Fibrillogenesis and Cytotoxic Activity of the Amyloid-forming Apomyoglobin Mutant W7FW14F*

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Ivana Sirangelo‡, Clorinda Malmo‡, Clara Iannuzzi‡, Antonio Mezzogiorno§, Maria Rosaria Bianco§, Michele Papa§, and Gaetano Irace‡‡

From the ‡Dipartimento di Biochimica e Biofisica, Seconda Università degli Studi di Napoli, via L. De Crecchio 7, 80138 Napoli, Italy and §Dipartimento di Medicina Pubblica-Anatomia, Seconda Università degli Studi di Napoli, via L. Armanni 5, 80138 Napoli, Italy

The apomyoglobin mutant W7FW14F forms amyloid-like fibrils at physiological pH. We examined the kinetics of fibrillogenesis using three techniques: the time dependence of the fluorescence emission of thioflavin T and 1-anilino-8-naphthalenesulfonate, circular dichroism measurements, and electron microscopy. We found that in the early stage of fibril formation, non-native apomyoglobin molecules containing β-structure elements aggregate to form a nucleus. Subsequently, more molecules aggregate around the nucleus, thereby resulting in fibril elongation. We evaluated by MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) the cytotoxicity of these aggregates at the early stage of fibril elongation versus mature fibrils and the wild-type protein. Similar to other amyloid-forming proteins, cell toxicity was not due to insoluble mature fibrils but rather to early pre-fibrillar aggregates. Propidium iodide uptake showed that cell toxicity is the result of altered membrane permeability. Phalloidin staining showed that membrane damage is not associated to an altered cell shape caused by changes in the cytoskeleton.

Most if not all proteins exist in two structurally different forms, a globular soluble form and an aggregated form that is often produced at extreme temperatures or pH values, at incompatible ionic strength or at a very high protein concentration (1–6). The structure of protein aggregates varies considerably and ranges from amorphous species with no defined intermolecular interactions to highly ordered amyloid fibrils (7–12). The transformation of soluble globular proteins into amyloid fibrils is closely associated with the so-called conformational diseases and is still not completely understood in physical terms (energetically and structurally). In amyloid fibrils, the structure of the polypeptide chain is dominated by hydrogen bonding between the atoms of the main chain that results in the formation of extended β-sheets rather than by the specific interactions of the side chains that govern the structures of globular proteins. Recently, studies have focused on the conformational state, i.e. the native, partially folded, and fully denatured state that precedes the formation of highly organized amyloid fibrils, in an attempt to understand the molecular events underlying protein aggregation.

The formation of insoluble protein aggregates is associated with Alzheimer’s disease, light chain amyloidosis, and familial amyloidosis (7), polyglutamine disorders, and Parkinson’s disease (13). In these conditions, a specific peptide or protein that is normally soluble is deposited, either intact or in fragmented form, in insoluble fibrils that accumulate in one or more types of tissues. This process leads to fibrous aggregates constituted by extracellular amyloid plaques, neurofibrillary tangles, and other intracytoplasmic or intranuclear inclusions.

In vitro, a variety of proteins, apparently unrelated to any human disease, form fibrils that are indistinguishable from fibrils of patients affected by sporadic and familial amyloidosis (14–19). Even myoglobin, an α-globular protein, forms fibrils containing β-strands under experimental conditions that favor the formation of partially folded states (20).

Experimental data indicate that some human genetic amyloid diseases are because of point mutations that cause incomplete protein folding, thereby increasing the propensity of the polypeptide chain to aggregate (21, 22). We previously demonstrated that the substitution of the two highly conserved tryptophanyl residues of myoglobin, i.e. Trp-7 and Trp-14, with phenylalanine results in the expression of a protein that does not undergo correct folding (23) but aggregates and forms amyloid-like fibrils at physiological pH (24). This mutated protein lends itself to investigations of the relationship between folding and misfolding at molecular level.

The mechanism by which protein aggregation and amyloid formation results in cell damage has been widely studied (25–33). Recent results suggest that the toxicity of amyloidogenic proteins lies not in the insoluble fibrils but rather in prefibrillar oligomeric intermediates that originate in the early stages of fibril formation (34–37). However, the accumulation of large quantities of aggregates may itself cause disease, particularly systemic non-neurological disorders (38). The toxicity of early aggregates has been attributed to their large exposed hydrophobic surfaces, which may inappropriately interact with a wide range of cellular components (34).

The aim of this study was to identify the conformational species involved in the process of W7FW14F apomyoglobin fibrillation. The toxicity and cell damage induced by the structural states that form during fibrillation were also investigated. We found that the aggregation process involves a non-native conformational state different from the well characterized partially folded state of this protein (39–44). Moreover, like other proteins, cell toxicity is not attributed to mature fibrils but to early pre-fibrillar aggregates. Finally, the cytotxic effect is primarily caused by pre-fibrils interacting with the cell membrane and altering its permeability.
ever, this early damage does not seem to cause changes in the shape of the cytoskeleton indicative of cell death.

EXPERIMENTAL PROCEDURES

Protein Purification—Wild-type and W7FW14F mutant myoglobin were expressed and purified essentially as described elsewhere (24). Proteins were expressed in Escherichia coli BL21 strain as N-terminal His-tagged forms and purified via affinity chromatography on Ni²⁺-nitrilotriacetic acid resin (Qiagen). The wild-type myoglobin was expressed in the soluble form and purified under native conditions. The heme was removed by the 2-butane extraction procedure (45). The W7FW14F mutant was sequenced into insoluble inclusion bodies and purified under denaturing conditions. Refolding was achieved by removing denaturant by dialysis against 10 mM NaH₂PO₄, pH 2.0, containing decreasing concentrations of urea. The pH of the protein solution was then adjusted at neutrality. This resulted in the formation of protein aggregates that were collected by centrifugation at 15000 g for 10 min. Pre-fibrillar aggregates were collected immediately after neutralization of the protein sample, and mature fibrils were collected 7 days later. Protein concentration was determined under denaturing conditions, and absorption was measured at 280 and 275 nm for wild-type and mutant apomyoglobin, respectively. The molar extinction coefficient calculated from tryptophan and tyrosine content (46) were ε280 = 13,500 M⁻¹ cm⁻¹ and ε275 = 3,750 M⁻¹ cm⁻¹.

Fluorescence Measurements—Fluorescence was measured with a PerkinElmer Life Sciences LS 55 spectrophotometer using cuvettes that have an optical path length of 1 cm. The temperature was maintained at 25 °C using an external bath circulating fluid. Fluorescence was measured at an excitation wavelength of 350 nm, and emission spectra were measured from 410 to 600 nm (excitation and emission slit width = 3.5 nm). Protein concentration was 8 μM with an ANS/protein molar ratio of 1:2. ThT fluorescence was recorded from 460 to 500 nm with excitation at 450 nm and slits of 10 nm for excitation and emission. Protein concentration was 5 μM with a ThT/protein molar ratio of 5:1. For each sample, the fluorescence intensity at 482 nm was corrected by subtracting the emission intensity recorded before the protein was added to the ThT solution.

Circular Dichroism Measurements—Far-UV CD activity was measured on homogeneous wild-type and W7FW14F apomyoglobin samples at a concentration of 10 μM. Spectra were recorded at 25 °C on a J-715 spectropolarimeter equipped with a temperature-controlled liquid system Neslab RTE-110. Cuvettes of 1-mm path length were used over the wavelength range between 250 and 200 nm. Spectral acquisition was taken at 0.2-nm intervals with a 4-s integration time and a bandwidth of 1.0 nm. An average of three scans was obtained for all of the samples at a multipler absorber to avoid temperatures greater than 5°C in the spectral region analyzed. Data were corrected for buffer contributions and smoothed using the software provided by the manufacturer (System Software, version 1.00). All of the measurements were performed under nitrogen flow. The results are expressed as mean residue ellipticity [θ] in units of degree cm⁻² dmol⁻¹. A mean residue weight of 115 was used to estimate the protein secondary structure.

Electron Microscopy—Fibril formation was monitored by electron microscopy to identify and characterize intermediate species appearing on the aggregation pathway. At time points of 4 and 12 h and 2, 4, 7, and 21 days, aliquots of 5 μl of protein were sampled from protein solution, deposited on colloidion-acylacetate-coated electron microscopy grids, and left to air-dry under germ-free conditions before being used for structural analysis. Samples were stained with 1% phosphotungstic acid for 30 s. Grids were blotted and again air-dried. Images were acquired with a Zeiss TM-109 electron microscope operating at an 80-kV electron voltage.

Amyloid Apomyoglobin Fibrils

Cell Culture and Incubation with Protein Aggregates—NIH-3T3 cells (mouse fibroblasts, American Type Culture Collection) were routinely cultured in Dulbecco's modified Eagle's medium-high glucose (Sigma) containing 10% bovine calf serum and 3.0 mM glutamine in a 5.0% CO₂ humidified environment at 37 °C. 50 units/ml penicillin and 50 μg/ml streptomycin were added to the medium. Cells were used for a maximum of five to six passages. Cells were plated at a density of 3000 cells/well on 12-well plates in 1 ml of fresh medium for MTT assay (3,4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and at a density of 30,000 cells on poly-L-lysine (50 μg/ml) coated coverslips for 24 h after plating. Cells were treated with phenol red-free Dulbecco's modified Eagle's medium containing 10% bovine calf serum. Early aggregates and mature fibrils of W7FW14F apomyoglobin, suspended in Dulbecco's modified Eagle's medium without phenol red, were immediately added to cell media at ~20 μM of final concentration. Cells incubated with culture medium without protein served as a control. A toxic control for propidium iodide uptake and phallolidin staining was obtained by adding tert-butyl hydroperoxide (TBHP) (Sigma) to the culture medium. Five separate experiments in triplicate were conducted for early aggregates, mature fibrils, and wild-type native folded apomyoglobin.

MTT and Cell Death Assays—Cell-mediated reduction of MTT (Sigma) was assessed according to the method of Hansen et al. (47). Briefly, 10 μl of a stock MTT solution (5 mg/ml in PBS) were then added to 900 μl of culture medium/well, and incubation was continued for an additional 3 h. The medium was aspirated, and cells were treated with isopropyl alcohol-0.1 N HCl for 20 min. Levels of reduced MTT were determined by measuring the difference in absorbance at 570 and 690 nm. Cell death was assessed by the trypan blue internalization test.

Propidium Iodide Uptake and Phallolidin Staining—Slides from the different groups of cultures were fixed by immersing them for 10 min in 4% paraformaldehyde in PBS. After several washes, cells were stained with 600 μM propidium iodide (Sigma) in PBS per 10 min. Cells were rinsed in PBS and then in 0.1 M glycine in PBS for 5 min, permeabilized in 0.5% Triton X-100 in PBS for 5 min, and incubated in fluorescein isothiocyanate phallolidin (Sigma) conjugate diluted 1:1000 in PBS for 30 min and rinsed three times in PBS (5 min/wash). Cells were rinsed twice in distilled water and mounted cell side down with a drop of Vectashield mounting solution (Vectorstain Laboratories, Burlingame, CA) on a microscope slide. Slides were preserved by sealing the edges of the coverslip and slide with clear nail polish. Propidium iodide uptake was monitored by fluorescence microscopy using a tetramethyl rhodamine isothiocyanate filter and a Hamamatsu f472-95 digital camera (Hamamatsu Photonics, Japan) and analyzed and classified numerically using MCID Analysis, version 7.0 Image software (Imaging Research Inc., St. Catharines, Ontario, Canada). Fields of 800,000 μm² of cultures were analyzed. In each field, the elements ranging from levels between 1 and 120 in a 1024 gray level scale were defined as propidium iodide-positive nuclei. All of the cells were counted as the elements ranging from levels between 1 and 512 in the 1024 level gray scale. Phallolidin staining was used to evaluate cell shape. The elements were classified as normal or not on the basis of the form factor, which is a standard estimate of circularity that relates perimeter length to area. The more convoluted (and longer) the perimeter, the less circular the target. This measurement varies from 0 and 1 with 1 being a perfect circle. Cells with a form factor between 0.6 and 0.8 were classified as normal. Cells with a form factor between 0.8 and 1 were considered necrotic. Further separate propidium iodide and phallolidin staining and analysis were performed to confirm the results of some experiments. Statistical analysis was carried out with a paired two-sample Student's t test for the means ± S.D.

RESULTS

In mildly acidic condition, i.e. pH 4.0, the W7FW14F apomyoglobin mutant adopts a soluble molten globule-like conformation similar to that of the wild-type protein (24). In this state, the A, G, and H helices are folded in the native conformation, whereas the rest of the molecule is essentially unfolded (39–44). We exploited the W7FW14F model to examine the mechanism and kinetics of fibril formation induced by raising the pH from mildly acidic to a neutral value and evaluated whether the structural states formed during fibrillogenesis affect cell viability.

We evaluated the pH dependence of apomyoglobin aggregation and amyloid-like fibril formation by monitoring the fluorescence emission of two probes, ANS and ThT. We also measured far-UV CD activity to correlate fluorescence variations to structural modifications. ANS has been widely used in apomyoglobin studies since the discovery that native apomyoglobin binds ANS with high affinity in the site normally occupied by heme (48). Upon binding, the quantum yield of the dye becomes

Footnote 1: The abbreviations used are: ANS, 1-anilino-8-naphthalenesulfonate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ThT, thioflavin T; PI, propidium iodide; TBHP, tert-butyl hydroperoxide; PBS, phosphate-buffered saline.
200-fold higher than in water and its fluorescence maximum
shifts from 510 to 460 nm. ANS also binds to the partially
folded molten globule-like species adopted by the protein when
the pH is lowered from 7.0 to 4.0, although the binding site is
different because of the unfolding of the heme region that
occurs in the partially folded form (39, 49). At pH 4.0, the
emission of ANS is greatly reduced (75–80%) and shifted to
longer wavelengths (39, 49). ANS has also been used to study
aggregation of non-native protein molecules such as the infect-
ious scrapie agent (50).

As shown in Fig. 1, the emission intensity of the ANS-
W7FW14F apomyoglobin complex at pH 4.0 peaked at 478 nm,
which is very near that obtained with ANS bound to the par-
tially folded species observed with the wild-type protein under
the same experimental conditions (39, 49, 51). At pH 6.5, the
emission of ANS bound to W7FW14F decreased and there was
a concomitant shift of the emission maximum toward longer
wavelengths, i.e. the emission maximum of the fluorophore was
practically the same as that of free ANS in water, i.e. 510–520
nm (39), which indicates fluorophore release. Under the same
experimental conditions, the pH increase determines an in-
crease of emission intensity of the wild-type protein and a shift
of the emission maximum from 478 to 460 nm, a value indicat-
ing restoration of the native structure of the heme site (39).

Fig. 2 shows the far-UV CD spectra of W7FW14F at pH 4.0,
5.0, and 6.5. At pH 4.0, the spectrum is typical of a polypeptide
containing a significant amount of α-helical structure as indi-
cated from the two characteristic minima at 208 and 222 nm.
The analysis of CD data by Yang’s algorithm (52) showed the
following: helix 20.2, β 0, turn 34.5, and random 45.2 (24). The
pH increase from 4.0 to 5.0 dramatically changed the spectrum
shape with a negative peak at 216 nm that is typical of a
polypeptide-containing β-structure. A further pH increase
caused a reduction of CD activity without substantially chang-
ing the spectrum shape.

Thioflavin T binds rapidly to amyloid fibrils, and this process
is accompanied by a dramatic increase of fluorescence at 485
nm upon excitation at 455 nm (53–55). We examined ThT
fluorescence emission in the presence of the W7FW14F apo-
myoglobin mutant at pH values from 4.0 to 7.0 (Fig. 3, lower
panel). ThT fluorescence was unchanged at pH values between
4.0 and 6.0, whereas it dramatically increased between pH 6.0
and 6.5. There was no further increase at higher pH values. We
then compared the pH dependence of ANS emission and CD
activity with changes in ThT emission (Fig. 3, upper and mid-
dle panels). Decreased ANS fluorescence and CD activity
clearly preceded increased ThT fluorescence, thus indicating
that the structural changes probed by ANS and CD are not
concomitant with those probed by ThT.

We next monitored the ANS emission spectra at various pH
values over time. There were no changes in the protein samples
at pH values below 6.5 (data not shown). At pH 6.5, ANS
fluorescence was dramatically time-dependent (Fig. 4). At day
4, ANS emission was enhanced and there was a concomitant blue shift of the emission maximum from 520 to 480 nm, which indicates re-binding of the probe to the protein. The increase of fluorescence continued throughout the 14 days of the experiment. As in the case of ANS, ThT fluorescence was time-dependent only above pH 6.5 (Fig. 5). We also monitored CD activity over time, but the measurements were not reliable because the protein samples had become turbid. However, the shape of the CD spectra at various time points did not differ from that recorded immediately after raising the pH from 4.0 to 6.5.

We monitored the morphology of the aggregates formed by W7FW14F at pH 6.5 using electron microscopy-negative staining (Fig. 6). Amorphous granular aggregates constituted by spherical particles between 4.0 and 7.0 nm in diameter predominated immediately after neutralization (Fig. 6A). 12 h later, larger globular aggregates between 20 and 50 nm in diameter appeared together with a few early pre-fibril formations (Fig. 6B). Two days after neutralization, pre-fibrillar elements emanated from small globular and pore-like structures (Fig. 6C). At day 4, there were very few aggregates, whereas the number of fibrils had increased and fibril extension continued for at least 1 week (Fig. 6, D and E). The amyloid fibrillar nature of these species was confirmed using Congo Red birefringence as reported elsewhere (24). Branching of fibrils was observed at 2 and 3 weeks after neutralization (Fig. 6, F and G, respectively). However, in some instances, the images may represent overlapping fibrils. The distribution of the various aggregated species at different time points is reported in Table I.

We compared the effect of the structural states formed during W7FW14F fibrillogenesis, i.e. amorphous aggregates and mature fibrils, on cultured mouse fibroblasts versus wild-type apomyoglobin. The cytotoxic effect of protein aggregates was determined by measuring cell viability, membrane permeability, and cytoskeleton changes that affected cell shape. The MTT assay is a rapid and sensitive indicator of amyloid-mediated toxicity (56–59). Changes in reduced MTT concentrations reflect alterations in cellular reductase activity and in the endocytosis and exocytosis pathways (59–61). Early W7FW14F aggregates significantly decreased levels of reduced MTT, whereas mature fibrils and wild-type apomyoglobin had no effect (Fig. 7). Trypan blue internalization showed that exposure of the cell culture to granular aggregates does not induce cell death (data not shown).

Alterations of plasma membrane permeability are usually identified from the uptake or release of molecules endowed with light-absorbing or fluorescent properties. Thus, viable
and damaged cells can be discriminated by differential staining. Consequently, we examined PI uptake by cultured mouse fibroblasts exposed to early pre-fibrillar aggregates and to mature W7FW14F fibrils (Fig. 8). Most cells exposed to pre-fibrillar aggregates were stained with PI. The extent of membrane damage induced by pre-fibrillar aggregates, measured by the PI-positive cells/total cells ratio, was comparable to that of TBHP-treated control fibroblasts, i.e. 0.94 and 0.80, respectively. Conversely, fibroblasts exposed to mature fibrils contained very few PI-positive elements (0.5 PI-positive/total cells), which is comparable to data obtained with the control cell culture (0.4 ratio of PI-positive/total cells).

We examined cell shape by phalloidin staining. As shown in Fig. 9, the ratio between normal and necrotic cells ranged from a very low value (0.8) for TBHP-treated cells, which indicates a high percentage of necrotic cells, to much higher values for control cells (4.8) and cells exposed to mature fibrils (3.96). Surprisingly, the cell culture treated with pre-fibrillar aggregates had a rather high ratio (3.1), which indicates a low percentage of necrotic cells.

**DISCUSSION**

Amyloid fibril formation is a nucleation-dependent process in which non-native, partially folded protein molecules gradually cluster together to form a nucleus. This is followed by fibril extension in which molecules are added to the nucleus. Interestingly, pre-formed fibrils accelerate this process (62–64). Increasing evidence suggests that a critical event in the amyloidogenesis of most proteins and peptides is the transition of the native conformer to a partially folded or fully unfolded conformation that has increased propensities for aggregation (65–68). However, the conformational species underlying amyloid fibril formation is still a matter of debate. Rigorous biophysical studies require protein preparations free of fibrillar material, which precludes studies of early fibrillogensis. In this respect, tryptophanyl-substituted apomyoglobin is an appropriate model of fibrillogenesis because it is soluble at a pH below 6.0 and aggregates only at pH 6.5 or higher (24).

The relationship between pH and ANS fluorescence observed by us shows that, in the case of the apomyoglobin mutant

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### Table I

| Time  | Granular aggregates | Globular aggregates | Pre-fibrils | Short fibrils | Elongated fibrils | Branching fibrils |
|-------|---------------------|---------------------|------------|--------------|-----------------|-----------------|
| 2 h   | 97                  | 2.5                 | 0.5        |              |                 |                 |
| 4 h   | 80                  | 7.5                 | 2.5        |              |                 |                 |
| 12 h  | 60                  | 27                  | 9.5        | 3.5          |                 |                 |
| 24 h  | 40                  | 29                  | 14         | 17           |                 |                 |
| 2 days| 29                  | 35                  | 15         | 21           |                 |                 |
| 4 days| 16                  | 19                  | 16         | 42           | 4               | 3               |
| 7 days| 9                   | 10                  | 7          | 34           | 28              | 12              |
| 14 days| 5                   | 5                   | 3          | 10           | 42              | 35              |
| 20 days| 4                   | 3.5                 | 2          | 7.5          | 39              | 44              |
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W7FW14F, the nucleation process is preceded by the release of ANS molecules. In fact, on increasing pH from 4.0 to 6.5, ANS fluorescence not only decreased but shifted from 478 to 520 nm, which is practically identical with that of the free-in-water dye. It is noteworthy that the wild-type protein showed a remarkable increase of ANS emission and a concomitant blue shift of the emission maximum. This finding indicates that ANS binds to the newly formed particles. Electron microscopy results with the W7FW14F apomyoglobin mutant confirm that pre-fibrillar amyloid aggregates of proteins unrelated to amyloid diseases are highly cytotoxic, whereas mature fibrils are harmless.

It is has been suggested that early aggregates exert their cytotoxic effect by interacting through their large exposed hydrophobic surface with cellular and membrane components. In support of this hypothesis, our PI-staining data show that protofibrillar intermediates formed during W7FW14F apomyoglobin fibrillogenesis consistently and significantly alter the permeability of cultured fibroblasts. It is noteworthy that Aβ peptides form ion channels in lipid bilayers, liposomes, neurons, and endothelial cells (72, 73). Consequently, it is conceivable that channel formation is a critical factor in determining cytotoxic effects also in the case of the W7FW14F apomyoglobin mutant. However, because the membrane damage does not seem to affect immediately the overall morphology of cells, we suggest that gain-of-function mechanisms trigger a late effect on cell viability.

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