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A Partially Structured Species of β_2-Microglobulin Is Significantly Populated under Physiological Conditions and Involved in Fibrillogenesis*

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The folding of β_2-microglobulin (β_2-m), the protein forming amyloid deposits in dialysis-related amyloidosis, involves formation of a partially folded conformation named I_2, which slowly converts into the native fold, N. Here we show that the partially folded species I_2 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_2 conformation is populated to ~14 ± 8% at equilibrium under conditions of pH and temperature close to physiological. In the presence of fibrils extracted from patients, the I_2 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 β_2-m *in vivo* involving the association of the preformed fibrils with the fraction of I_2 existing at equilibrium is proposed from these results. The possibility of isolating and quantifying a partially folded conformer of β_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 β_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 β_2-microglobulin (β_2-m).[1] In its native form, this 99-residue protein is constituted by two β-sheets packed against each other to form a fold typical of the immunoglobulin superfamily (5). The two β-sheets, constituted by three and four strands, respectively, interact by means of hydrophobic interactions and a disulfide bridge that stabilizes further the β-sandwich structure.

β_2-m constitutes the light chain of the major histocompatibility complex class I (MHCI). A significant pool of β_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 β_2-m to increase significantly (6). In addition, β_2-m cannot be filtered efficiently through the artificial membrane, resulting in an increase of the levels of soluble β_2-m from 0.3 to 30 μg/ml, the range of concentrations observed within healthy individuals, to ~40 μg/ml (7). The increase of free circulating β_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 β_2-m concentration and clinical improvement, no dialysis membrane has yet been designed to reduce the β_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 β_2-m into amyloid fibrils. The ability of β_2-m to form amyloid fibrils *in vitro* 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 β_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: β_2-m, β_2-microglobulin; ThT, thioflavine T; MHCI, major histocompatibility complex class I; GdnHCl, guanidinium chloride; CE, capillary electrophoresis; CR, Congo Red.

since the disulfide bridge is found to be intact in the β_2-m constituents of natural amyloid fibrils (11).

The characterization of the folding process from denatured β_2-m with an intact disulfide bridge has revealed a complex folding scheme (12),

$$U \overset{\text{very fast}}{\rightleftharpoons} I_1 \overset{\text{fast}}{\rightleftharpoons} I_2 \overset{\text{slow}}{\rightleftharpoons} N$$

SCHEME 1

The I_1 ensemble forms from the unfolded state on the submillisecond time scale. The I_2 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 I_1 and I_2 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 I_2 species, is very unusual for a protein of the size of β_2-m. In this study, we investigate the role of this folding intermediate in the amyloidogenesis process of β_2-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 β_2-m is very likely to arise from the fraction of protein in this conformation.

EXPERIMENTAL PROCEDURES

Materials—β_2-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 β_2-m, stabilized by an intermolecular disulfide bridge, was obtained as a by-product of the expression system used to produce recombinant β_2-m (9). This covalently linked dimeric β_2-m is detected as an acidic band in native electrophoresis of solubilized recombinant β_2-m inclusion bodies. This species was separated from monomeric β_2-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 β_2-m was obtained as described (13). The identity and purity of the β_2-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 Na_2HPO_4 and NaH_2PO_4 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* β_2-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 β_2-m composition and microscopic pattern (11). By SDS-polyacrylamide gel electrophoresis and immunoblot, more than 90% of the extracted material appears to be β_2-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 I_2 into N ($k_{I_2 \to N}$) and of global unfolding (k_{unf}) in the absence of denaturant were determined from the intercepts of plots of ln $k_{I_2 \to N}$ and ln k_{unf} *versus* guanidinium chloride (GdnHCl) concentration. 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 I_2 species or by an alternative pathway reaching the U conformation without formation of I_2. If these two unfolding mechanisms are both possible, their $k_{unf} = k_{N \to U} + k_{N \to I_2}$, where $k_{N \to I_2}$ is the rate constant of unfolding when this occurs via the I_2 species (in this pathway, the conversion of N into I_2 is the rate-determining step, since the following steps are much faster) and $k_{N \to U}$ is the rate constant of the alternative unfolding process. Under strong refolding conditions, $k_{I_2 \to N}$ and k_{unf} are 1 order of magnitude faster than $k_{I_1 \to I_2}$, $k_{I_2 \to I_1}$, $k_{I_1 \to U}$, and $k_{U \to I_1}$ (12). Moreover, $k_{I_1 \to I_2}$ is much faster than $k_{I_2 \to I_1}$, and $k_{U \to I_1}$ is much faster than $k_{I_1 \to U}$ (data not published). The analytical inspection of this reaction leads to Equation 1,

$$[I_2]_{eq}/[\beta_2\text{-m}]_0 = k_{N \to I_2} + k_{N \to U}/(k_{N \to I_2} + k_{N \to U} + k_{I_2 \to N}) \qquad \text{(Eq. 1)}$$

where $[I_2]_{eq}/[\beta_2\text{-m}]_0$ has the same meaning as in Equation 2 (*i.e.* the ratio between the equilibrium concentration of I_2 and that of total protein). If the two mechanisms of unfolding we have considered above are both possible, then $k_{unf} = k_{N \to U} + k_{N \to I_2}$ and Equation 1 becomes Equation 2. If the direct conversion of N into U represents a negligible contribution to the overall unfolding process, then $k_{N \to U} \sim 0$ and $k_{unf} = k_{N \to I_2}$. On the other hand, if unfolding through formation of I_2 is negligible, then $k_{N \to I_2} \sim 0$ and $k_{unf} = k_{N \to U}$. 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 millibars 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 Na_2HPO_4, 1.5 mM KH_2PO_4, 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 every conformational population as a percentage of total protein.

Folding Kinetics Followed by Far UV Circular Dichroism—A Jasco 710 spectropolarimeter was used to monitor the change of mean residue ellipticity of β_2-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 a dilution to 18% acetonitrile. Final protein concentration was 0.2 mg ml^{-1}. Measurements were performed at 20 °C in cells with 0.1-cm path length. The folding rate constant was measured as described (12).

Congo Red Affinity of β_2-m—β_2-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 β_2-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} β_2-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 Na_2HPO_4 and 254 mM NaH_2PO_4 in the presence or absence of β_2-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 β_2-m is in the I_2 conformation (12). Fluorescence values of suitable blanks obtained in each case using identical solutions without protein were subtracted from the measured values. In a second set of experiments, the initial β_2-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 cut-off membrane. Soluble β_2-m and fibrils appeared to be 3.3 times more concentrated than immediately after dilution. For the ThT test 15 μl of protein sample

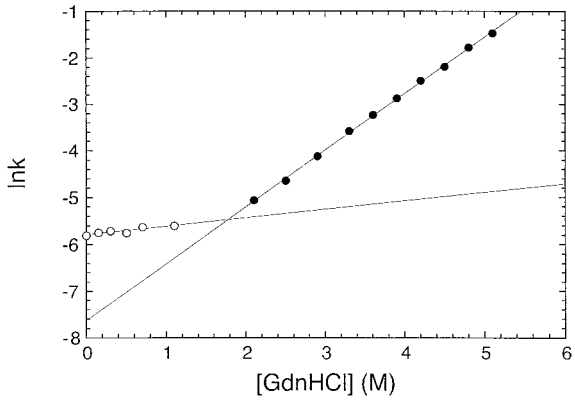

FIG. 1. **Folding and unfolding rate of β_2-m.** Dependence of the natural logarithm on the rate constant for the conversion of I_2 into N (*empty circles*) and for unfolding (*filled circles*) on GdnHCl concentration. The *solid lines* represent the linear equations best fitted to the two sets of data points.

were mixed with 485 μl of ThT solution. All other conditions are as described for the first set of experiments.

Electron Microscopy—Images were acquired using a Jeol JEM 1200 EX electron microscope operating at 80 kV excitation voltage. In each case, 3.0 μl of protein solution was placed on a Formvar- and carbon-coated grid and air-dried after 2–3 min. The sample was then stained with 2% uranyl acetate, air-dried, and observed at a magnification of × 6000–30,000.

RESULTS

Folding and Unfolding of β_2-m—Conversion of I_2 into N is the third exponential phase observed during folding of oxidized β_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 1 order of magnitude faster than the conversion of I_2 into N and global unfolding (12). In this mechanism, Equation 2 can be demonstrated,

$$[I_2]_{eq}/[\beta_2\text{-m}]_0 = k_{unf}/(k_{unf} + k_{I_2 \rightarrow N}) \quad \text{(Eq. 2)}$$

where $[I_2]_{eq}$ is the equilibrium concentration of I_2, and $[\beta_2\text{-m}]_0$ 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 ± 0.08 for $[I_2]_{eq}/[\beta_2\text{-m}]_0$ 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 β_2-m represents therefore a mixture of the real N state and of the residual I_2 species, the latter representing ~14 ± 8% of total β_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 β_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

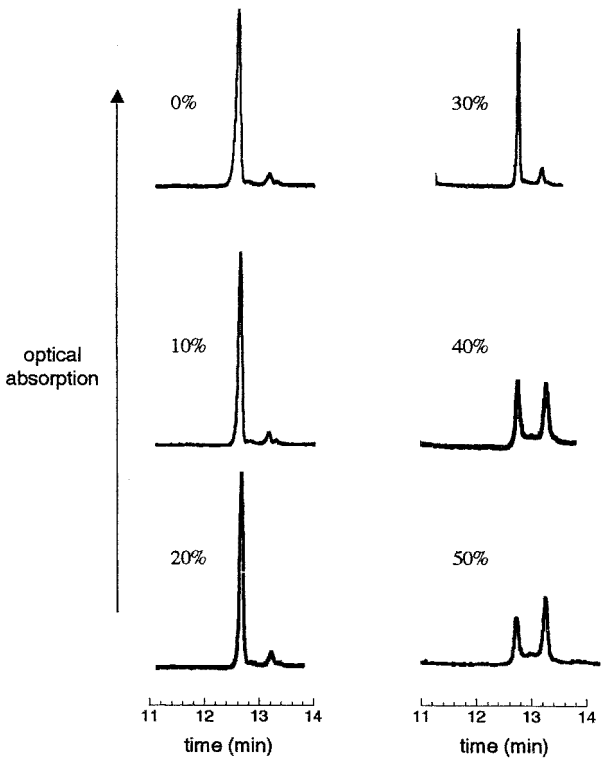

FIG. 2. **Profiles of CE obtained from β_2-m preincubated at various concentrations of acetonitrile.** β_2-m was equilibrated at the indicated percentages of acetonitrile and then injected into the capillary equilibrated in the absence of acetonitrile. Other details regarding procedures and analytical conditions are given under "Experimental Procedures."

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 β_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, β_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 β_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

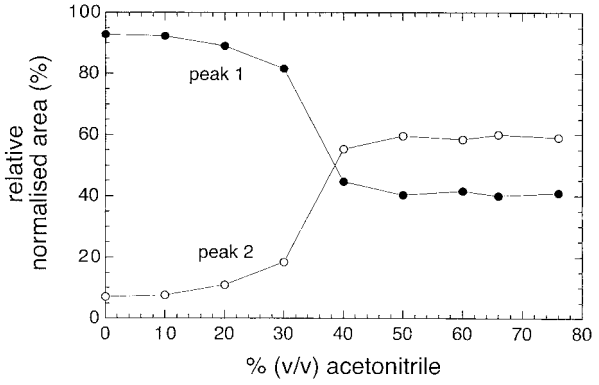

FIG. 3. **Change of the relative normalized area of the two CE peaks monitored during unfolding.** Shown is the change of the percentage of peak 1 (*filled circles*) and peak 2 (*empty circles*) detected in CE following preincubation at increasing acetonitrile concentrations. Procedures and analytical conditions are given under "Experimental Procedures."

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. 4*A* 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. 4*B*) and previously attributed to the conversion of I_2 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 I_2. 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 I_2 ($[I_2]_{eq}/[\beta_2\text{-m}]_0$), is lower than that calculated from the kinetic analysis (~14%), an observation consistent with the lower operating capillary temperature (10 *versus* 30 °C).

The electrophoretic mobility of a β_2-m dimer stabilized by an intermolecular disulfide bridge was also analyzed (Fig. 5). The main peak obtained when dimeric β_2-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 β_2-m, are attributable to "monomeric impurities" in the sample of the dimer. However, the low mobility major peak obtained with dimeric β_2-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 β_2-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 β_2-m, obtained by reduction of the unique disulfide bridge of the protein

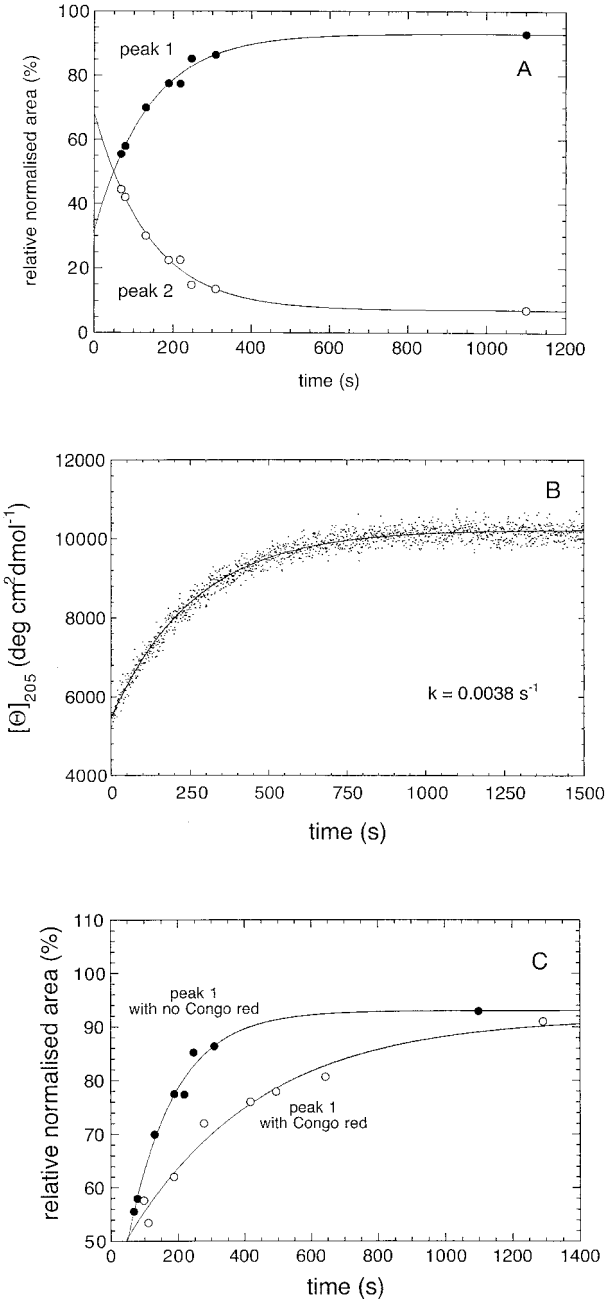

FIG. 4. **Change of the relative normalized area of CE peaks monitored during folding.** *A*, the time reported on the *x* axis corresponds to the delay time (*i.e.* the time elapsed from sample dilution to injection into the capillary). *B*, change of mean residue ellipticity of β_2-m at 205 nm during folding. The rate constant value (*k*) is reported in the *figure*. *C*, change of the relative normalized area of the first CE peak monitored during folding in the presence (*empty circles*) and in the absence (*filled circles*) of 1 mM Congo Red in the β_2-m sample. The time reported on *x* axis corresponds to the delay time, *i.e.* the time elapsed from sample dilution to injection into the capillary.

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 I_2 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

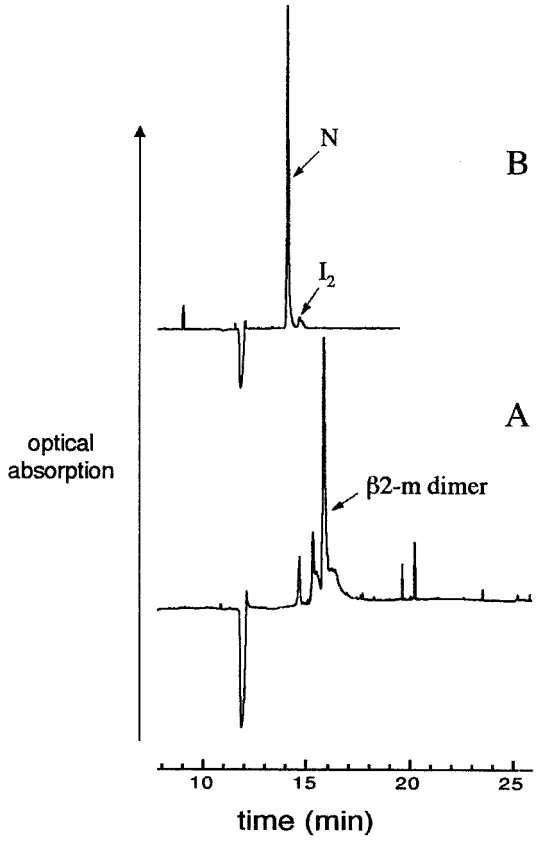

FIG. 5. **CE profiles of dimeric (*A*) and native (*B*) β_2-m.** Effective mobilities are as follows: dimer, -0.67×10^{-4} cm^2 V^{-1} s^{-1}; native, -0.51×10^{-4} cm^2 V^{-1} s^{-1}; I_2, -0.60×10^{-4} cm^2 V^{-1} s^{-1}.

previously and named β_2-m$_s$ 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. 4*C*). 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 refolding procedure has been excluded by measuring spectrophotometrically β_2-m (centrifuged at $10{,}000 \times g$) 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 hemodialyzed 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 containing such material. Fig. 7*A* shows an electron micrograph with fibrils obtained under such conditions. By comparison, Fig. 7*B* shows an electron micrograph of *ex vivo* preformed fibrils that, as previously shown (11), appears as a densely

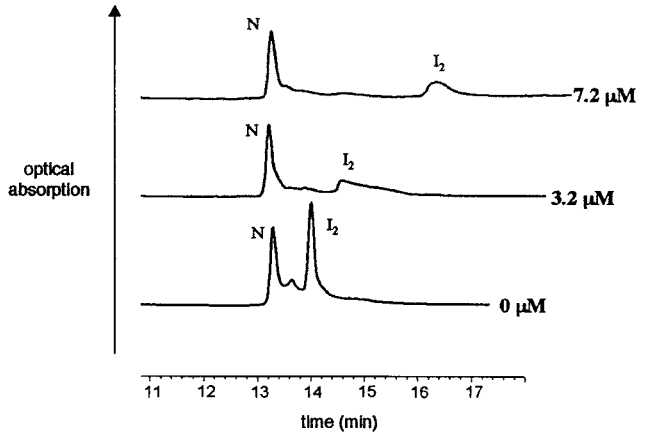

FIG. 6. **Binding of I_2 to Congo Red.** Shown is CE analyses of β_2-m sample initially denatured in 50% acetonitrile. Electrophoretic separation was carried out in the absence and in the presence of 3.2 and 7.2 μM Congo Red in the running buffer. Other details regarding procedures and analytical conditions are given under "Experimental Procedures."

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 incubation 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 discriminate 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),

$$v = k_{agg}\,[S]\,[M] - k_{disagg}\,[S] \qquad \text{(Eq. 3)}$$

where v is the rate of extension of preexisting fibrils; k_{agg} and k_{disagg} are the rate constants for aggregation and disaggregation, respectively; [S] is the concentration of fibrils; and [M] is the concentration of monomeric β_2-m. Strong aggregating conditions 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. 7*C*, *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. 7*C*, *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 weak increase of fluorescence was observed regardless of the β_2-m conformation (Fig. 7*C*, *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,

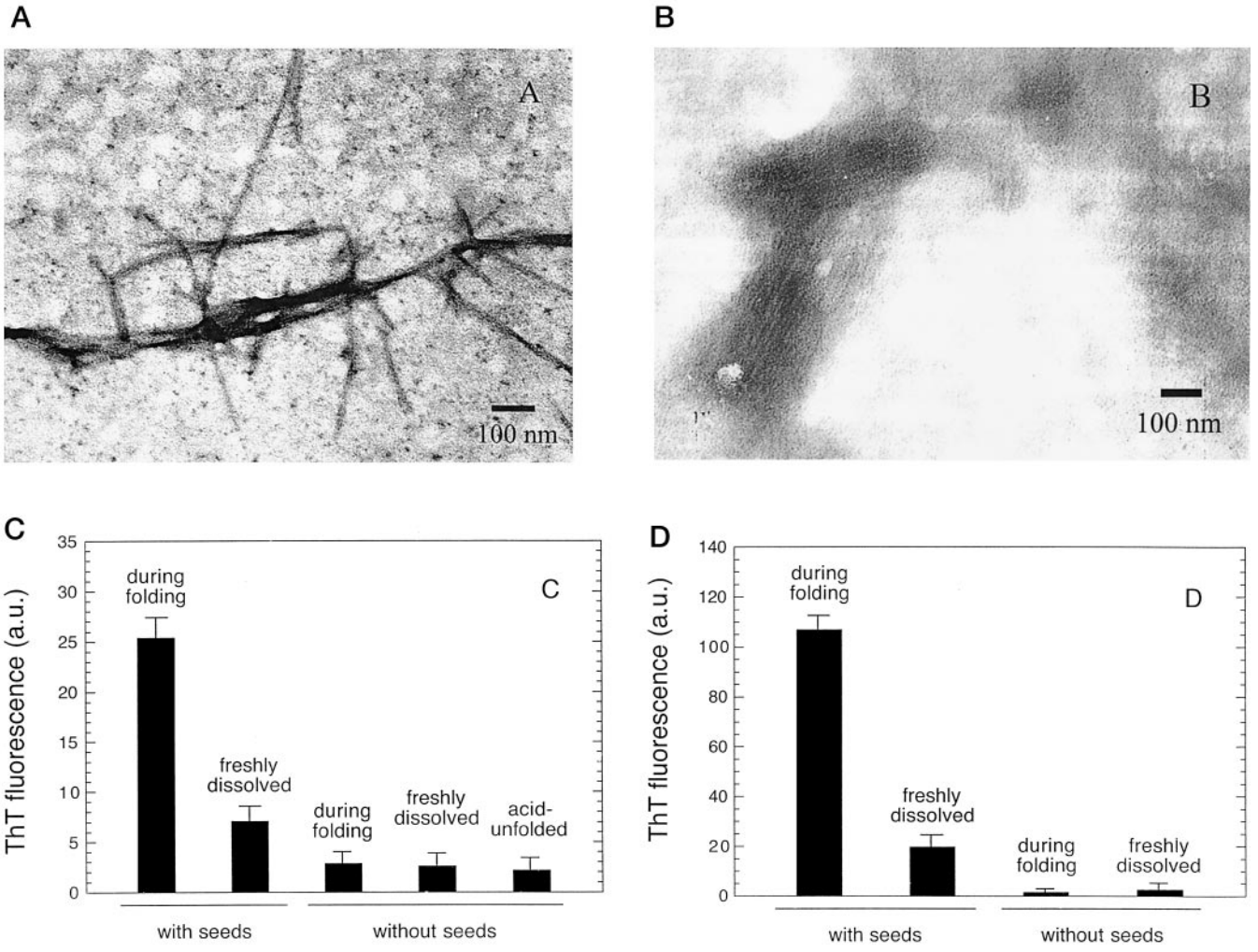

FIG. 7. **Propensity to aggregate of β_2-m and I_2.** *A*, electron microscopy (*EM*) image of fibrils grown from soluble β_2-m at pH 7.4 in the presence of fibrils extracted from a patient. *B*, EM image of natural preformed fibrils before any *in vitro* fibril elongation process has occurred. *C*, increase of ThT fluorescence induced by β_2-m during folding, when most of the protein is in the I_2 conformation (*first* and *third bars*), and of the freshly dissolved protein, when N is instead the most represented conformation (*second* and *fourth bars*). The ThT test was also carried out for the acid-denatured protein to rule out that the aggregates originated in the initial denaturing conditions (*fifth bar*). The experiments were done in the presence (*first* and *second bars*) and absence (*third*, *fourth*, and *fifth bars*) of fibrils extracted from the bones of a patient. *D*, the data have the same significance as in *A*, but the experiment was carried out using a procedure of ultrafiltration, immediately after the initiation of folding, to concentrate both monomeric β_2-m and fibrils (when present).

a procedure of rapid ultrafiltration following initiation of folding was employed as described under "Experimental Procedures." Both fibrils and soluble β_2-m appeared concentrated 3.3 times with respect to the initial conditions. The results, reported in Fig. 7*D*, are qualitatively similar to those described in Fig. 7*C*. 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 β_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 β_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 β_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 β_2-m. Here we have shown that, although to a lesser

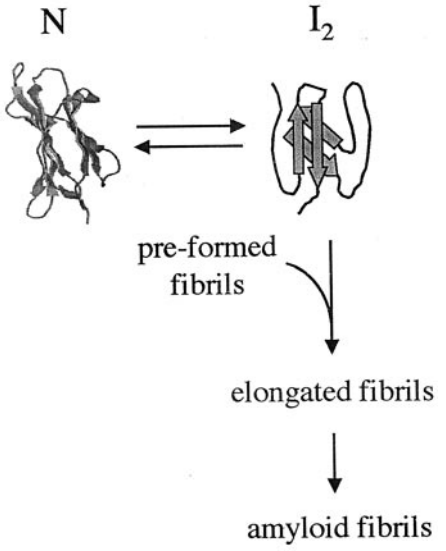

FIG. 8. **Proposed mechanism of fibril elongation of β_2-m in dialysis-related amyloidosis.** The fully native state (N) is in a reversible equilibrium with a partially folded conformation (I_2). The I_2 species is populated to ~15% (over total β_2-m) under conditions close to physiological and similar to those present in the plasma, where amyloid formation by β_2-m occurs. Preformed fibrils interact preferentially with I_2, allowing the aggregates to grow. The equilibrium between N and I_2 is fast compared with the process of amyloid growth. The same fraction of monomeric β_2-m is therefore available, in the form of I_2, as a substrate for the aggregation process.

extent, such a species is also present under conditions close to physiological in the absence of any denaturant.

The I_2 conformation appears less structured than N. Spectroscopic investigation indicates the presence of a lower level of β-sheet structure and a less packed hydrophobic core (12, 18). The higher unfolding rate observed previously (12) and the lower electrophoretic mobility observed here also indicate that I_2 presents a degree of folded structure lower than N. The second peak in the CE electropherogram of β_2-m refolded from acetonitrile, now identified with I_2, was also shown to have a preferential and higher affinity for the Congo Red amyloid-specific dye and for 8-anilino-1-naphthalene sulfonic acid than N (15, 18). Partially folded structures of this type are very susceptible to aggregation, since part of the hydrophobic residues and polypeptide backbone are available for intermolecular interactions (8, 20). A few seconds after the initiation of folding, when most of β_2-m is in the I_2 conformation, the protein appears to be more prone to aggregation than the freshly dissolved one, in which N is instead the most represented species. Importantly, the increase of ThT fluorescence induced by the freshly dissolved protein is 17 ± 6% (or 20 ± 7%) of that measured during folding. This value is very similar to the estimated fraction of I_2 in the freshly dissolved protein sample under these conditions of temperature (14 ± 8%), suggesting that the aggregation of the freshly dissolved protein may also arise from the fraction of I_2 present in the sample. Importantly, the peak area of the electropherogram corresponding to the I_2 species appears reduced when a β_2-m sample is analyzed after preincubation with natural fibrils, indicating preferential interaction of the fibrils with I_2.[2]

There seem to be a general consensus that β_2-m is relatively stable *in vitro* at pH values close to those found in the plasma and in the extracellular spaces and that *in vivo* particular events are required to destabilize the native fold of β_2-m and therefore initiate the aggregation process (9, 10, 19, 21). Our results are in agreement with these findings, since fibril 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 β_2-m molecules by proteolytic cleavage of the six N-terminal residues (9), uptake into lysosomes of macrophages with consequent exposure of the protein to acidic pH values at which aggregation is more favored (10, 19), and destabilization by Cu^{2+} ions possibly released by hemodialyzed membranes (21).

While a particular environment is required to nucleate formation of the β_2-m fibrils, other observations indicate that elongation of preformed fibrils from soluble β_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 β_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 β_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 β_2-m that is significantly populated at equilibrium (Fig. 8). This species, corresponding to a partially folded conformation that forms during folding of oxidized β_2-m, is likely to represent a significant fraction of the MHCI-dissociated circulating pool of β_2-m in the plasma and to drive the conversion of β_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 singling 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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A Partially Structured Species of β_2-Microglobulin Is Significantly Populated under Physiological Conditions and Involved in Fibrillogenesis

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