**–RhB] = 1.1×10^{-4} unit M; amino acid unit) were measured after incubation for 3 days in water–TFE (8:2 v/v, pH 4.0). As a result, efficient FRET was found to occur for the mixture of myoglobin–NBD and **1**–RhB, as evidenced by a quenching of the donor emission at 533 nm and an increase in the acceptor fluorescence at 568 nm, as compared with the pure myoglobin–NBD (fluorescence emission was only observed at 533 nm) observed under the same condition (data not shown). These results clearly show the complexation between peptide **1** and myoglobin under this condition, and therefore it can be considered that the obtained fibrils are composed of both myoglobin and peptide **1**.

In order to investigate the higher-order structure of these **1**/myoglobin fibrils, amyloidophilic dye Congo red (CR) binding and birefringence studies were subsequently carried out on the **1**/myoglobin fibril. Fig. 4 shows the absorption spectra of CR with and without **1**/myoglobin fibril. CR alone had an absorption spectrum with a maximum at 494 nm (Fig. 4b). When the fibrils were present, the absorption maximum shifted to 505 nm and the absorption newly appeared at around 530–540 nm (Fig. 4a). The difference spectrum (Fig. 4, inset) exhibited a clear peak at 537 nm, indicating that the dye bound to the **1**/

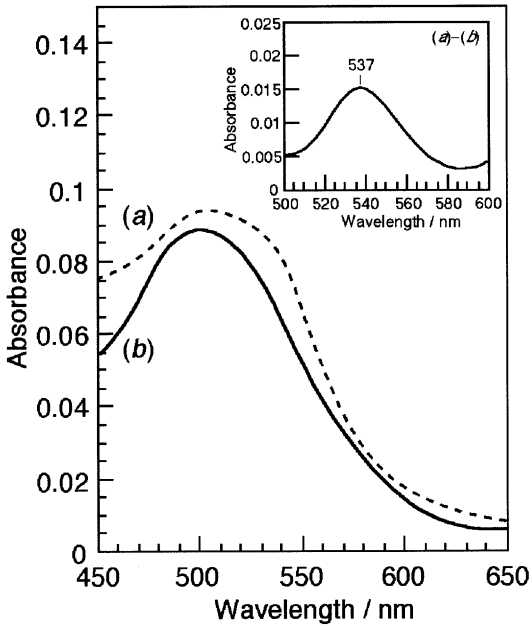


Fig. 4. Absorption spectra of Congo red with (*a*) and without (*b*) **1**/myoglobin mixture. The peptide **1**/myoglobin mixture was incubated for 5 months in water–TFE (8:2 v/v) at pH 4.0. The inset shows the differential spectrum of (*a*)−(*b*). [CR] = 3 μM.

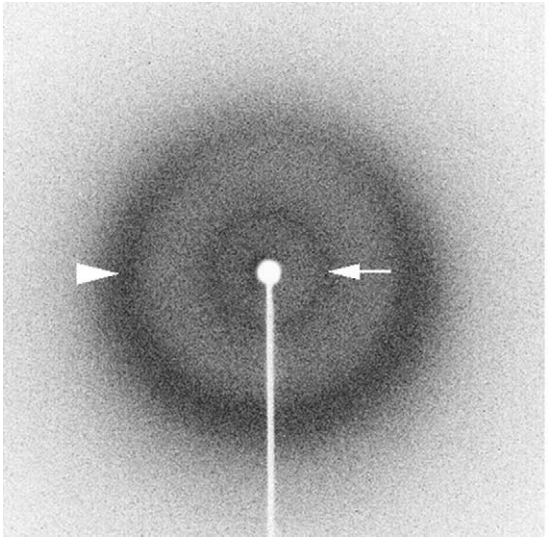


Fig. 5. X-ray diffraction pattern of **1**/myoglobin mixed fibril. Arrowhead indicates the 4.65-Å reflection; arrow indicates the 10.65-Å reflection.

myoglobin fibril, similar to the case with the authentic amyloid fibril [19]. In addition, these CR-stained peptide assemblies exhibited a yellow birefringence under cross-polarized light (data not shown), which showed their anisotropy. These findings indicate that **1**/myoglobin fibrils possess a regular quaternary structure. Moreover, the X-ray diffraction pattern of the **1**/myoglobin fibril showed the characteristic 'cross-β structure' reflections at 4.65 Å and 10.60 Å (Fig. 5) [20]. The 4.65 Å reflection arises from the inter-strand spacing between β-strand, and the 10.60 Å reflection corresponds to the inter-sheet spacing. These results obviously indicate the presence of an amyloid-like structure, in which continuous β-sheets lie parallel to the long axis of a fiber while their constituent β-strands run perpendicular to this axis.

The possibility as to whether other non-amyloidogenic proteins also induced the formation of amyloids as the myoglobin did under these conditions is now unclear. These results, however, strongly suggest the concept that the inter-molecular interaction of myoglobin with the amyloidogenic artificial peptides triggered the amyloid formation and that amyloid formation is one of the fundamentally common properties of peptides and proteins, although the appropriate conditions are required. Furthermore, these results provide a new method for regulating a high-order structure of protein assembly, namely, the conformation and morphology of a native protein could be controlled by utilizing the inter-molecular interaction between the protein and an artificial peptide having the desired structural properties. We believe that this kind of work opens a new vista in the study of the mechanisms underlying the folding of proteins and the formation of amyloid fibrils.

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