Role of the C-Terminal 28 Residues of \( \beta_2 \)-Microglobulin in Amyloid Fibril Formation†

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ABSTRACT: \( \beta_2 \)-microglobulin (\( \beta_2 \)m) is the major protein component of the fibrillar amyloid deposits isolated from patients diagnosed with dialysis-related amyloidosis (DRA). While investigating the molecular mechanism of amyloid fibril formation by \( \beta_2 \)m, we found that the \( \beta_2 \)m C-terminal peptide of 28 residues (c\( \beta_2 \)m) itself forms amyloid fibrils. When viewed by electron microscopy, c\( \beta_2 \)m aggregates appear as elongated unbranched fibers, the morphology typical for amyloids. C\( \beta_2 \)m fibers stain with Congo red and show apple-green birefringence in polarized light, characteristic of amyloids. The observation that the \( \beta_2 \)m C-terminal fragment readily forms amyloid fibrils implies that \( \beta_2 \)m amyloid fibril formation proceeds via interactions of amyloid forming segments, which become exposed when the \( \beta_2 \)m subunit is partially unfolded.

Amyloidosis is the process of protein aggregation associated with a variety of human degenerative diseases (1). These pathological amyloid aggregates consist of elongated fibers, resistant to disruption. The components of any given amyloid fibril are primarily of a single-type protein and may form from wild-type, mutant, or truncated proteins. Similar fibrils can be formed in vitro from oligopeptides and denatured proteins (2–4).

\( \beta_2 \)-microglobulin (\( \beta_2 \)m)1 has been used as a model system to study the mechanism of amyloid fibril formation (5–8). Normally, \( \beta_2 \)m is the light chain of the type I major histocompatibility complex responsible for the presentation of peptides to the immune system. In its native state, \( \beta_2 \)m adopts a typical immunoglobulin fold consisting of seven \( \alpha \)-strands organized into two \( \beta \)-sheets connected by a single disulfide bridge (9) (Figure 1a). In addition, \( \beta_2 \)m has a pathological role. It was discovered to be the major component of the amyloid deposits taken from patients diagnosed with dialysis-related amyloidosis (DRA), a serious complication of long-term hemodialysis (10, 11).

Different segments of \( \beta_2 \)m were isolated from ex vivo amyloid fibrils consisting of \( \beta_2 \)m truncated at residues Ile7, Ser11, Gly18, Leu87 (12), and Ser20 (13). Also, two amyloid forming segments of \( \beta_2 \)m were identified to be the Ser20 to Lys41 peptide (14) and the Asp59 to Ala79 peptide (15).

In this paper, we show that yet another \( \beta_2 \)m segment (c\( \beta_2 \)m) encompassing the F and G \( \alpha \)-strands (Figure 1; \( \beta_2 \)m residues 72–99) aggregates to form fibrils, which also have properties common to amyloids.

EXPERIMENTAL PROCEDURES

\( \beta_2 \)m and c\( \beta_2 \)m Overexpression and Purification. The gene encoding \( \beta_2 \)m was subcloned from the pALUW31 vector into pET3a (Novagen). Four \( \beta_2 \)m segments (\( \beta_2 \)m residues

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1 Abbreviations: DRA, dialysis-related amyloidosis; \( \beta_2 \)m, \( \beta_2 \)-microglobulin; c\( \beta_2 \)m, a \( \beta_2 \)m segment spanning residues 72–99; GST, glutathione-S-transferase; CD, circular dichroism; EM, electron microscopy.

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1–32, 33–71, 72–99, and 1–71) were cloned into the pGEX-4T vector (Amersham Pharmacia) to be expressed as glutathione-S-transferase (GST) fusion proteins. The plasmids were transformed into *Escherichia coli* BL21(DE3) (Novagen). The cells were grown at 37 °C in LB media with 100 μg/mL ampicillin (Fischer) and induced at OD₆₀₀ 0.6 with 0.5 mM isopropyl β-d-thiogalactopyranoside (Fischer) for 3 h to produce protein.

β2m was refolded using the protocol described in ref 16. After refolding, β2m was purified on a size-exclusion silica G3000 column (Toso Haas). Cβ2m fused to GST was immobilized onto glutathione sepharose 4B (Amersham Pharmacia) and cleaved with thrombin (Amersham Pharmacia; 40 units per 1 mL bed volume of glutathione sepharose; 16 h at room temperature). The cleaved cβ2m was further purified on a size-exclusion G3000 column (Toso Haas).

Electron Microscopy. Specimens were applied directly onto hydrophilic carbon-coated parlodion support films mounted on copper grids, allowed to adhere for 2 min, rinsed with distilled water, and stained with 1% uranyl acetate (Ted Pella, Inc.). Grids were examined in a Hitachi H-7000 electron microscope at an accelerating voltage of 75 kV.

Circular Dichroism (CD). CD experiments were performed on a Jasco J-715 spectropolarimeter. Samples of 45 μM cβ2m were used to record spectra at room temperature in a 1 mm path-length cell with a 0.5 nm bandwidth, 0.5 nm resolution, 20 nm/min interval speed, and 8 s response time.

Congo Red Binding Assays. For birefringence analysis, the fibers were incubated with 120 μM Congo red in 150 mM NaCl, 10 mM HEPES pH 8.0 for 30 min, sedimented by centrifugation at 20 000g for 1 min, washed three times with water, resuspended with 10 μL of water, and dried on a glass slide to be examined by a light microscope. Spectroscopic assays were done as described in ref 17.

Polymerization and Dissociation Assays. Both assays were done at 37 °C. Fibrils were formed with shaking after dissolving 180 μM lyophilized protein in 1.5 M NaCl, 25 mM phosphate buffer, pH 2.0. For fibril dissociation studies, preformed fibers (180 μM total protein amount) were spun at 20 000g for 5 min and resuspended with 125 mM NaCl, 25 mM HEPES, pH 7.4.

RESULTS

Identifying cβ2m as an Amyloid Forming Peptide. Initially, we partitioned the β2m sequence into three segments (1–32; 33–71; 72–99) each about 30 residues in length. All segments when fused to GST were soluble, but we were able to cleave only the cβ2m segment (β2m residues 72–99).

Then, we tested if the β2m 1–71 segment could be purified, but it was expressed as inclusion bodies both when fused to GST and when not fused to GST. Consequently, we were not able to investigate if the 1–32, 33–71, and 1–71 segments could aggregate into amyloid fibrils. In contrast, cβ2m fused to GST was soluble and easily cleaved. After screening various conditions, we found that cβ2m forms fibrils at higher salt concentrations (1.0 M NaCl; 25 mM phosphate, pH 2.0) and a higher concentration (160 μM) than β2m. For comparison, β2m forms fibrils at concentrations as low as 10 μM and in solutions with as little as 50 mM NaCl (18).

Characterization of cβ2m Fibrils. Cβ2m fibrils are elongated and unbranched, morphology typical for amyloids. On the basis of their diameters, fibrils of cβ2m can be divided into two groups: thin (T) (5.0 ± 0.6 nm) and wide (W) (11.5 ± 1.2 nm), Figure 2a. The thin fibrils may intertwine to form the thicker fibrils. β2m fibrils can also be classified into two groups by their diameters: 7.2 ± 0.6 nm thin (T) and 13.1 ± 0.7 nm wide (W). Notice that cβ2m fibrils are straighter than β2m fibrils. However, both β2m and cβ2m fibrils are intertwined with alternating thinner and wider regions (arrows marked with *).

In neutral pH buffer, cβ2m exists primarily as a coil (Figure 4), a conformation different from the mostly β-sheet β2m. However, as typical for amyloids, an increase in β-sheet...
content is observed in the CD spectrum taken immediately after the lyophilized cβ2m is dissolved into buffer promoting fibril formation (2.5 M NaCl, 25 mM phosphate, pH 2.0; Figure 4). This increase in β-sheet content becomes even more pronounced after one week of incubation at 37 °C (Figure 4). Thus, the estimated β-sheet content of the week-old cβ2m is about 37%, opposed to 20% for cβ2m stored at neutral pH. There is a minimum at 218–220 nm, characteristic for the β-sheet, in the spectrum of a week-old cβ2m sample incubated in a high salt and low pH solution.

Monitoring cβ2m Fibril Formation. Fibrils form upon transfer of β2m into a low pH buffer containing high salt (1.5 M NaCl, pH 2.0), and there is no lag phase in the aggregate formation (open rhombs, Figure 5). Similarly, McParland et al. (18) and Hong et al. (21) observed that β2m forms fibrils rapidly without a lag phase. In contrast, cβ2m incubated at the same conditions does not appear to aggregate until more than 90 min after the initiation of the reaction (inset and filled circles, Figure 5). Cβ2m fibers form with a lag time of 90 min, as opposed to wild-type β2m, which aggregates immediately upon transfer into 1.5 M NaCl and pH 2.0 buffer.

Stability of β2m and cβ2m Fibrils in Physiological Buffers. After transfer into pH 7.4 buffer, most of the cβ2m fibrils/aggregates dissociate in the first 30 min, as judged by light scattering at 340 nm. The inset gives the time course of cβ2m aggregation. Cβ2m fibers form with a lag time of 90 min, as opposed to wild-type β2m, which aggregates immediately upon transfer into 1.5 M NaCl and pH 2.0 buffer.

Electron micrographs of cβ2m and β2m samples incubated for 3 days at pH 7.4 are shown in Figure 7. A sheetlike
Figures 2 and 3 show that cβ2m aggregates form long fibrils and that these fibers bind Congo red, both properties commonly observed in amyloids. The importance of cβ2m in the context of β2m fibril formation is supported by the finding that an antibody raised against β2m residues 92–99 inhibits fibril formation in vitro (22). In contrast, antibodies against 20–41 and 63–75 do not inhibit fibril formation (22), which suggests that cβ2m (β2m residues 72–99) is the determinant of the propensity of β2m to aggregate into amyloid fibrils.

Our observation that the F and G β-strands (cβ2m; Figure 1) are sufficient for amyloid fibril formation implies their importance in β2m fibril formation. Jones et al. (15) showed that neither the F β-strand nor the G β-strand forms fibrils. Therefore, only when F and G β-strands are fused together can they form fibrils. In conditions favorable for fibril formation, the G β-strand, but not the F β-strand, is solvent exposed (7, 8). Thus, the fiber formation may be due to exposure of residues in the F and G β-strand connecting segment (His84 to Pro90).

Besides cβ2m, segments β2m 20–41 (14), 59–71 (15), and 59–79 (15) were found by others to form fibrils. To compare these four amyloid forming segments of β2m, we examined properties that have been found by others to correlate with the ability of the peptide to form amyloid fibrils, such as hydrophobicity (23), β-sheet propensity (24), the arrangement of the hydrophobic and hydrophilic residues (25), the net charge (26), and the number of aromatic residues (27). All these segments and the full-length β2m have similar propensities to form a β-sheet (28, 29). Depending on the hydrophobicity scale, we found that each segment has at least a four-residue-long segment with alternating hydrophobic/hydrophilic residues (30, 31). The 21–40 segment (14) has the longest segment (six residues—Kyte and Doolittle scale (30) and seven residues—Eisenberg et al. scale (31)) of alternating hydrophobic/hydrophilic residues. Other than alternating hydrophobic/hydrophilic residues, there seems to be little in common among all four segments.

Cβ2m fibrils form less efficiently than β2m fibrils (Figure 5). One explanation of the slower fibril growth is that β2m nucleates faster than cβ2m, which might be due to other amyloid determining factors, such as a missing amyloid forming segment from the cβ2m peptide (14, 15). An alternative explanation is that prior to or during nucleation, cβ2m undergoes a slow transition from coil to β-sheet (Figure 4). In contrast, β2m has high β-sheet content in its native state (Figure 1), and there is no evidence of conformational changes in the protein subunits during fibril assembly. A similar conformational transition was proposed to be one of the contributing factors to the lag phase of the Alzheimer’s Aβ fibril formation (32).

Fibrils of the cβ2m protein dissociate faster than β2m fibrils (Figure 6). This may be explained by the lack of stabilizing interactions from the rest of the protein. A number of different segments of β2m have been proposed to be important in the fibril assembly including the B and C β-strands (14), the edge of strand D (33), and the E β-strand...
(15) (Figure 1). These segments of β2m, in addition to elements of αβ2m, may participate in β2m fibril formation.

The knowledge of the β2m regions that are involved in fibril formation may aid in the design of compounds that could then be inhibitors of amyloid formation. A similar strategy was successfully used to delay the onset of Alzheimer’s disease in mice (34), where antibodies raised against a fragment of Aβ (residues Phe4 to Tyr10) have strong antifibrillogenic properties.

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REFERENCES