A Partially Structured Species of \(\beta_2\)-Microglobulin Is Significantly Populated under Physiological Conditions and Involved in Fibrillogenesis*

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The folding of \(\beta_2\)-microglobulin (\(\beta_2\)-m), the protein forming amyloid deposits in dialysis-related amyloidosis, involves formation of a partially folded conformation named I\(\beta\), which slowly converts into the native conformation, involves formation of a partially folded conformer of these results. The possibility of isolating and quantifying a partially folded conformer of \(\beta_2\)-m can be separated from N by capillary electrophoresis. Data obtained with this technique and analysis of kinetic data obtained with intrinsic fluorescence indicate that the I\(\beta\) conformation is populated to \(-14 \pm 8\% \) at equilibrium under conditions of pH and temperature close to physiological. In the presence of fibrils extracted from patients, the I\(\beta\) conformer has a 5-fold higher propensity to aggregate than N, as indicated by the thioflavine T test and light scattering measurements. A mechanism of aggregation of \(\beta_2\)-m in vivo involving the association of the preformed fibrils with the fraction of I\(\beta\) existing at equilibrium is proposed from these results. The possibility of isolating and quantifying a partially folded conformer of \(\beta_2\)-m involved in the amyloidogenesis process provides new opportunities to monitor hemodialytic procedures aimed at the reduction of such species from the pool of circulating \(\beta_2\)-m but also to design new pharmaceutical approaches that consider such species as a putative molecular target.

Dialysis-related amyloidosis represents an inevitable and severe complication of long term hemodialysis (1–4). Under this pathological condition, protein aggregates known as amyloid fibrils, accumulate in essential tissues, such as the skeletal muscle, interfering with their normal functions. A major constituent of the amyloid fibrils related to this pathological condition is \(\beta_2\)-microglobulin (\(\beta_2\)-m). In its native form, this 99-residue protein is constituted by two \(\beta\)-sheets packed against each other to form a fold typical of the immunoglobulin superfamily (5). The two \(\beta\)-sheets, constituted by three and four strands, respectively, interact by means of hydrophobic interactions and a disulfide bridge that stabilizes further the \(\beta\)-sandwich structure.

\(\beta_2\)-m constitutes the light chain of the major histocompatibility complex class I (MHCI). A significant pool of \(\beta_2\)-m is also normally present in the plasma as a consequence of the constant release from the MHCI to allow the process of catabolic degradation in the kidney (1, 2). In chronic dialysis patients, the artificial membrane induces an inflammatory reaction, which causes the production and release of \(\beta_2\)-m to increase significantly (6). In addition, \(\beta_2\)-m cannot be filtered efficiently through the artificial membrane, resulting in an increase of the levels of soluble \(\beta_2\)-m from 0.3 to 30 \(\mu\)g/ml, the range of concentrations observed within healthy individuals, to \(-40 \mu\)g/ml (7). The increase of free circulating \(\beta_2\)-m, the preferential substrate for amyloid deposition by this protein, is responsible, at least in part, for this form of amyloidosis in these patients (1, 2). Big efforts have been expended to improve the biocompatibility and performance of dialysis approaches. Although high performance membranes have led in many cases to the reduction of \(\beta_2\)-m concentration and clinical improvement, no dialysis membrane has yet been designed to reduce the \(\beta_2\)-m levels to the normal range (2, 4).

Very little is known about the complex process of conversion of the soluble and native conformation of \(\beta_2\)-m into amyloid fibrils. The ability of \(\beta_2\)-m to form amyloid fibrils in vivo indicates that this process may take place without the assistance of other protein or cellular factors. It has been shown that in order to aggregate, a protein has to undergo at least partial denaturation as the native conformation is very stable as a soluble state (8). This is likely to be the case also for \(\beta_2\)-m, since partially unfolded states, generated by either decreasing the pH to values of 3–4 or by removing the six N-terminal residues, have been shown to be more prone to fibrillar aggregation than the wild-type protein under physiological conditions (9, 10). Complete denaturation, such as that created by reduction of the unique disulfide bridge, is not required for fibril formation.

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1 The abbreviations used are: \(\beta_2\)-m, \(\beta_2\)-microglobulin; ThT, thioflavin T; MHCI, major histocompatibility complex class I; GdnHCl, guanidinium chloride; CE, capillary electrophoresis; CR, Congo Red.
Aggregation of β₂-Microglobulin

since the disulfide bridge is found to be intact in the β₂-m constituents of natural amyloid fibrils (11).

The characterization of the folding process from denatured β₂-m with an intact disulfide bridge has revealed a complex folding scheme (12),

\[
\text{U} \quad \text{→ I}_1 \quad \text{→ I}_2 \quad \text{→ N}
\]

**Scheme 1**

The I₁ ensemble forms from the unfolded state on the sub-millisecond time scale. The I₂ species forms subsequently with a time constant of ~200 ms, at pH 7.4 and 30 °C, but converts very slowly into the fully native state (N) with a time constant of ~6 min under the same conditions. Scheme 1 does not imply that I₁ and I₂ are necessarily on-pathway intermediates of folding but simply indicates their sequential formation prior to the attainment of N. Indeed, it is not unlikely that these partially folded species have to unfold to some degree before folding can progress (12). The accumulation of a slow folding structure, such as the I₂ species, is very unusual for a protein of the size of β₂-m. In this study, we investigate the role of this folding intermediate in the amyloidogenesis process of β₂-m. We will provide experimental evidence that such a species remains significantly populated at equilibrium when most of the protein is in the native state, that it has a remarkably higher propensity to aggregate than the fully folded conformation, and that the amyloidogenicity of β₂-m is very likely to arise from the fraction of protein in this conformation.

**EXPERIMENTAL PROCEDURES**

**Materials—** β₂-m was purified as described previously (9). The solution containing the pure protein was freeze-dried and dissolved in the desired solution immediately before use. Dimeric β₂-m, stabilized by an intermolecular disulfide bridge, was obtained as a by-product of the expression system used to produce recombinant β₂-m (9). This covalently linked dimeric β₂-m is detected as an acidic band in native electrophoresis of solubilized recombinant β₂-m inclusion bodies. This species was separated from monomeric β₂-m by ion exchange chromatography as previously described (9). The presence of a disulfide intermolecular interaction in the purified species was assessed by parallel runs of SDS-polyacrylamide gel electrophoresis under reducing and nonreducing conditions. Reduced and carboxymethylated monomeric β₂-m was obtained as described (13). The identity and purity of the β₂-m species used in this study were checked by electron spray mass spectrometry. Uncoated fused silica capillaries (50-μm inner diameter) were from Polymicro Technologies (Phoenix, AZ). Phosphate buffer stock solutions (0.1 M) were prepared by mixing analytical grade Na₂HPO₄ and NaH₂PO₄ solutions to give a pH of 7.3. Prior to use, all solutions were filtered through a 0.45-μm Millipore membrane filter (Bedford, MA) and degassed by sonication. Ex vivo β₂-m fibrils were obtained by the water extraction procedure from an amyloidoma surgically removed during a hip replacement operation and were previously characterized in terms of β₂-m composition and microscopic pattern (11). By SDS-polyacrylamide gel electrophoresis and immunoblot, more than 90% of the extracted material appears to be β₂-m (both monomeric and aggregated).

**Folding and Unfolding Rate Measurements—** Folding and unfolding rate constants were measured as described (12). The rate constants for the conversion of I₂ into N (k₁₂→N) and of global unfolding (k₃→N) in the absence of denaturant were determined from the intercepts of plots of ln [N]/[I₂] vs. time. Equation 2 (see "Results") was derived assuming that global unfolding (i.e. the conversion of N into U) can occur by either transiting through the I₂ species or by an alternative pathway reaching the U conformation without formation of I₂. If these two unfolding mechanisms are both possible, their k₃→N = k₁₂→I₂ + k₃→I₂, where k₁₂→I₂ is the rate constant of unfolding when this occurs via the I₂ species (in this pathway, the conversion of N into I₂ is the rate-determining step, since the following steps are much faster) and k₃→I₂ is the rate constant of the alternative unfolding process. Under strong refolding conditions, k₁₂→I₂ and k₃→I₂ are 1 order of magnitude faster than k₃→I₂ and k₃→I₂, and k₁₂→I₂ (12).

Moreover, k₁₂→I₂ is much faster than k₁₂→I₂, and k₃→I₂ is much faster than k₃→I₂ (data not published). The analytical inspection of this reaction leads to Equation 1,

\[
\frac{[I]_2/[I_2]}{[I_2]} = k_{3\rightarrow N} + k_{3\rightarrow N} \cdot (k_{3\rightarrow N} + k_{3\rightarrow N} + k_{3\rightarrow N}) - (Eq. 1)
\]

where [I]₂/[I₂] has the same meaning as in Equation 2 (i.e. the ratio between the equilibrium concentration of I₂ and that of total protein).

If the two mechanisms of unfolding we have considered above are both possible, then k₃→N = k₁₂→I₂ + k₃→I₂ and Equation 1 becomes Equation 2. If the direct conversion of N into U represents a negligible contribution to the overall unfolding process, then k₃→N = 0 and k₃→N = 0, and on the other hand, if unfolding through formation of I₂ is negligible, then k₃→N = 0 and k₃→N = k₃→N. In all cases, Equation 1 becomes Equation 2.

**Capillary Electrophoresis—** A Hewlett Packard three-dimensional capillary electrophoresis (CE) system (Waldbronn, Germany) with built-in diode-array detector was employed. The CE instrument control was performed with an HP Vectra XA/166 computer utilizing a Chemstation A.05.01 software. The capillary (57 cm total length) was thermostatted with circulating air at 10 °C, and separations were carried out at 15 kV with the anode at the sample injection end. For all experiments, the running background electrolyte consisted of 100 mM sodium phosphate buffer at pH 7.3. Sample hydrodynamic injection was performed by applying 50 millilbars for 8 s. Throughout all experiments, the operative current varied from 59 to 62 μA. The capillary was rinsed after electrophoresis with 0.1 M NaOH for 2 min, water for 2 min, and electrophoresis buffer for 4 min. The protein was dissolved in phosphate-buffered saline buffer (6.5 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, pH 7.4), and the final concentration was estimated by measurements of the 280-nm absorbance using an extinction coefficient of 1.7 ml mg⁻¹ cm⁻¹. The initial protein solutions were diluted with water in order to obtain 100-μl sample solutions containing a 0.09 mg/ml concentration of the protein and different amounts of acetonitrile (0, 10, 20, 30, 40, 50, 66, and 76%). The final samples containing acetonitrile were incubated for 20 min at room temperature and then injected. For the kinetic experiments, the protein was unfolded in solutions containing 50% acetonitrile for 20 min. The refolding process was initiated by diluting these samples down to 18% acetonitrile. These were injected into the capillary after different time intervals had elapsed from dilution. Peak areas obtained for all experiments have been divided by their corresponding retention time in order to express them as normalized areas (14). Normalized areas are further divided by the total peak areas to express each conformational population as a percentage of total area.

**Folding Kinetics Followed by Far UV Circular Dichroism—** A Jasco 710 spectropolarimeter was used to monitor the change of mean residue ellipticity of β₂-m at 205 nm during folding. The protein, dissolved in phosphate-buffered saline, was initially denatured at equilibrium in the presence of 60% acetonitrile and subsequently refolded by 18% acetonitrile. Final protein concentration was 0.2 mg ml⁻¹. Measurements were performed at 20°C in the presence of CR by monitoring the change of mean residue ellipticity at 205 nm. Congo Red Affinity of β₂-m—β₂-m was evaluated for its interaction with Congo Red (CR) by affinity CE following the experimental procedure described by Heegaard et al. (15) but with different capillary temperature (10°C). Unfolded β₂-m in 50% acetonitrile was used in the presence of CR concentration in the running buffer up to 7.2 μM.

**Thioflavine T Fluorescence—** In a first set of experiments, 2 mg ml⁻¹ β₂-m was incubated for 20 min in 100 mM HCl (or pure water for a control experiment) at 30°C. Refolding was then started by a 1:1 dilution into a 30°C equilibrated refolding buffer containing 26 mM Na₂HPO₄ and 254 mM NaH₂PO₄ in the presence or absence of β₂-m fibrils extracted from the bones of a patient. Final pH was 7.3 in all cases. 25 μl of the protein sample were mixed in a 500-μl fluorescence cell with 475 μl of 2.5 mM thioflavine T (ThT), 25 mM phosphate buffer, pH 6.0, 30°C. The resulting fluorescence at 485 nm (excitation 440 nm) was measured on a 30°C thermostatted PerkinElmer Life Sciences 50 B fluorimeter. The measurement was carried out after 1 min from the initiation of folding, when over 80% of β₂-m is in the I₂ conformation (12). Fluorescence values of suitable blanks obtained in each case using 25 μl of the same buffer but without protein were subtracted from measured values. In a second set of experiments, the initial β₂-m concentration in the unfolding solution (or pure water) was 1 mg ml⁻¹. After dilution in the refolding buffer, the solution was concentrated for 2–3 min with a 50-ml ultrafiltration system using a 10,000-cutoff membrane. Soluble β₂-m and fibrils appeared to be 3.3 times more concentrated than immediately after dilution. For the ThT test 15 μl of protein sample
RESULTS

Folding and Unfolding of $\beta_2$m—Conversion of I$_2$ into N is the third exponential phase observed during folding of oxidized $\beta_2$m. The rate constant for such conversion ($k_{I_2 \rightarrow N}$) was determined at pH 7.4 and 30°C at various GdnHCl concentrations ranging from 0 to 1.2 M (Fig. 1, empty circles). The rate constant observed for global unfolding ($k_{unf}$), was measured within the range of GdnHCl concentration of 2–5.2 M, under the same conditions of pH and temperature (Fig. 1, filled circles). Within these ranges of denaturant concentration, linear correlations were observed between both the natural logarithms of $k_{I_2 \rightarrow N}$ and $k_{unf}$ and GdnHCl concentration. This allowed the values of $k_{I_2 \rightarrow N}$ and $k_{unf}$ to be determined in the absence of denaturant by linear extrapolation to 0 M GdnHCl. Under these strong refolding conditions, the first two steps of Scheme 1 are irreversible and more than one order of magnitude faster than the conversion of I$_2$ into N and global unfolding (12). In this mechanism, Equation 2 can be demonstrated,

$$[I_2]_{eq}/[\beta_2m]_0 = k_{unf}/k_{I_2 \rightarrow N}$$  (Eq. 2)

where $[I_2]_{eq}$ is the equilibrium concentration of I$_2$, and $[\beta_2m]_0$ is the concentration of total protein (see “Experimental Procedures” for the derivation of this equation). From the plots reported in Fig. 1, values of $3.0 \pm 0.4 \times 10^{-3}$ s$^{-1}$ and $4.9 \pm 1.8 \times 10^{-4}$ s$^{-1}$ were obtained for $k_{I_2 \rightarrow N}$ and $k_{unf}$ respectively (12). Equation 2 allows a value of $0.14 \pm 0.08$ for $[I_2]_{eq}/[\beta_2m]_0$ to be determined. Hence, from this kinetic analysis, it appears that the I$_2$ species does not extinguish completely when the folding process is complete. The native state of $\beta_2$m represents therefore a mixture of the real N state and of the residual I$_2$ species, the latter representing $\sim 14 \pm 8$% of total $\beta_2$m under these conditions of temperature and pH.

Identification of I$_2$ by Capillary Electrophoresis—CE, a technique that has the potential to separate different conformations based on their radii of gyration or charge state (16), allowed the N and I$_2$ species to be resolved. The electropherograms of $\beta_2$m, preincubated in the presence of different percentages of acetonitrile, have been obtained (Fig. 2). The electrophoretic profile obtained from the protein freshly dissolved in the absence of acetonitrile shows two distinct peaks, possibly representing the equilibrium between the N (peak 1) and I$_2$ (peak 2) species (Fig. 2). The traces obtained from the samples preincubated in the presence of high concentrations of acetonitrile ($>35$%) confirm the electrophoretic pattern previously obtained, which was hypothesized to represent two distinct conformations of $\beta_2$m, namely the native protein (peak 1) and a conformational variant (peak 2) (15). As described under “Experimental Procedures,” the capillary is equilibrated, for technical reasons and regardless of the initial conditions of preincubation, with a buffer that does not contain acetonitrile and therefore favors refolding. In the initial denaturing conditions, $\beta_2$m is largely denatured. Nevertheless, under the folding conditions of the capillary, the largely denatured state U and the rapidly forming I$_1$ species (Scheme 1) deplete far more rapidly than the time required for electrophoretic separation. The two species identified from the samples containing initially high concentrations of acetonitrile are therefore very likely to be the I$_2$ and N conformations, the former representing a residual population that has not yet converted into N.

Within the range of 30–40% acetonitrile, a remarkable decrease in the area ratios between the first and the second peak occurs. By adding acetonitrile percentages higher than 50%, the ratio between the two peak areas remains constant. Such results are summarized in the plot reported in Fig. 3. The CD data obtained under these conditions clearly confirm that within the range of 30–40% acetonitrile, a conformational transition from a structured state to a globally unfolded one takes place (data not shown). The CD data show that above 50% acetonitrile $\beta_2$m is totally unfolded, whereas in CE, as is evident from Fig. 3, the native form is still detected. This discrepancy is due to the refolding environment of the capillary, a medium that induces rapid refolding from the initial
unfolded state as described above.

In order to study further the origin of the second peak, the electrophoretic profile was obtained by inserting progressively longer delays between dilution of the acetonitrile-denatured protein into refolding buffer and injection into the capillary. Fig. 4A illustrates the variation of normalized areas of the two peaks as a function of the delay time. Since the CE system is operating on an intermediate time regime, the relative normalized areas detected after the time required for separation cannot be taken as accurate measures of the population fractions of the two species immediately before or after the electrophoretic run (17). A rigorous kinetic analysis cannot therefore be performed from these data. Nor can the fractions of the two species attributable to the first and second peak be quantified during the development of the observed kinetics.

Despite these technical difficulties, it is clear that while the first peak increases in intensity, the second peak decreases. The observed time scale of such change overlaps with the change of CD signal obtained under similar conditions (Fig. 4B) and previously attributed to the conversion of I₂ into N (12). This further suggests the idea that the second peak identified in the CE profile is the intermediate of folding previously identified as I₂. Importantly, the second peak reaches a relative normalized area of ~8%, a value significantly higher than zero and similar to that obtained from the freshly dissolved protein. This value, which represents the population fraction of I₂ ([I₂]/[I₂-m]), is lower than that calculated from the kinetic analysis (~14%), an observation consistent with the lower operating capillary temperature (10 °C versus 30 °C).

The electrophoretic mobility of a I₂-m dimer stabilized by an intermolecular disulfide bridge was also analyzed (Fig. 5). The main peak obtained when dimeric I₂-m is injected in the capillary has a migration time higher than those of the two peaks obtained from the freshly dissolved protein (Fig. 5). Some minor peaks, having mobilities similar to those of the two peaks obtained with fresh samples of I₂-m, are attributable to “monomeric impurities” in the sample of the dimer. However, the low mobility major peak obtained with dimeric I₂-m is absent in the electropherograms obtained from the protein freshly dissolved in the presence or absence of acetonitrile. This rules out that the second peak of the various electropherograms shown in Fig. 2 originates from dimeric forms of I₂-m.

Furthermore, it can also be excluded that such a low intensity peak represents a fraction of fully unfolded protein. Indeed, injection in the capillary of the fully unfolded conformation of I₂-m, obtained by reduction of the unique disulfide bridge of the protein and further carboxymethylation of the resulting free cysteine residues, resulted in a very broad band at long migration times in the electropherogram due to aggregation. Indeed, the fully unfolded protein aggregates on a time scale faster than that required for electrophoretic separation under the conditions used here, making it impossible to detect it as a sharp peak.

**Binding of I₂ to Congo Red**—The electrophoretic pattern obtained from the acetonitrile-denatured protein is identical to that obtained previously by Heegaard et al. (15, 18). This makes it possible to identify the slow running species observed...
CE that CR has a higher affinity for I$_2$ than for N (Fig. 6). In addition, as described by these authors, we have found by migration shift of the I$_2$ peak, whereas the migration of N is concentrations of CR to the running buffer results in an anodic Indeed, it is clear from Fig. 6 that addition of increasing con-

further the conversion of this species into N. The possibility affinity to CR, which is therefore responsible for decelerating was determined following the addition of CR (Fig. 4)

suggests that the I$_2$ species exhibits a significant binding under conditions of pH and temperature close to physiological

previously and named β$_2$-m, with the slow refolding species I$_2$. In addition, as described by these authors, we have found by CE that CR has a higher affinity for I$_2$ than for N (Fig. 6). Indeed, it is clear from Fig. 6 that addition of increasing concentrations of CR to the running buffer results in an anodic migration shift of the I$_2$ peak, whereas the migration of N is almost unaffected. In order to verify the effect of CR on the rate of conversion of I$_2$ into N state, we have refolded β$_2$-m from 50% to 18% acetonitrile in the presence of 1 mM CR, as described under “Experimental Procedures.” A 40% reduction of $k_{I_2 \rightarrow N}$ was determined following the addition of CR (Fig. 4C). This suggests that the I$_2$ species exhibits a significant binding affinity to CR, which is therefore responsible for decelerating further the conversion of this species into N. The possibility that CR could induce protein precipitation during the refold-

ing procedure has been excluded by measuring spectrophotometrically β$_2$-m (centrifuged at 10,000 × g) before and after refolding in the presence of CR. Furthermore, the conditions recorded in the CE runs exclude any protein precipitation. Indeed, no current drop or capillary clogging was registered. Nor did the peak area and peak shape of the native protein appear altered upon refolding in the presence of CR relative to that obtained after refolding in its absence.

Propensity of I$_2$ and N to Aggregate—β$_2$-m was incubated under conditions of pH and temperature close to physiological in the presence and absence of fibrils extracted from hemodia-

lyzed patients. While no substantial aggregation was observed in the samples devoid of preformed fibrils, conversion from the soluble to the fibrillar state was obtained in the samples con-

taining such material. Fig. 7A shows an electron micrograph with fibrils obtained under such conditions. By comparison, Fig. 7B shows an electron micrograph of ex vivo preformed fibrils that, as previously shown (11), appears as a densely packed fibrillar material. It is clear that fibrils grown in vitro are morphologically distinguishable from those present initially to seed fibril elongation. In addition, the fluorescence of ThT undergoes a slow but significant increase during the incu-

bation of β$_2$-m with ex vivo fibrils, indicating that the fibrillar material formed following such incubation represents, to a large extent, newly formed fibrils.

The extension of the initial fibrils is, however, a very slow process at pH values close to physiological. In order to discrimin-

ate between the propensity to aggregate of I$_2$ and that of N, a much faster aggregation process is required as aggregation of I$_2$ needs to be evaluated from 5 s to 1–2 min after the initiation of folding, when I$_2$ is maximally populated. Our test therefore required particularly strong aggregating conditions. Aggregation of β$_2$-m was reported to follow a kinetic equation of the following type (19),

$$v = k_{agg} [S] [M] - k_{disagg} [S]$$

(Eq. 3)

where $v$ is the rate of extension of preexisting fibrils; $k_{agg}$ and $k_{disagg}$ are the rate constants for aggregation and disaggrega-

tion, respectively; [S] is the concentration of fibrils; and [M] is the concentration of monomeric β$_2$-m. Strong aggregating con-

ditions were therefore achieved by allowing folding of β$_2$-m at relatively high protein concentrations and in the presence of amyloid fibrils extracted from a patient (see “Experimental Procedures” for further details).

During folding in the presence of preformed fibrils, β$_2$-m has a considerable tendency to aggregate, as deduced by the ThT test (Fig. 7C, first bar). That such aggregates originate under the conditions used to initially denature the protein can be ruled out, since the increase of ThT fluorescence following incubation under unfolding conditions was largely negligible. Aggregation was also measured for the protein freshly dissolved under the same conditions of pH, temperature, protein, and fibril concentration (Fig. 7C, second bar). A significant increase of ThT fluorescence was observed, although this was 20 ± 7% of that measured during folding (the nonspecific increase caused by the protein in the absence of preformed fibrils was subtracted from the measured fluorescence values to obtain this estimate). When the experiments were repeated in the absence of preformed fibrils, a very strong increase of fluorescence was observed regardless of the β$_2$-m conformation (Fig. 7C, third to fifth bar).

In order to minimize further the problems related to aggregation in the initial denaturing conditions and within the first milliseconds immediately after dilution in the refolding buffer,
a procedure of rapid ultrafiltration following initiation of folding was employed as described under “Experimental Procedures.” Both fibrils and soluble $\beta \_2$-m appeared concentrated 3.3 times with respect to the initial conditions. The results, reported in Fig. 7D, are qualitatively similar to those described in Fig. 7C. While in the absence of preexisting fibrils no significant aggregation appears to occur, in their presence the increase of ThT is remarkable and is considerably higher during folding. The increase of ThT fluorescence induced by the freshly dissolved protein corresponds to 17 ± 6% of that measured during folding.

**DISCUSSION**

The results reported here show that the native state of the MHCI-dissociated form of $\beta \_2$-m consists of an equilibrium of two conformations, a largely populated one, which we call N, and another, which we call $I \_2$, that represents only ~15% of total $\beta \_2$-m at 30 °C and pH 7.4. The latter conformation corresponds to a partially folded species that forms during the folding process of $\beta \_2$-m and converts into the fully folded conformation N very slowly. Such conversion is only marginally faster than the unfolding reaction, and therefore the $I \_2$ species remains populated to a small but significant extent after completion of the folding reaction. That $I \_2$ is populated at equilibrium is indicated by two independent observations (i.e. from the ratio of folding and unfolding rate constants determined in the absence of denaturant and from the two distinct peaks detected in the electropherogram of the protein freshly dissolved in a physiological buffer). The relative normalized area of the peak corresponding to $I \_2$ does not depend on whether the protein is freshly dissolved in buffer or is folded from an acetonitrile-denatured state to completion. Furthermore, the existence of $I \_2$ at equilibrium can be deduced from a pure kinetic analysis, indicating that the presence of this partially folded species is due to a real chemical equilibrium and does not arise from a fraction of protein that has lost irreversibly the ability to reach the N conformation. The $I \_2$ species corresponds to the abnormally folded conformation previously identified by Heegaard et al. (15, 18) with the slow running species of a CE profile obtained from the acetonitrile- or trifluoroethanol-denatured $\beta \_2$-m. Here we have shown that, although to a lesser
and in the extracellular spaces and that in vivo particular events are required to destabilize the native fold of $\beta_2$-m and therefore initiate the aggregation process (9, 10, 19, 21). Our results are in agreement with these findings, since fibrillar formation does not occur in the absence of preformed fibrils at pH 7.4. Various hypotheses have been put forward as possible mechanisms nucleating aggregation in vivo. These include destabilization of a fraction of $\beta_2$-m molecules by proteolytic cleavage of the six N-terminal residues (9), uptake into lysosomes of macropathies with consequent exposure of the protein to acidic pH values at which aggregation is more favored (10, 19), and destabilization by Cu$^{2+}$ ions only released by hemodialyzed membranes (21).

While a particular environment is required to nucleate formation of the $\beta_2$-m fibrils, other observations indicate that elongation of preformed fibrils from soluble $\beta_2$-m under physiological conditions is possible, in the absence of any other factors, at a rate that is slow but relevant on the time scale at which dialysis-related amyloidosis occurs (9, 19, 22). Our observation that $\beta_2$-m has the ability to elongate preexisting fibrils at physiological pH values with a consequent increase of ThT fluorescence supports this view. The role of components other than $\beta_2$-m within the extracted material (23), such as glycosaminoglycans, ApoE, collagen, etc., in priming the fibril elongation by $I_2$ is a matter of future investigation. However, we propose that a plausible mechanism of fibril elongation occurring in dialysis-related amyloidosis involves a partially folded structure of $\beta_2$-m that is significantly populated at equilibrium (Fig. 8). This species, corresponding to a partially folded conformation that forms during folding of oxidized $\beta_2$-m, is likely to represent a significant fraction of the MHC-I-dissociated circulating pool of $\beta_2$-m in the plasma and to drive the conversion of $\beta_2$-m from the soluble to the amyloid state. Structural characterization of $I_2$ will be the object of future work, since this species may represent an important target for drug design. In addition, the possibility of singing out and quantifying this fraction by CE opens new opportunities to test hemodialytic procedures in which the equilibrium between N and $I_2$ could be modified by factors such as pH, interaction with artificial membranes, hydrostatic pressures, and composition of hemodialysis solution.

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