Structure, Folding Dynamics, and Amyloidogenesis of D76N β2-Microglobulin

**ROLES OF SHEAR FLOW, HYDROPHOBIC SURFACES, AND α-CRYSTALLIN**

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**Background:** We recently discovered the first naturally human β2-microglobulin variant, D76N, as an amyloidogenic protein. The α-crystallin chaperone inhibits variant-mediated co-aggregation of wild type β2-microglobulin.

**Results:** Fluid flow on hydrophobic surfaces triggers its amyloid fibrillogenesis. The α-crystallin chaperone inhibits variant-mediated co-aggregation of wild type β2-microglobulin.

**Conclusion:** These mechanisms likely reflect in vivo amyloidogenesis by globular proteins in general.

**Significance:** Our results elucidate the molecular pathophysiology of amyloid deposition.

Systemic amyloidosis is a fatal disease caused by misfolding of native globular proteins, which then aggregate extracellularly as insoluble fibrils, damaging the structure and function of affected organs. The formation of amyloid fibrils in vitro is poorly understood. We recently identified the first naturally occurring structural variant, D76N, of human β2-microglobulin (β2m), the ubiquitous light chain of class I major histocompatibility antigens, as the amyloid fibril protein in a family with a new phenotype of late onset fatal hereditary systemic amyloidosis. Here we show that, uniquely, D76N β2m readily forms amyloid fibrils in vitro under physiological extracellular conditions. The globular native fold transition to the fibrillar state is primed by exposure to a hydrophobic-hydrophilic interface under physiological ion strength shear flow. Wild type β2m is recruited by the variant into amyloid fibrils in vitro but is absent from amyloid deposited in vivo. This may be because, as we show here, such recruitment is inhibited by chaperone activity. Our results suggest general mechanistic principles of in vivo amyloid fibrillogenesis by globular proteins, a previously obscure process. Elucidation of this crucial causative event in clinical amyloidosis should also help to explain the hitherto mysterious timing and location of amyloid deposition.

β2m (mass, 11,729 Da), the invariant light chain of the human HLA class I complex, is produced at ~200 mg/day in adults and is cleared only via the kidney. In patients with end stage renal failure on dialysis, the plasma concentration of β2m therefore rises from the normal 1–2 mg/liter to persistently raised values of ~50–70 mg/liter, leading to the serious and intractable condition of dialysis-related amyloidosis with β2m amyloid fibrils deposited in bones and joints, causing painful arthropathy, bone cysts, pathological fractures, and rarely visceral β2m amyloid deposits. The normal structure and function of β2m are well characterized, and although wild type β2m is poorly amyloidogenic in vitro, its fibrillogenesis and its tissue-specific deposition have been intensively investigated (1). Despite much progress, there is neither general agreement about the underlying molecular mechanisms nor an understanding of the forces involved in vivo during the destabilization and subsequent amyloid aggregation of either β2m or any of the other natively folded globular proteins that form amyloid fibrils.

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‡‡ This abbreviations used are: β2m, β2-microglobulin; ΔG, free energy change; U, unfolded state; I, intermediate state; N, native state; subscript C, cis; His11-Pr0; subscript T, trans-His-Pro; T5, transition state; ΔG*, free energy barrier; τm, mixing time for TOCSY, NOESY, and ROESY; BLU-Tramp, Bio-physics Laboratory University of Udine temperature ramp; Gdn-HCl, guanidine hydrochloride; THt, thioldavitin T; AFM, atomic force microscopy; ΔN6 β2m, truncated β2m isof orm lacking the 6 N-terminal residues; TOCSY, total correlation spectroscopy; ROESY, rotating frame nuclear Overhauser enhancement spectroscopy.
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in disease. We later reported (2) the first naturally occurring structural variant of β₂m, D76N, discovered in members of a French family who developed progressive bowel dysfunction with extensive visceral β₂m amyloid deposits despite normal renal function and normal circulating β₂m concentrations and with none of the osteoarticular deposits characteristic of dialysis-related amyloidosis. Here we elucidate in detail the biophysical parameters of amyloid fibrillogenesis by this uniquely tractable protein and develop a paradigm that could be applicable generally to the in vivo pathophysiology of amyloidogenesis by the whole range of globular proteins that cause clinically significant systemic amyloidosis.

EXPERIMENTAL PROCEDURES

Production of Recombinant Proteins—Recombinant wild type and variant β₂m were expressed and purified as described previously (2).

Differential Scanning Calorimetry—Differential scanning calorimetry experiments were carried out with a VP-DSC instrument (MicroCal, Northampton, MA) with protein at 0.5 mg/ml in 25 mM sodium phosphate buffer, pH 7.4 and scans from 10 to 90 °C at a scanning rate of 60 °C/h. The reversibility of protein denaturation was assessed by repeating heating and cooling cycles. After normalization and base-line subtraction, the thermal unfolding curves were analyzed using MicroCal Origin 7.0 software with a two-state unfolding model.

Equilibrium Denaturation Experiments and Folding Kinetics—Guanidine hydrochloride (Gdn-HCl) equilibrium denaturation, unfolding, and refolding kinetics were performed as described previously (3). All experiments were carried out at 30 °C in 20 mM sodium phosphate buffer, pH 7.4 at a 0.02 mg/ml final protein concentration. Refolding of acid-denatured protein and double jump experiments were performed at 4 °C as described previously (4).

Energy Diagram—All free energy changes (ΔG) were determined in J mol⁻¹ and then converted into kcal mol⁻¹ through-out we use the following abbreviations: U, unfolded state; I, intermediate state; N, native state; subscript C, cis-His³¹-Pro³²; subscript T, trans-His³¹-Pro³²; TS, transition state. The Uᵣ state was arbitrarily given a free energy (G) of 0 J mol⁻¹ and was considered as a reference for all reported ΔG values. The ΔG from the Uᵣ to the Nᵣ state was determined from Gdn-HCl unfolding equilibrium curves as reported (3). The ΔG from the Uᵣ to the Nᵣ states was determined using ΔG = –RTln(kᵣ/kᵣ) where R is the universal gas constant, T is the absolute temperature, and kᵣ and kᵣ are the rate constants (in s⁻¹ units) for unfolding and for the slow phase of folding, respectively, extrapolated to the absence of Gdn-HCl. The ΔG from the Iᵣ to the Uᵣ states was determined by plotting the fluorescence of the Iᵣ state (corresponding to the fluorescence at time 0 of a kinetic trace of folding) against Gdn-HCl concentration and by plotting the fluorescence of the Uᵣ state against Gdn-HCl concentration (in the latter case, the values at low Gdn-HCl concentration were obtained by linear extrapolation from the values at high Gdn-HCl concentration). The fluorescence of the Iᵣ state decreased with increasing Gdn-HCl concentration until it approached the fluorescence of the Uᵣ state, thus providing an approximate measure of the conformational stability of the Iᵣ state relative to Uᵣ. The ΔG from the Iᵣ to the TSᵣ state was determined using ΔGᵣ = –RTln(kᵣ/kᵣ) where ΔGᵣ is the free energy barrier, kᵣ is the rate constant for the fast phase of unfolding, and kᵣ is the pre-exponential term taken as 4.8 × 10⁸ s⁻¹ as reported (5, 6). Similarly, the ΔG values from Nᵣ to TS₂ and from Nᵣ to TS₂ were determined using ΔGᵣ = –RTln(kᵣ/kᵣ) and ΔGᵣ = –RTln(kᵣ/kᵣ), respectively, where kᵣ and kᵣ are the rate constants for the slow phase of folding and for unfolding, respectively. The ΔG values from Uᵣ to TS₂ and from Iᵣ to TS₁ were not determined. All other ΔG values not explicitly mentioned in the study can be determined by arithmetic linear combination of the ΔG parameters described above.

NMR Measurements—NMR spectra were obtained at 500.13 MHz with a Bruker Avance 500 spectrometer on 0.1–0.3 mM protein samples dissolved in H₂O/D₂O 90:10 or 95:5 with 20–70 mM sodium phosphate buffer and pH* (pH meter reading without isotope effect correction) in the range 6.6–7.2. Unlabeled and uniformly¹⁵N- or¹⁵N,¹³C-labeled protein samples, expressed as described previously (2), were used. The spectra were collected mostly at 25 °C with only a few experiments obtained also at 30 or 37 °C. Homonuclear two-dimensional TOCSY (7), NOESY (8), and ROESY (9) spectra were acquired. The adopted experimental schemes included solvent suppression by WATERGATE (10) and excitation sculpting (11); 1-s steady state recovery time; mixing times (tᵉ) of 40–50 ms for TOCSY, 100–150 ms for NOESY, and 100 ms for ROESY; τ₁ quadrature detection by time-proportional phase incrementation (12); and gradient-assisted coherence selection (echo-antiecho) (13). The spin-lock mixing of the TOCSY and ROESY experiments was obtained with MLEV17 (14) pulse trains or single long pulse, respectively, at γB₂/2π ~ 0.1 kHz (TOCSY) and ~ 2.5 or 5 kHz (ROESY). The acquisitions were performed over a spectral width of 8012.82 Hz in both dimensions with matrix size of 1024–2048 points in τ₂ and 256–400 points in τ₁ and 32–64 scans per each τ₁ free induction decay. The BLUU- Tramp experiments were conducted using the procedure described previously over the temperature range 22–42 °C (15, 16). Measurements were performed on samples that had undergone complete deuterium substitution for hydrogen with two cycles of exchange at 4 °C in D₂O containing 10 mM NH₄HCO₃ and subsequent lyophilization. The solvent for the back-exchange was used for the preliminary thinning to enable quick start after dissolving the protein (~5 min dead time before starting the acquisition). The ¹⁵N[¹H] NOE data were obtained at 25 and 37 °C by standard sequence using a 3-s relaxation interval. The spectra with (NOE) and without (no NOE) proton saturation were acquired in an interleaved manner.

Three-dimensional HNCA (17–19) and HNCOCA (19, 20) were typically acquired with 64 scans and 64 × 40 × 1024 data points in τ₁ (¹³C), τ₂ (¹⁵N), and τ₃ (¹H), respectively, over spectral windows of 40, 33.5, and 16 ppm for ¹³C, ¹⁵N, and ¹H, respectively. HNCO spectra (17, 19) were acquired using 128 × 40 × 1500 data points and only 32 scans for each τ₁ × τ₂ experiment over spectral windows of 22.1 (¹³C), 33.5 (¹⁵N), and 14 (¹H) ppm. Processing of three-dimensional data ended up with real matrices of 512 × 256 × 1024 points in F₁, F₂, and F₃, respectively, except for the HNCO spectra where the carbon
dimension (F1) was limited to 256 points. All data, except those from BLUU-Tramp, were processed with Topspin (Bruker Bio- 
spin) and analyzed with Sparky (T. D. Goddard and D. G. 
Kneller, University of California). BLUU-Tramp data were pro-
cessed using NMRPipe and analyzed by NMRView (21).

Electrostatic Calculations—For the calculation of both sur-
face potential and pK_a shifts, we used the recently developed 
program BLUESES (22) available also as a server utility (23). For 
the calculation of isopotential surfaces, we used the program 
UHBD, and we displayed the isopotential surfaces using the

program VMD. To assess effects that could arise from slightl 
different arrangement in the structural models used for cal-

culation, an alternative structure for the D76N variant was gener-
ated using the program SCWRL4.0 by alternative schemes: (i) 
only the side chain of the mutated residue is allowed to change 
conformation, and (ii) only the side chain of the mutated

residues and contacting residues are allowed to change con-
formation. Despite numerical differences, the results from 
the homology-modeled structures are in agreement with the

experimental data reported in the study, confirming that the 
effects are mainly due to the mutation rather than other

minor conformational differences.

Molecular Dynamics Simulations—The force field used in 
the simulations was CHARMM v.27 (24) with the CMAP (two-
dimensional dihedral energy grid correction map approach) 
correction (25). The minimized system was further relaxed, 
keeping the solute molecules (including ions) fixed, by molecu-

lar dynamics simulation. The system was heated to 47 °C in 2

ps, and a further 18-ns simulation was run to let water mol-
ecules reorient, consistent with the average lifetime of a hydro-
gen bond in water of 1−2 ps (26). The system without restraints 
on solute molecules was energy-minimized by 300 conjugate 
gradient minimization steps. The system was then heated to 
47 °C in 2 ps, and a further 3.0-ns simulation was run to reach 
equilibrium. The simulations lasted 250 ns, and snapshots were 
saved every 0.1 ns. All molecular dynamics simulations were 
performed in the NPT ensemble using the Nosé-Hoover Lan-
gevin piston method (27, 28). The Langevin damping coeffi-
cient for temperature control was 10 ps \(^{-1}\). For all simulations, 
the size of the box was fluctuating around its average value 
within fractions of Å.

H-bond Network Analysis—The mutation D76N within the

β_m sequence is likely to affect the molecular hydrogen bond

network. To identify indirect effects of the mutation, 2500 
networks of hydrogen bonds. On the other hand, it 
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![Diagram](https://example.com/diagram.png)

**RESULTS**

**Structural Basis of Amyloidogenicity of D76N β₂m Variant—**

The Asp⁷⁶ mutation to Asn substantially destabilizes β₂m, and differential scanning calorimetry reveals a melting temperature 10.26 °C lower than that of the wild type, corresponding to mean ± S.D. (n = 3) ΔH values of 63.9 ± 1.2 and 86.2 ± 1.5 kcal mol⁻¹ for D76N and wild type β₂m, respectively. The calculated ΔG values (46) for unfolding of the D76N variant at 37 and at 30 °C were thus 2.73 and 2.86 kcal mol⁻¹ lower, respectively, than for wild type β₂m.

The complex folding mechanisms shared by wild type and D76N β₂m involve multiple intermediates and parallel folding routes with two major exponential phases observed during refolding (3): an initial fast phase that was 3 times slower for the D76N variant at 0.2 M Gdn-HCl and a subsequent prolyl trans-cis isomerization-dependent slow phase (4) (data not shown). Unresolved fluorescence changes take place in the dead time of the experiments, showing that a burst phase occurs on the submillisecond time scale with the same amplitude for both pro-
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| TABLE 1 | Thermodynamic and kinetic values |
|---------|----------------------------------|
| All values are mean ± S.D. (n = 3) Cm, midpoint concentration of Gdn-HCl; ΔG°(H2O) (kcal mol⁻¹), free energy of unfolding in the absence of denaturant; m (kcal mol⁻¹ M⁻¹), dependence of ΔG°(H2O) on denaturant concentration; k (s⁻¹), value of rate constant extrapolated to zero denaturant concentration. These values were used to calculate the free energy changes shown in Fig. 1. In addition, ΔG values from the I1 to the U2 states, 3.25 ± 2.3 and 0.55 ± 0.3 kcal mol⁻¹ for wild type and D76N β2m, respectively, were determined with the procedure described under “Experimental Procedures.” |

| Gdn-HCl denaturation at equilibrium | Cm | ΔG°(H2O) | m |
|------------------------------------|----|----------|---|
| Wild type                          | 2.0 (± 0.2) | 5.7 (± 0.4) | 2.73 (± 0.7) |
| D76N                               | 1.15 (± 0.1) | 3.0 (± 0.15) | 2.64 (± 0.3) |

Chevron plots

|                     | Unfolding | Fast phase | Slow phase |
|---------------------|-----------|------------|------------|
|                     | k₀         | mᵤ         | k₀ | mfast | k₀ | mslow |
| Wild type           | 1.52 (± 0.2) x 10⁴ | 1.34 (± 0.17) | 5.17 (± 1.1) | -2.37 (± 0.47) | 3.01 (± 1.3) x 10⁻³ | 0.076 (± 0.03) |
| D76N                | 6.52 (± 1.3) x 10⁴ | 1.20 (± 0.24) | 1.78 (± 0.37) | -2.26 (± 0.50) | 1.95 (± 0.8) x 10⁻³ | 0.070 (± 0.03) |

Proteins. In contrast to folding, unfolding appears to be a monophasic process. D76N β2m unfolded faster than wild type with a 2-fold increase at 5.4 M Gdn-HCl. The free energy diagrams of (un)folding of the two proteins at pH 7.4, 30 °C in the absence of any denaturant (Fig. 1) derived from combined equilibrium unfolding and kinetic data (Table 1) show that the Nc native state of the variant is (mean ± S.D.; n = 3) 2.7 ± 0.25 kcal mol⁻¹ less stable than that of native wild type β2m, thereby promoting the population of partially folded, typically amyloidogenic states of the variant (47). The native-like state of β2m with the His₃¹-Pro₃² peptide bond in a non-native trans configuration (Nₚ), previously shown to be highly related to the amyloidogenic pathway of wild type β2m and populated at (mean ± S.D.; n = 3) 4.8 ± 3.0% at equilibrium, was remarkably more abundant in the D76N variant (mean ± S.D.; n = 3) at ~25 ± 9%.

Despite the notably reduced stability of D76N β2m, its solution structure did not differ significantly from wild type. Other than obvious changes at the mutation site and neighboring residues, the NMR signature of the variant at 25 °C was nearly the same as that of the wild type protein (Fig. 2).

Unequivocal assignment at 11.7 teslas (500-MHz ¹H frequency) could be obtained for 85% of the backbone ¹H, ¹⁵N, and ¹³C nuclei, but no major chemical shift difference, that is no major structure deviation, was observed compared with the wild type protein, consistent with the crystallographic findings (2). For instance, the average difference in deviation from random coil values of the Hν chemical shifts between D76N and wild type β2m, Δ(Δδ), is ~0.012 ± 0.017 ppm with a value of ~0.01 ppm even at the mutation site. This reflects essentially invariant local secondary and tertiary structure. One relevant effect related to the mutation is that the same chemical shifts are observed for the carboxamide resonances of both Asn₄² and Asn₇⁶ (Fig. 3A), suggesting that the two side chains share the same chemical environment, consistent with the occurrence of reciprocal H-bonds between the carboxamides. The presence of an interaction between the side chains of residues 42 and 76 in the variant protein is supported by several lines of evidence including two-dimensional ¹H NOESY (Fig. 3B) and ROESY cross-peaks (data not shown).

In the wild type protein, the side chain amide of Asn₄² is H-bonded to the Asp₇⁶ side chain carboxylate that also forms salt bridges with the side chain ammoniums of Lys₄¹ and Lys₇⁶. The latter salt bridges contribute electrostatic stability and a computed pKₐ shift of ~1.2 units for the Asp₇⁶ carboxylate with respect to the standard value (Table 2). Despite the survival of the residue 42-76 interaction, the asparagine substitution for aspartate has a substantial impact in the variant protein. At pH
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![Image of NMR patterns of Asn42 and Asn76 carboxyamides.](image)

**TABLE 2**

| Residue | pK_a (limit) | pK_a WT β2-m | pK_a D76N β2-m |
|---------|-------------|--------------|---------------|
| Ile1    | 8.000       | 7.388        | 7.401         |
| His13   | 6.500       | 6.078        | 6.006         |
| Glu16   | 4.500       | 3.679        | 3.668         |
| His21   | 6.500       | 4.641        | 4.630         |
| Asp34   | 3.800       | 3.117        | 3.127         |
| Glu69   | 4.500       | 4.211        | 4.207         |
| Asp38   | 3.800       | 3.492        | 3.495         |
| Glu44   | 4.500       | 4.678        | 4.599         |
| Glu47   | 4.500       | 4.119        | 4.120         |
| Glu50   | 4.500       | 4.927        | 4.940         |
| His51   | 6.500       | 6.967        | 6.956         |
| Asp53   | 3.800       | 3.564        | 3.589         |
| Asp59   | 3.800       | 3.727        | 3.711         |
| Glu69   | 4.500       | 4.579        | 4.535         |
| Asp76   | 3.800       | 4.191        | 4.100         |
| Asp53   | 3.800       | 2.619        |               |
| Glu77   | 4.500       | 4.056        | 4.089         |
| His84   | 6.500       | 4.471        | 4.525         |
| Asp96   | 3.800       | 5.350        | 5.333         |
| Asp98   | 3.800       | 4.374        | 4.408         |
| Met99   | 3.200       | 4.863        | 4.836         |
| Lys101  | 10.500      | 11.173       | 10.470        |

The lower thermal resistance of D76N β2-m compared with wild type as shown by microcalorimetry was confirmed at single residue resolution by NMR using BLUUE-Trapmp (15, 16) even under critical conditions for the variant due to its instability (Fig. 5).

The lower thermal resistance of D76N β2-m can be explicitly tracked from 15N-[1H] NOE data measured at 25 and 37 °C, respectively (Fig. 6). These data indicate an extended loss of rigidity consistent with the H-bond analysis (Fig. 7). The average NOE values at 25 °C are consistent with a higher mobility of the variant compared with the wild type. The loss of rigidity observed when temperature increases at 37 °C is slightly more marked for the wild type protein (Fig. 6). However, the fluctuations of the thermally induced mobility increment that derive

7.0, the net charge of D76N β2-m is +0.3 units, whereas wild type carries an average −1.4 elementary charge (Fig. 4). This is consistent with a decreased intermolecular repulsion that facilitates aggregation at pH around neutrality. In addition, the lower stability of D76N compared with wild type as shown by microcalorimetry was confirmed at single residue resolution by NMR using BLUUE-Trapmp (15, 16) even under critical conditions for the variant due to its instability (Fig. 5).

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![Image of Electrostatic properties of wild type and D76N β2-m.](image)

Values were calculated with the software BLUUES (22) and compared with the corresponding random coil model values (limit). The pK_a values for Lys41 side chain, which establishes an electrostatic interaction with Asp76 in wild type β2-m, are also given. Asp38 is the lowest titrating residue with a pK_a of 2.6, corresponding to a pK_a shift of 1.2 compared with the free amino acid pK_a of 3.8. This shift is primarily due to the interaction with Lys41 and Lys75. Among other groups with a significant shift of pK_a are Asp21 (more exposed to the solvent than Asp38) and Asp34 (similar degree of exposure limitation and interactions as Asp38). Among histidine residues, His13 and His51 both have a pK_a shifted toward acidic pH by ~2 pK_a units.

Values for groups titrating below pH 10 in wild type and D76N β2-m are given in Table 2.

![Image of NMR patterns of Asn42 and Asn76 carboxyamides.](image)

**FIGURE 3.** NMR patterns of Asn42 and Asn76 carboxyamides. A, region of 15N-[1H] heteronuclear single quantum correlation spectrum of D76N β2-m. The boxed extremes of the black line correspond to the locations expected for the carboxamide resonances of Asn42 in the wild type protein spectrum; the dashed red lines cross through the NOE of the carboxamides NHs at 7.80 and 7.21 ppm that are attributed to Asn38 and Asn76 and tentatively assigned as H2m and H4m, respectively. To avoid notation crowding, the relevant connectivities are highlighted by numbers (1, Asn42 NH-Asn38 H2m 9.75 ppm and Asn76 NH-Asn42 H2m 9.75 ppm). The blue line indicates the new signals observed for D76N β2-m. The new signal pair can be attributed to Asn76 and Asn42 side chain amides from NOESY evidence. B, details from 1H two-dimensional NOESY spectrum of D76N β2-m. The dashed red lines cross through the backbone amide NOEs of Asn96 (9.75 ppm) and Asn76 (7.31 ppm), whereas the dashed blue lines cross through the NOEs of the carboxamide NHs at 7.80 and 7.21 ppm that are attributed to Asn38 and Asn76 and tentatively assigned as H2m and H4m, respectively. To avoid notation crowding, the relevant connectivities are highlighted by numbers (1, Asn42 NH-Asn38 H2m 9.75 ppm and Asn76 NH-Asn42 H2m 9.75 ppm). The black line corresponds to the locations expected for the carboxamide resonances of Asn76 and Asn42 as well as for the carboxamide resonances at 7.21 and 7.80 ppm support the attribution of the latter signal pair to the carboxamide resonances of both Asn76 and Asn42 suggests that the two side chains experience the same environment, which in turn is consistent with the occurrence of reciprocal H-bonds between Asn42 and Asn76 carboxyamides.

![Image of Electrostatic properties of wild type and D76N β2-m.](image)

**FIGURE 4.** Electrostatic properties of wild type and D76N β2-m. A, isopotential curves are displayed at +0.5kT/q (blue) and −0.5kT/q (red). The regions around Asp34 and Asn42 are circled. The molecular structure of β2-m (Protein Data Bank code 3HLA, chain B) (74) and the D76N β2-m homology model were prepared for electrostatic calculations using the program PDB2POVR, which adds hydrogens and assigns charges and atomic radii according to different force fields. The CHARMM force field parameters were used except that the pKa values had been calculated using BLUUES (22).
from the NOE$_{25 \degree C}$/NOE$_{37 \degree C}$ ratio are uniformly spread over the whole molecule of the wild type protein compared with a distinctive uneven pattern in the variant. When related to the spatial structure, this pattern delineates an instability propagation path (illustrated by the backbone mobility changes with temperature shown in Fig. 8). Decreased conformational stability and reduction of repulsive electrostatic interactions make D76N/H9252$_{2m}$ extremely sensitive to aqueous boundary conditions where the preferential interface partitioning of the protein and the subsequent surface tension fluctuations overcome the determining role for the effective force field of hydrophobic folding drives, thereby enhancing unfolding and fibrillogenic nucleation events.

**Fibrillogenesis of D76N β$_2$-Microglobulin Occurs under Physiological Conditions**—In marked contrast to the wild type protein, D76N β$_2$-m rapidly aggregates as shown by ThT fluorescence (Fig. 9A) and atomic force microscopy (Fig. 9B) when agitated at pH 7.4 and 37 °C in the presence of an air-water interface. We had already shown that neither protein aggregates in the absence of agitation (2). Furthermore, replacement of the air-water interface with Teflon-water, which reduces the interfacial tension from about 70 to 50 millinewtons/m (43) at fixed interfacial area, also completely suppressed aggregation of the D76N variant (Fig. 9A). The water-air boundary is known to behave as a hydrophobic interface (43, 48, 49). To evaluate interfacial effects on D76N/H9252$_{2m}$ fibrillogenesis, the air-water interface was removed and replaced with a hydrophilic-hydrophobic interface. We used the prototypic hydrophobic surface provided by graphite or elastin, the very hydrophobic ubiquitous insoluble fibrillar component of the extracellular matrix (50) (Fig. 10). In the absence of the air boundary, graphite triggered fibril formation on the sheet surfaces (Fig. 10A) without massive conversion of the bulk β$_2$-m in solution (data not shown). Ultrasonication triggered fibrillogenesis by D76N/H9252$_{2m}$ even in the absence of an air-water interface (Fig. 10B, blue lines), and aggregation was accelerated by addition of carbon nanotubes (Fig. 10B, green lines). In these conditions, ultrasonication had no effect on aggregation of wild type β$_2$-m (red and dashed black lines). Inclusion of fibrillar elastin in the protein solution, kept at 37 °C under agitation in the absence of an air boundary, strongly promoted fibril formation by D76N β$_2$-m (Fig. 10C, red triangles). Elastin did not promote aggregation by wild type β$_2$-m under the same conditions (Fig. 10C, black triangles).

Under conditions that suppress aggregation, the absence of an air-water interface or any agitation of the protein solution, the consistent enhancement of D76N β$_2$-m fibrillogenesis by graphite or elastin clearly demonstrates the impact of an
increased interfacial area. In addition to the crucial role of hydrophobic-hydrophilic interfaces, shaking of the solution is required for amyloid conversion of D76N/H9252 2m in the bulk, and the mechanism by which agitation influences the kinetics of fibril formation is clearly important.

Role of Shear Forces and Hydrophobic Surfaces in β2m Amyloidogenesis—Agitation of a protein solution applies hydrodynamic shear stress, which in principle could also contribute to protein destabilization leading to denaturation (51). We have therefore calculated the shear forces acting on the β2m molecule using the equation (52)

\[ T = F_s / A = \mu * (dv/dx) \]  
(Eq. 4)

where \( T \) is the shear stress, \( F_s \) is the shear force, \( A \) is the cross-sectional area of the molecule, \( \mu \) is the dynamic viscosity of the fluid, and \( dv/dx \) is the shear rate, that is the fluid velocity gradient. \( F_s \) clearly depends greatly on molecular shape (for example, see Refs. 51 and 53). With uniform fluid flow, the major forces acting on a molecule in the bulk are elongational forces along the flow axis that depend on both protein length and shape. We have used the model proposed by Shankaran and Neelamegham (54) that assumes that the molecule has a dumbbell shape, which yields a force coefficient derived from the radius of the two ends of the dumbbell and the distance between them. \( F_s \) is then calculated from

\[ F_s = \alpha \cdot \mu \cdot \gamma \cdot R^2 \]  
(Eq. 5)

where \( \alpha \) is the force coefficient, \( \mu \) is the dynamic viscosity, \( \gamma \) is the shear rate \((dv/dx)\), and \( R \) is the radius of the molecule. The shear rate of the flow can be calculated as

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**FIGURE 6. Temperature and heteronuclear NOE.** $^{15}\text{N}\{^1\text{H}\}$ NOE values measured at 11.7 teslas (500.13-MHz $^1\text{H}$ frequency) at 25 °C (A) and 37 °C (B) for wild type and D76N β2m. The horizontal lines represent the average values for wild type (solid line) and variant (dashed line) species; error bars indicate S.E. The abscissa axes do not include the positions 5, 14, 32, 72, and 90 corresponding to proline residues, therefore lacking in secondary amides. Only the pairs of well resolved cross-peaks observed at both temperatures were selected for each species. The strand naming scheme is drawn parallel to abscissas between the two panels.
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FIGURE 7. Hydrogen bonding relationship of the residue 76 side chain. Shortest hydrogen bond path lengths involving at least one side chain atom for residue 76 in wild type (solid line) and D76N (dashed line) β2m from the Floyd-Warshall algorithm are shown. The variant shows only minor differences from wild type, specifically with a slightly stronger connection of residue 76 to loop AB and overall slightly weaker connection to other residues of the loop EF itself, to loop CC, and to the C terminus. The wild type Asp76 residue is strongly linked to Asn42 with the hydrogen bond Asn42 HN-Asp76 Oε1, which is found in 2222 of 2500 snapshots. In turn, Asn42 is hydrogen-bonded with Glu77 via two nearly completely conserved hydrogen bonds (Asn42 HN-Glu77 O and Glu77 HN-Asn42 Oε1). The connection to the C-terminal residue Lys54 is due to the salt bridge with Glu57. The connection with the loop AB is weaker and involves the hydrogen bonds of Asp76 with Thr73 (Thr73 H32-Asp76 Oε1), the fluctuating hydrogen bond of Arg59 side chain with Thr73 backbone, and the salt bridge of Arg59 with Glu77. The connection between Thr73 and Arg59 is very weak. In D76N β2m, all connections are weaker except for the path to loop AB with a hydrogen bond of the side chain of Arg59 to the side chain of Asn76 and the hydrogen bond of the side chain of Arg59 to the side chain of Asn17.

FIGURE 8. Changes in backbone mobility. The mobility of wild type and D76N β2m based on the heteronuclear NOE ratios derived from values shown in Fig. 6 with increments (red intensity) and decrements (blue intensity) is shown. The weaker connection between EF and CC loops of the variant spreads all around the underlying region with flexibility gains involving the intervening edges of strands A, B, E, and F. The solution structure of β2m (Protein Data Bank code 1JNJ) was also used to model the D76N variant. The central diagram shows the strand naming scheme.

\[
\gamma = \frac{V}{L} \tag{Eq. 6}
\]

where \(V\) is the translational velocity of the fluid and \(L\) is the half-length of the cell. For our system (Fig. 9A), \(\gamma = 94.2/s\), and using an \(\alpha\) value of 10 as reported previously for a similar system (54), the calculated shear force, \(F_s\), of \(3.3 \times 10^{-17}\) newton is much lower than the force of \(-10^{-12}\) newton typically required to unfold proteins (44, 45). Shear stress alone is thus unlikely to destabilize native β2m, but liquid agitation increases the sampling frequency of natively folded monomers at the hydrophobic-hydrophilic interface and facilitates the exchange of misfolded monomers and interface-formed nuclei of aggregation with the bulk solution. All these entities are then available for the recruitment of other protein units, thereby increasing the efficiency of aggregation (51).

At the hydrophobic-hydrophilic interface, the native protein fold is perturbed by the combined action of interfacial, intermolecular, and hydrophobic interactions (31–34) of which the latter is apparently dominant (35–37) with much evidence that exposed hydrophobic domains trigger the interaction of a protein with an apolar surface (37–40). Based on the known exposed hydrophobic domains present on β2m, we estimate that the forces acting on the protein at the hydrophobic-hydrophilic interface are in the range of 5–100 piconewtons and are sufficient to perturb its three-dimensional structure (2, 44, 45) (see “Experimental Procedures” for details).

The crucial role of interfacial forces in protein destabilization and fibrillogenesis must vary with the different electrostatic charges and thermodynamic stabilities of individual proteins because efficient adsorption at a hydrophobic-hydrophilic interface depends on overcoming the energy barriers of surface pressure and electrostatic repulsion (39, 55–58). The almost neutral D76N β2m molecule is likely to adsorb more rapidly than the more charged wild type β2m, and once at the interface, it should undergo larger structural perturbations as it is thermodynamically less stable (59, 60) (Fig. 9A).

D76N β2m Primes the Fibrillar Conversion of Wild Type β2m in Vitro—The D76N β2m variant in solution in physiological buffered saline converts into fibrils at the highest rate ever reported for an amyloidogenic globular protein under these conditions. When mixed in equimolar proportions with native wild type β2m, all the latter was also transformed into insoluble fibrils (Fig. 11A) with a much shorter lag phase than reported previously for seeding by the truncated isoform lacking the six N-terminal residues (ΔN6 β2m) (61) (Fig. 11B). Fibrillogenesis was monitored by quantifying the soluble protein by native 1% agarose gel electrophoresis in which the soluble forms of wild type, D76N, and ΔN6 β2m are readily distinguished by their different respective electrophoretic mobilities (Fig. 12). The duration of the lag phase depended on the aggregation state of D76N variant, which can potently promote aggregation of wild type β2m only when it is assembled into elongated oligomers and filaments (Fig. 11C).

Unexpectedly, our previous proteomic characterization of ex vivo natural amyloid fibrils from the tissue deposits of patients carrying the amyloidogenic D76N mutation showed only the presence of full-length variant protein (2). Because wild type β2m is intrinsically amyloidogenic in vivo and forms abundant amyloid fibrils in patients affected by dialysis-related amyloidosis, the absence of any wild type β2m in the hereditary variant β2m deposits indicates that in vivo fibrillogenesis is more complex than the simple in vitro experiment containing just wild type and variant β2m. A likely physiological factor modulating misfolding, aggregation, and fibrillogenesis could be the presence of extracellular chaperones. Indeed, we show here that α-crystallin (62, 63) prevented amyloid conversion of wild type β2m induced by D76N β2m fibrils without interfering with fibrillogenesis of the variant at the lowest chaperone concentration used (1 μM). However, at 40 μM α-crystallin, even the conversion of D76N β2m is significantly reduced (Fig. 13). The effect of this prototypic chaperone strongly suggests mecha-
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DISCUSSION

β₂m is among the most extensively studied globular protein precursors of human amyloid fibrils. The discovery of the first natural variant of human β₂m as the cause of hereditary systemic amyloidosis uniquely enables a very informative comparison of two different types of β₂m amyloidosis with distinctly different clinical and pathological features. The D76N residue substitution allows a fully folded three-dimensional structure almost identical to that of the wild type protein that forms amyloid fibrils in dialysis-related amyloidosis. However, dissection of the mechanism of D76N β₂m fibrillogenesis confirmed our previously established paradigm that the amyloidogenicity of monomeric globular proteins is intimately connected to destabilization of the native fold (64). Importantly, a specific intermediate of the folding pathway of wild type β₂m, which was previously structurally characterized and shown to play a crucial role in priming the amyloid transition (47), is particularly abundantly populated by the D76N variant. It is therefore possible that this specific residue substitution facilitates the molecular mechanism responsible for the inherent amyloidogenicity of wild type β₂m and thereby enables the variant to cause clinical pathology even at a normal plasma concentration rather than the grossly increased abundance of wild type β₂m responsible for dialysis-related amyloidosis.

Our elucidation of the structural properties and folding dynamics of the highly amyloidogenic D76N variant has validated several earlier interpretations of the molecular basis of the amyloid transition of the wild type β₂m. The characterization of conditions for rapid fibrillogenesis of the variant in a physiological milieu is therefore particularly significant. D76N β₂m forms amyloid fibrils within a few hours in physiological buffers in vitro that is enhanced by fluid agitation and exposure to a hydrophobic surface. In contrast, fibrillogenesis of wild type β₂m is extremely slow under physiological conditions, being minimal or absent after 100 days of incubation (61). Fluid agitation has been shown previously to be crucial in priming amyloid fibrillogenesis of other polypeptides including amyloid β (31), insulin (65), apolipoprotein C-II (66), and α-synuclein (67), but all these precursors were either natively unfolded (apolipoprotein-CII, amyloid β, and α-synuclein) or induced to unfold by a denaturing buffer (insulin).

Our present demonstration that the interfacial forces, acting in a physiologically relevant fluid flowing over natural hydrophobic surfaces, can prime fibrillar conversion of D76N β₂m monomers identifies this protein as a genuine paradigm for amyloidogenic globular proteins causing systemic amyloidosis. Although critically destabilized by comparison with the wild
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extracellular environment (51), the variant’s propensity to misfold and aggregate as amyloid fibrils becomes evident. Within the extracellular space where amyloid is deposited in the systemic amyloidoses, the interstitial fluid flows over the extensive surfaces of the fibrous network of elastin, collagen, and leucine-rich proteoglycans (68), the high hydrophobicities of which play a key role in promoting local unfolding of globular proteins. We have previously reported the capacity of collagen to

FIGURE 10. Fibrillogenesis of D76N and wild type β2m in presence of hydrophilic-hydrophobic interfaces. A, tapping mode AFM images of fibrils formed by D76N β2m in the presence of graphite sheets under stirring conditions (ⅰ) or without agitation (ⅱ). B, time course of fibril formation by D76N β2m under ultrasonication (light blue lines show replicate experiments) in contrast to the absence of fibrillogenesis by wild type β2m (red line) under the same conditions. D76N β2m fibril formation was accelerated in the presence of carbon nanotubes (green lines), whereas wild type β2m (dashed black line) did not aggregate. C, fibrillogenesis of D76N (red triangles) and wild type β2m (black triangles) carried out under stirring conditions at 37 °C in the presence of a Teflon-water interface with 6 μM human elastin. αα, arbitrary units.

FIGURE 11. Wild type β2m elongates D76N fibrils in vitro. A, soluble fractions of wild type β2m either alone (black empty circles; WT) or in an equimolar mixture with the variant (black filled circles; WT+D76N) and of D76N variant either alone (red empty circles; D76N) or in the mixture (black empty triangles; D76N+WT). WT+D76N. Values are mean and S.D. (error bars) from three independent experiments. B, surface plots of AFM images showing different steps of the aggregation process of D76N β2m. At 1 h, oligomers are present; at 2 h, they coexist with short prefibrillar aggregates; and at 8 h, filaments can be observed, whereas at 24 h, fibrils and complex fibril assemblies are seen. The surface plots were obtained from topographic tapping mode AFM images (Fig. 9B).

FIGURE 12. Residual soluble β2m during aggregation. Agarose gel electrophoresis analysis of supernatants from fibrillogenesis of wild type β2m alone (lane 1), D76N β2m alone (lane 2), ΔN6 β2m alone (lane 3), an equimolar mixture of wild type and D76N β2m (lane 4), and an equimolar mixture of wild type and ΔN6 β2m (lane 5) is shown. The arrows show the electrophoretic mobility of each isoform.

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extracellular environment (51), the variant’s propensity to misfold and aggregate as amyloid fibrils becomes evident. Within the extracellular space where amyloid is deposited in the systemic amyloidoses, the interstitial fluid flows over the extensive surfaces of the fibrous network of elastin, collagen, and leucine-rich proteoglycans (68), the high hydrophobicities of which play a key role in promoting local unfolding of globular proteins. We have previously reported the capacity of collagen to
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FIGURE 13. Modulation by α-crystallin of fibril formation by wild type and D76N β2m. Soluble fractions of wild type and D76N variant β2m from an equimolar mixture in the presence and absence of α-crystallin quantified at 96 h by native agarose gel electrophoresis are shown. Values are mean ± S.D. (error bars) from three independent experiments.

prime the formation of wild type β2m amyloid fibrils stacked on the collagen surface, and here we show that elastin is a potent promoter of massive amyloid conversion of the D76N variant in solution. Massive enhancement by graphite nanotubes of variant β2m amyloid fibrillogenesis further confirms the role of hydrophobic surfaces. Although Linse et al. (69) have previously noted an effect of hydrophobic surfaces on fibrillization of wild type β2m with accelerated nucleation induced by nanoparticles covering a range of sizes and hydrophobicity patterns, their experiments were done at the grossly non-physiological pH of 2.5.

The kinetics of fibril formation by wild type β2m and its truncated form ΔN6 β2m depend on a critical nucleation step and can be accelerated by the presence of amyloid fibril seeds. In particular, the truncated form ΔN6 β2m can catalyze the oligomerization of the wild type (70) and even prime the fibrillogenesis of the wild type protein in physiological buffer (61) although with a slower rate and lower yield than when primed by D76N β2m. The D76N variant is also much more potent than ΔN6 β2m in promoting formation of actual amyloid fibrils by wild type β2m. The apparent capacity of monomeric ΔN6 β2m to induce conformational rearrangement of the wild type protein structure has previously been ascribed to a prion-like effect (71). In our hands, however, monomeric D76N variant and ΔN6 β2m do not prime fibrillogenesis by wild type β2m, which only occurs when it is exposed to filaments and fibrils of the priming species. Such a mechanism is more consistent with a surface nucleation process (72) rather than a genuine prion-like effect.

The contrast between the potent in vitro priming and enhancement by D76N β2m of amyloid fibril formation by wild type β2m and the proteomic evidence that the wild type protein is not present in the in vivo amyloid deposits are intriguing, especially as wild type β2m clearly does form amyloid in vivo in dialysis-related amyloidosis. Furthermore, in other types of hereditary systemic amyloidosis in which the wild type precursor protein is mildly amyloidogenic, for example transthyretin, most patients are heterozygotes for the causative mutation, expressing both amyloidogenic variant and wild type, and both proteins are present in the amyloid fibrils. However, as we have shown here, the capacity of D76N β2m to catalyze fibrillogenesis by wild type β2m can be modulated and even blocked by typical chaperones such as α-crystallin, and this inhibition depends on the stoichiometric chaperone/β2m ratio. A role for extracellular chaperone-like proteins in the inhibition of wild type β2m amyloidogenesis has been proposed previously (73), and it is plausible that the persistent, extremely high concentration of wild type β2m in renal failure patients on dialysis may overcome the natural protective role of physiological chaperones that otherwise protect against deposition of this rather weakly amyloidogenic protein when it circulates at its normal serum concentration. In addition to illuminating the critically important biophysical features of the physiological milieu where amyloid formation takes place, our results thus open up novel avenues for exploration of hitherto unanswered questions about amyloidosis: why only a handful of all proteins ever form amyloid in vivo, and when, why, and where amyloid is deposited in disease.

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