Role of the single disulphide bond of β_2 -microglobulin in amyloidosis in vitro

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Abstract

The aggregation of β_2 -microglobulin (β_2 m) into amyloid fibrils occurs in the condition known as dialysis-related amyloidosis (DRA). The protein has a β -sandwich fold typical of the immunoglobulin family, which is stabilized by a highly conserved disulphide bond linking Cys25 and Cys80. Oxidized β_2 m forms amyloid fibrils rapidly in vitro at acidic pH and high ionic strength. Here we investigate the role of the single disulphide bond of β_2 m in amyloidosis in vitro. We show that reduction of the disulphide bond destabilizes the native protein such that non-native molecules are populated at neutral pH. These species are prone to oligomerization but do not form amyloid fibrils when incubated for up to 8 mo at pH 7.0 in 0.4 M NaCl. Over the pH range 4.0–1.5 in the presence of 0.4 M NaCl, however, amyloid fibrils of reduced β_2 m are formed. These fibrils are ~10 nm wide, but are shorter and assemble more rapidly than those produced from the oxidized protein. These data show that population of non-native conformers of β_2 m at neutral pH by reduction of its single disulphide bond is not sufficient for amyloid formation. Instead, association of one or more specific partially unfolded molecules formed at acid pH are necessary for the formation of β_2 m amyloid in vitro. Further experiments will now be needed to determine the role of different oligomeric species of β_2 m in the toxicity of the protein in vivo.

Keywords: β_2 -Microglobulin; amyloidosis; disulphide bond; protein fibril

The term amyloid has been used to describe extracellular and intracellular fibrillar protein deposits associated with disease (Glenner 1980). The amyloid plaque is the end result of a dynamic process in which insoluble fibrils are assembled from initially soluble protein monomers, often together with other constituents, such as serum amyloid P component and glycosaminoglycans (Floege and Ehlerding 1996). Currently, ~20 proteins are known to be associated with human amyloid (Sunde et al. 1997). Even though the structure of the soluble form of the known human amyloido-

genic proteins varies widely and includes α -helical, β -sheet, and mixed α/β proteins, the fibrils they produce exhibit similar structural characteristics. Electron microscopy (Sunde and Blake 1997; Jimenez et al. 1999), dye-binding assays (Naiki et al. 1989; Klunk et al. 1999), and X-ray diffraction studies (Sunde et al. 1997) have established that amyloid fibrils are long, unbranching protein fibers with a cross- β structure.

Dialysis-related amyloidosis (DRA) involves the aggregation of β_2 -microglobulin (β_2 m) into amyloid deposits and is a serious complication of long-term haemodialysis (Gejyo et al. 1986). β_2 m is a small (99-residue) protein that has a seven-stranded β -sandwich fold typical of proteins belonging to the immunoglobulin superfamily (Bjorkman et al. 1987) (Fig. 1). The two β -sheets are held together by a single buried disulphide bond that links Cys25 and Cys80 in β -strands 2 and 6, respectively. The disulphide bond is highly conserved in the immunoglobulin superfamily and stabilizes the native fold of these proteins (Isenman et al., 1975). In vivo, β_2 m is continuously shed from the surface of

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Abbreviations: β_2 m, human β_2 -microglobulin; thio-T, thioflavin-T; ANS, 1-anilinonaphthalene-8-sulphonic acid; DRA, dialysis-related amyloidosis; ESI MS, electrospray ionisation mass spectroscopy; GuHCl, guanidinium chloride; red- β_2 m, β_2 m in which the single disulphide bond is reduced; ox- β_2 m, β_2 m in which the single disulphide bond is oxidized.

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Fig. 1. Ribbon diagram of the structure of human β_2m taken from the crystal structure of the MHC class I complex (PDB 3HLA) (Bjorkman et al. 1987). The position of the β -strands were based on NMR measurements of the isolated monomeric protein in solution (Okon et al. 1992) and the crystal structure of the protein (Bjorkman et al. 1987). The inter-sheet disulphide bond connecting Cys25 and Cys80 is shown. The figure was drawn using Swiss-PDB Viewer (Guex and Peitsch 1997).

cells displaying MHC class I molecules. It is then carried in the plasma to the kidneys where it is degraded and excreted. As a consequence of renal failure, the concentration of β_2m in the plasma increases 25– to 35-fold (Gejyo et al. 1986), which ultimately leads to the deposition of the protein into amyloid fibrils in the musculoskeletal system (Ritz and Zeier 1996). Full-length wild-type β_2m , as well as modified and truncated versions of the protein, have been found in β_2m fibrils *ex vivo*, although no natural mutations of the gene sequence have been associated with the disease (Floegel and Ehlerding 1996; Esposito et al. 2000). The role of sequence modifications in the mechanism of amyloidosis of β_2m *in vitro* and the influence of these and other factors in the development of the disease *in vivo* are currently poorly understood.

β_2m amyloidosis *in vitro* has been shown to be critically dependent on the pH and ionic strength of the solution (Connors et al. 1985; Ono and Uchino 1994; Naiki et al. 1997; Bellotti et al. 1998; McParland et al. 2000). Fibrils of full length β_2m have not been generated at neutral pH to date in the absence of additional factors (Ono and Uchino

1994). The addition of other factors, however, including serum amyloid P component (Ono and Uchino 1994), copper (Morgan et al. 2001), or air drying the protein onto dialysis membranes, has been shown to induce fibril formation at pH 7 (Connors et al. 1985). In contrast, fibrils form rapidly *in vitro* at low pH and high ionic strength (Naiki et al. 1997; McParland et al. 2000). Akin to studies of other proteins (Kocisko et al. 1996; Lai et al. 1996; Booth et al. 1997; Raffin et al. 1999), conditions favoring fibrillogenesis of β_2m lead to partial unfolding of the native monomeric protein (McParland et al. 2000). Based on this result, and the observation that the rate of β_2m fibrillogenesis *in vitro* correlates closely with the concentration of partially unfolded molecules, it has been suggested that one or more partially unfolded species are key to β_2m amyloidosis (McParland et al. 2000). On average, these species retain substantial secondary structure, lack the fixed tertiary structure characteristic of the native protein, bind the hydrophobic dye 1-anilinonaphthalene-8-sulphonic acid (ANS), and are weakly protected from hydrogen exchange (McParland et al. 2000; Esposito et al. 2000). The fibrils produced by incubation of β_2m at pH 3.6 in 0.4 M NaCl at 37°C are ~10 nm wide, short (50–200 nm), and have a curvilinear morphology (McParland et al. 2000). On further acidification (to pH 1.6), the fibrils formed have the same width and morphology as those produced at pH 3.6, but extend to greater than 600 nm in length. Under these conditions a second acid-denatured species is generated that is less structured than partially unfolded β_2m at pH 3.6 (McParland et al. 2000).

The data obtained so far are consistent with a model for β_2m fibrillogenesis *in vitro* in which one or more partially unfolded molecules of the protein associate to form ordered fibrillar assemblies (McParland et al. 2000). To test this hypothesis further and to examine the role of protein stability in β_2m amyloidosis, we performed a study of the amyloidogenic properties of reduced β_2m (red- β_2m) *in vitro*, in which the single disulphide bond linking Cys25 and Cys80 has been disrupted (Fig. 1). We show that red- β_2m is non-native even at neutral pH. Nevertheless the protein does not form amyloid fibrils under these conditions. Like its oxidized counterpart, red- β_2m forms fibrils at acidic pH and high ionic strength. The data indicate that destabilization of β_2m by reducing its disulphide bond is not sufficient for amyloidosis, but association of specific partially unfolded molecules formed at acidic pH are necessary for amyloidosis of this protein *in vitro*.

Results

Spectroscopic studies of red- β_2m as a function of pH

β_2m was reduced by a procedure involving denaturation of the protein in 6 M guanidinium chloride (GuHCl) in the

presence of DTT (Isenman et al. 1975). The reduced protein was then refolded by rapid removal of the denaturant using gel filtration (see Materials and Methods). Under the conditions used, the protein was shown to be fully reduced using mass spectrometry and HPLC (see Materials and Methods).

Characterization of the conformational properties of red- β_2 m as a function of pH was performed using a variety of spectroscopic techniques. The far UV CD spectra of ox- β_2 m and red- β_2 m at pH 7.0 are shown in Figure 2A. Ox- β_2 m at

pH 7.0 and 10°C is native (Okon et al. 1992; McParland et al. 2000). The far UV CD spectrum of this species is characteristically weak in intensity, as is often observed for β -sheet proteins (Woody 1995). Positive bands at 270 and 290 nm are observed in the near UV CD spectrum of ox- β_2 m at pH 7.0, consistent with the packing of aromatic rings in a fixed conformation in the native protein (Fig. 2D). On reduction of the disulphide bond, there is a large increase in intensity of the negative ellipticity in the far UV CD, suggesting that β -sheet secondary structure persists in red- β_2 m

Fig. 2. Far and near UV CD spectra of red- β_2 m and ox- β_2 m. (○) Red- β_2 m; (●) ox- β_2 m. All spectra were acquired at 10°C and a protein concentration of 0.2 mg/ml. Far UV CD spectra at (A) pH 7.0, (B) pH 3.6, (C) pH 1.5. Near UV CD spectra at (D) pH 7.0, (E) pH 3.6 and (F) pH 1.5.

at neutral pH (Fig. 2A). In addition, there is a small, but significant, decrease in intensity of the near UV CD spectrum on reduction of the protein at pH 7.0, suggesting that the tertiary structure of the reduced protein is altered relative to that of its native, oxidized counterpart (Fig. 2D). Analytical ultracentrifugation studies showed that although the oxidized protein is monomeric at pH 7, the reduced protein forms a mixture of monomers and tetramers at this pH (Table 1). Changes in tertiary structure (involving aromatic rings buried in the core of the protein), or quaternary structure (for example, involving Trp 60, which is solvent exposed in native oxidized β_2m), could account, at least in part, for the differences in the far UV CD spectra of the oxidized and reduced proteins at neutral pH. Nevertheless, the data show that red- β_2m is non-native at neutral pH.

Binding of the hydrophobic dye ANS was also used to probe the structural properties of red- β_2m . At pH 7, red- β_2m binds ANS, resulting in a small increase in the fluorescence intensity of the dye and a blue shift in its λ_{max} . In contrast, ox- β_2m does not bind ANS under these conditions (Fig. 3A). These data suggest that red- β_2m at pH 7 retains significant secondary and tertiary structure but exposes non-native hydrophobic surface area, consistent with the population of non-native species in the reduced protein under these conditions.

The conformational properties of ox- and red- β_2m were also compared at pH 3.6. At this pH, the oxidized protein is partially unfolded and highly amyloidogenic (McParland et al. 2000). Red- β_2m is also partially unfolded at pH 3.6, as judged by its relatively weak intensity in the near UV CD and significant intensity in the far UV CD. Interestingly, the far UV CD spectra of the reduced and oxidized proteins differ significantly in intensity, suggesting that the proteins have distinct conformational properties at this pH (Fig. 2B,E). Ox- and red- β_2m bind ANS at pH 3.6, indicating that a significant proportion of both species expose hydrophobic surface area at this pH (Fig. 3B). Ultracentrifugation studies indicate that both ox- and red- β_2m at pH 3.6 are polydispersed in the mixed buffer used (see Materials and Meth-

ods), forming a number of species ranging from monomers to large oligomers (Table 1). Because the oxidized and reduced proteins are polydisperse at this pH under the conditions used (Table 1), a more detailed interpretation of the CD spectra in terms of the nature of residual secondary structure present in each protein is not possible.

When both ox- and red- β_2m are acidified to pH 1.5, further denaturation of the proteins occurs, as judged by far and near UV CD (Fig. 2C,F). The far and near UV CD spectra of red- β_2m at pH 1.5 suggest that the protein retains residual secondary structure, but lacks significant fixed tertiary interactions under these conditions. At this pH, the oxidized protein has a similar near UV CD spectrum to its reduced counterpart above 260 nm, but has a distinct far UV CD spectrum, suggesting that the conformational properties of the two proteins differ in detail at this pH. Both ox- β_2m and red- β_2m bind ANS at pH 1.5 (Fig. 3C), indicating that both proteins expose hydrophobic surface area at this pH. Ultracentrifugation studies demonstrated that both ox- β_2m and red- β_2m form specific trimers at pH 1.5 (Table 1).

The amyloidogenic properties of red- β_2m

The above data indicate that reducing the disulphide bond of β_2m destabilizes the protein such that non-native species are populated at neutral pH. To investigate the role of these, and other partially unfolded species in amyloidosis in vitro, ox- β_2m and red- β_2m were incubated at different pH values and fibrillogenesis was initiated by the addition of 0.4 M NaCl. The structural properties of the protein were measured immediately after the addition of NaCl by far UV CD (Fig. 4), and the presence of fibrils was determined after incubation under these conditions for three days using thio-T binding and negative stain EM (Figs. 5 and 6). Increasing the ionic strength has little effect on the far UV CD spectra of ox- β_2m at pH 7.0 and pH 3.6, and red- β_2m at pH 7.0 (Fig. 4A,B). In contrast, the addition of NaCl to red- β_2m at pH 3.6 causes a rapid decrease in intensity in the far UV CD (Fig. 4B), which occurs concomitantly with the production

Table 1. *The effect of conditions on amyloid formation of ox- and red- β_2m*

pH	Redox state	Sedimentation coefficient (S)	State	MW after modification with iodoacetate	Rate of fibril formation ^a	Thio-T binding ^b	Fibril length (nm)	Fibril morphology
7.0	red	1.314/5.18	monomer/tetramer	11,978	43.6	yes	—	—
3.6	red	1.3–34	multiple species	—	108.5	yes	10–40	very short curved
1.5	red	3.871	trimer	—	35.6	yes	50–200	short curved
7.0	ox	1.55	monomer	11,860	0.0	no	—	—
3.6	ox	1.55–20	multiple species	—	85.3	yes	50–200	short curved
1.5	ox	3.35	trimer	—	28.4	yes	200–>600	long curved

All experiments were carried out at a protein concentration of 0.2 mg/ml.

^a Measured by Thio-T fluorescence (arbitrary units s⁻¹). The data in replicate experiments was reproducible to within $\pm 10\%$.

^b Thio-T binds to small soluble oligomers of red- β_2m under these conditions.

Fig. 3. Fluorescence emission spectra of ANS in the presence of ox- β_2 m and red- β_2 m. Red- β_2 m and ox- β_2 m were mixed with ANS at the pH values shown and the fluorescence emission spectrum of the dye was monitored. (○) Red- β_2 m; (●) ox- β_2 m; (▲) ANS alone at (A) pH 7.0, (B) pH 3.6, and (C) pH 1.5. All spectra were acquired at 10°C.

of material that scatters light (measured by optical density; data not shown). At longer incubation times under these conditions, insoluble material that sedimented in the CD cuvette was produced. Both ox- β_2 m and red- β_2 m appear to partially refold on the addition of NaCl at pH 1.5 (Fig. 4C). Therefore, the addition of NaCl to ox- β_2 m at pH 1.5 results in partial refolding of the molecule such that a species with

a CD spectrum similar to that of acid unfolded β_2 m at pH 3.6 is formed (Fig. 4B,C) (McParland et al. 2000). In addition, the decrease in intensity of the far UV CD signal of red- β_2 m at <215 nm on the addition of NaCl is consistent with reformation of secondary structure in this partially unfolded state.

Ox- β_2 m rapidly forms fibrils below pH 4.5 as measured by thio-T binding (Fig. 5; Table 1) (McParland et al. 2000). Under these conditions, the fibrils formed are ~10-nm wide and have a curvilinear morphology (Fig. 6C,D). The fibrils

Fig. 4. Ionic strength dependence of the far UV CD spectra of red- β_2 m and ox- β_2 m at (A) pH 7.0, (B) pH 3.6, and (C) pH 1.5. The protein concentration was 0.2 mg/ml and spectra were acquired at 10°C. (○) Red- β_2 m in buffer alone; (●) ox- β_2 m in buffer alone; (△) red- β_2 m in the presence of 0.4 M NaCl; and (▲) ox- β_2 m in 0.4 M NaCl.

formed from the oxidized protein at pH 3.6 are relatively short (ranging from 50–200 nm in length), whereas those formed at pH 1.5 have the same width and morphology as those produced at higher pH, but extend to >600 nm in length (Table 1) (McParland et al. 2000). Interestingly, incubation of red- β_2 m in 0.4 M NaCl results in the formation of species capable of binding thio-T over the entire pH range studied (pH 8.0–1.0) (Fig. 5). Despite examining a large number of grids, fibrillar material was only observed by negative stain EM in samples incubated below pH 4.0 (Fig. 6A,B). At these pH values, curved fibrils with a diameter of ~10 nm are formed. Fibrils of the reduced protein are shorter in length than those formed from ox- β_2 m under the same growth conditions (Table 1). At pH 4.5, the formation of amorphous aggregates from red- β_2 m results in light scattering that accounts for the apparent large increase in thio-T fluorescence at this pH. Because red- β_2 m at neutral pH does not form high molecular-weight oligomers (as judged by ultracentrifugation, light scattering, and negative stain EM), binding of thio-T to samples of red- β_2 m incubated above pH 4.5 most probably reflects an interaction of the dye with the tetrameric species formed from the reduced protein at this pH. At pH 7, ox- β_2 m does not form tetrameric species and no thio-T binding is evident.

Fibril morphology is dependent on the rate of fibril growth

To examine the influence of growth rate on fibril length, the kinetics of fibrillogenesis of ox- β_2 m and red- β_2 m at pH 7.0, pH 3.6, and pH 1.5 were measured using a continuous thio-T binding assay (see Materials and Methods). At pH 7.0, monomeric ox- β_2 m does not bind thio-T, consistent with previous observations that the native protein is not amyloidogenic (Fig. 5; Table 1) (McParland et al. 2000). Conversely, red- β_2 m shows a significant rate of increase in

Fig. 5. The pH dependence of fibril growth of β_2 m measured by thio-T fluorescence. (○) Red- β_2 m; (●) ox- β_2 m.

Fig. 6. Negative stained EM images of amyloid fibrils formed from reduced (A,B) and oxidized (C,D) β_2 m. Fibrils were formed at pH 1.5 (A,C) and pH 3.6 (B,D) by incubating β_2 m (0.2 mg/ml) for 3 days at 37°C (see Materials and Methods). Scale bar, 100 nm.

thio-T fluorescence at pH 7.0, which presumably reflects the formation of the small oligomeric species detected by ultracentrifugation (see above and Table 1). Most interestingly, there is a correlation between the rate of fibril growth and fibril length (Table 1). Conditions that favor rapid growth (red- β_2 m in 0.4 M NaCl at pH 3.6) result in the formation of numerous short fibrils, whereas those resulting in slower growth (ox- β_2 m at pH 1.5) produce fewer, longer fibrils. Fibril length is also independent of the redox state of β_2 m (fibrils of comparable length are formed from ox- β_2 m at pH 3.6 and red- β_2 m at pH 1.5). The observation that the length of fibrils increases when the association rate is lowered has long been established for a number of assembling systems (Whitesides et al. 1991; Bowden et al. 2001). Despite the apparent independence of fibril morphology on the redox state of the precursor protein, however, there is no reason to assume that the mechanism of β_2 m amyloid formation from the oxidized and reduced proteins will also be similar. Further experiments will be needed to determine the

nature of the nucleus and the mechanism of elongation of the oxidized and reduced proteins.

Discussion

The amyloidogenic properties of red- β_2 m

Destabilization of the native state of transthyretin (Lai et al. 1996), immunoglobulin light chains (Helms and Wetzel 1996; Raffin et al. 1999), lysozyme (Booth et al. 1997), SH3 domains (Guijarro et al. 1998), acylphosphatase (Chiti et al. 1999), and β_2 m (McParland et al. 2000) has been shown to be a crucial feature in amyloidosis. Reduction of the disulphide bond in an amyloidogenic Bence Jones protein (a light chain dimer) (Klafki et al. 1993) and the human prion protein (Swietnicki et al. 2000) has also been shown to favor the formation of amyloid fibrils in vitro. The inter-sheet disulphide bond of the immunoglobulin domains has an important role in stabilizing their β -sandwich fold (Isenman et al. 1975; Frisch et al. 1996). Accordingly, this disulphide bond is highly conserved in the immunoglobulin superfamily and natural mutations of the Cys residues involved are rare (Stevens et al. 2000). Interestingly, a missense mutation of Cys23 in λ cDNA is associated with a human light chain amyloid disease (Perfetti et al. 1998). In this paper we have shown that reducing the single disulphide bond of human β_2 m results in the destabilization of the native protein, such that non-native species are populated at neutral pH. Despite this, only partially unfolded species formed below pH 4.0 are capable of forming amyloid in vitro under the conditions investigated here. These results indicate that destabilization of native β_2 m by reduction of its disulphide bond is not sufficient for the formation of amyloid in vitro. Instead, one or more partially unfolded conformers formed at low pH appear to be necessary for amyloidosis of β_2 m in vitro. The symptoms of DRA are known to be brought about by the stimulation of macrophages to produce bone-resorbing cytokines, such as interleukin 1 β , tumor necrosis factor- α , and interleukin 6, resulting in bone degradation (Hou et al. 2001b). It is also known that β_2 m modified with advanced glycation end products may induce a local inflammatory response associated with DRA (Hou et al. 2001a). Whether the immature or mature fibrils of β_2 m are responsible for different facets in the evolution of DRA is currently unknown. Moreover, it is also possible that soluble oligomeric species of β_2 m, such as the trimeric and tetrameric species observed here under different conditions, could also be involved. In accord with this view, studies of other proteins have indicated that nonfibrillar species could be the culprits of these types of disease (for review, see Lansbury 1999).

The characteristics of the fibrils formed from ox- β_2 m and red- β_2 m depend on the pH at which they are formed. Although fibrils of both ox- β_2 m and red- β_2 m formed in vitro

have a common highly curved morphology and are ~10-nm wide, the length of fibrils produced depends on the initial rate of their formation but is independent of the redox state of the protein. Fibrils formed from both ox- β_2 m and red- β_2 m at acidic pH in vitro are shorter and more curved in morphology than those formed from most other proteins (Brancaccio et al. 1995; Goldsbury et al. 1997; Sunde and Blake 1997). β_2 m fibrils have also been formed in vitro by extension of amyloid fibrils from patients with DRA with full-length β_2 m (Naiki et al. 1997), as well as with β_2 m truncated by six residues at its amino terminus (Esposito et al. 2000). Under such conditions, the fibrils formed are ~10-nm wide, but are longer and straighter than those formed de novo in vitro. The latter may therefore represent immature fibrils or, in the case of the very short fibrils formed from red- β_2 m at pH 3.6, early assembly intermediates. Interestingly, however, further assembly of the curved immature fibrils formed in vitro to the mature fibrils observed ex vivo has not been observed to date, despite exploring a range of conditions (Naiki et al. 1997; McParland et al. 2000). Consistent with these results, incubation of immature fibrils assembled from both ox- β_2 m and red- β_2 m de novo in vitro at pH 3.6 in 0.4 M NaCl for up to 8 mo at 37°C have not yielded mature fibrils akin to those formed in vivo. Further experiments will be needed to determine the factors that influence the extent of β_2 m fibril assembly in vitro, the role of the curved immature fibrillar species in the assembly of mature amyloid fibrils and the influence of these different species in the evolution of the pathogenicity of β_2 m amyloid in vivo.

Implications for β_2 m amyloidosis in vivo

The incidence of dialysis related amyloidosis in patients receiving haemodialysis for more than two decades is 100% (Floege and Ehlerding 1996). Studies of the epidemiology of haemodialysis-related amyloidosis offer clues as to its possible causes. Fibrils formed from β_2 m in vivo are often located in areas rich in collagen, such as the joints (Gejyo et al. 1995; Floege and Ehlerding 1996), resulting in the crowding of macrophages in these areas (Ohashi et al. 1992; Ayers et al. 1993). β_2 m amyloid has been observed directly within macrophage lysosomes (Van Ypersele and Drucke 1996), raising the possibility that uptake of the protein into lysosomes may be involved in the development of β_2 m amyloidosis. In contrast with the neutral pH of serum, the acidic pH within lysosomes would promote partial unfolding of β_2 m to species capable of assembly into amyloid fibrils. In addition, it has been discovered recently that the enzyme gamma-interferon-inducible lysosomal thiol reductase is responsible for the enzymatic reduction of disulphide bonds in lysosomes (Arunachalam et al. 2000), raising the possibility that reduction of disulphide bonds could be in-

volved the initiation of protein aggregation within this cellular compartment.

Native monomeric β_2m has been recovered from amyloid deposits of patients with DRA (Gejyo et al. 1986; Campistol et al. 1996; Bellotti et al. 1998). Analysis of this material has suggested that at least a significant proportion of the protein in amyloid deposits *ex vivo* is in the oxidized form. In addition, the observation that amyloid can be formed from immunoglobulin light chains (Helms and Wetzel 1996; Raffin et al. 1999), lysozyme (Booth et al. 1997), insulin (Bouchard et al. 2000), and β_2m (Connors et al. 1985; Ono and Uchino 1994; Naiki et al. 1997; Bellotti et al. 1998; McParland et al. 2000) in their oxidized forms suggests that reduction is not a prerequisite for amyloidosis. The rapid formation of small oligomers or immature protofibrils of red- β_2m within lysosomes could seed polymerization of partially unfolded ox- β_2m in the acidic pH environment therein. Assembly of truncated β_2m into fibrils from such seeds would also be possible in the neutral pH of serum (Esposito et al. 2000). Further experiments will be needed to elucidate the role of disulphide bond reduction in β_2m amyloidosis *in vivo*. Nevertheless, the results presented here indicate that population of partially unfolded conformers of β_2m in an acidic environment are important features of β_2m amyloidosis in common with many other human amyloid diseases (Lai et al. 1996; Swietnicki et al. 1997; Guijarro et al. 1998).

Materials and methods

Materials

The *Escherichia coli* strain BL21(DE3) was obtained from Promega. Dithiothreitol (DTT), Q-Sepharose, and all other reagents were purchased from Sigma-Aldrich Chemical Company. Spectrapore membrane (molecular weight cut-off 3500 Da) was obtained from Spectrum Laboratories Inc. Butyl-Sepharose and Superdex 75 were purchased from Pharmacia. Carbenicillin was from Melford Laboratories Ltd. PD10, NAP10, and NAP5 disposable columns containing Sephadex G25 medium were purchased from Amersham Pharmacia Biotech.

β_2m overexpression and purification

The gene encoding human β_2m was obtained from the plasmid BJ192 (kindly provided by B. Jakobson, Institute of Molecular Medicine, Oxford, UK). The gene was subcloned into the vector pET23a (Promega), and the resulting plasmid pINKwt was transformed into BL21(DE3) cells. The cells were grown in LB media containing 0.25 mg/ml carbenicillin and protein expression was induced by the addition of 1 mM IPTG when the optical density had reached an OD_{600} of 0.6 (McParland et al. 2000). After 12 h, the bacteria were harvested. Overexpressed β_2m was sequestered in the cells as inclusion bodies. These were isolated and the protein purified as described in McParland et al. (2000). A yield of 35 mg pure β_2m /L culture was obtained. The protein was shown to be >95% pure by SDS PAGE and of the expected molecular weight

by ESI MS (expected M_r = 11,860, observed M_r = 11,860 $\pm$ 0.72). The protein was stored at -20°C as a lyophilized powder.

Preparation and analysis of reduced β_2m

Lyophilized β_2m was dissolved at room temperature in 25 mM Tris HCl containing 6 M GuHCl, 10 mM DTT, pH 8.0. After 1 h at room temperature, the sample was cooled to 4°C and the protein was purified by rapid gel filtration using Pharmacia PD10 columns equilibrated with 10 mM Tris HCl, 10 mM DTT, pH 8.0. Any precipitate was removed by centrifugation and the sample was then passed through a 0.2 μm filter and used immediately. To determine the extinction coefficient of the reduced protein, the protocol was repeated using a known amount of ox- β_2m at a protein concentration of 0.2 mg/ml. Under these conditions, no precipitate was formed on removal of the denaturant. The extinction coefficient of reduced refolded β_2m at 280 nm in 10 mM Tris HCl, 10 mM DTT, pH 8.0 was determined to be 26,827 $\text{M}^{-1}\text{cm}^{-1}$. The extinction coefficient of the oxidized protein under these conditions was taken as 19,850 $\text{M}^{-1}\text{cm}^{-1}$ (Berggard and Bearn 1968).

To demonstrate that β_2m was fully reduced, two assays were performed. First, the sample (0.2 mg/ml in 25 mM Tris HCl, 25 mM sodium acetate, 25 mM glycine, 25 mM MES, 10 mM DTT, pH 8.0) was modified with iodoacetic acid. After an initial 15-min incubation at 25°C with 100 mM iodoacetate at pH 8.0 (to modify the DTT in the sample buffer), the protein was unfolded by the addition of 6 M GuHCl and the sample was incubated in the presence of iodoacetate for a further 20 min. The sample was then refolded by 10-fold dilution into 10 mM Tris HCl buffer, pH 8.0, dialyzed against water and the mass of the protein determined by ESI MS. The majority of the ox- β_2m was not modified by iodoacetic acid (observed M_r = 11,860 $\pm$ 1.5). A fraction of molecules (25%), however, contained modification of a single surface residue (M_r = 11,918.5 $\pm$ 0.53), possibly the solvent exposed histidine, H13. Red- β_2m was fully reduced using this protocol, 62% of molecules containing two carboxymethyl moieties (M_r = 11,978 $\pm$ 0.64). The remainder showed modification of a third residue (M_r = 12,035 $\pm$ 3.50). Repeating the experiment on the reduced, refolded protein after storage at a range of pH values (1.5–8.0) at 37°C for up to 6 days under an atmosphere of N_2 showed that no re-oxidation of the two Cys residues had occurred.

In the second assay, ox- β_2m and red- β_2m were analyzed by reverse-phase HPLC using a C18, 300Å column (Brownlee Aquapore OD-300 100 mm $\times$ 2.1 mm). All HPLC separations used an acetonitrile- H_2O gradient in the presence of 0.1% trifluoroacetic acid (TFA). Comparisons of elution times were used to ascertain the redox state of the protein. Peaks were identified by their relative intensities. The gradient used for the final separations was A: 0.1% TFA, B: 0.1% TFA/90% CH_3CN ; 0 min 10% B, 2 min 10% B, 7 min 30% B, 37 min 60% B, 45 min 90% B, 47 min 90% B, 52 min 10% B. Under these conditions, the retention time of ox- β_2m and red- β_2m were 28.7 min and 40.8 min, respectively. The data confirmed that the protein was fully reduced using the procedure outlined above.

Ultracentrifugation

Sedimentation velocity experiments were carried out using a Beckman Optima XL-I analytical ultracentrifuge (Beckman, Palo Alto, CA) fitted with a scanning absorption optical system. To determine the sedimentation coefficients of red- β_2m , the protein was prepared as described above. Double-sector cells were filled with 280 μl of 0.2 mg/ml protein solution and 300 μl of reference solvent

(25 mM Tris HCl, 25 mM sodium acetate, 25 mM glycine, 25 mM MES, 10 mM DTT) in their respective channels. Solutions were run at 60,000 rpm at 13°C and solute distributions were scanned at 280 nm. A series of 51 scans were taken at fixed time intervals over 2.5 h. Scans were analyzed using the program XLAVEL (Colfen et al. 1996), giving the sedimentation coefficient for each protein.

Thioflavin-T binding studies

Aliquots of red- β_2 m in 10 mM Tris HCl buffer, pH 8.0 were diluted into buffers consisting of 25 mM Tris HCl, 25 mM sodium acetate, 25 mM glycine, 25 mM MES, and variable concentrations of NaCl to adjust the ionic strength at each pH to 400 mM. The samples were then passed through a 0.2 μ M filter and diluted into the desired final solution conditions. A final β_2 m concentration of 0.2 mg/ml was used throughout. Protein was incubated at 37°C for 72 h without stirring, after which time the samples were diluted 100-fold into the assay buffer (10 μ M Thio-T, 50 mM Tris HCl, pH 8.5) before measurement. Thio-T fluorescence was measured using a PTI Quantamaster C-61 spectrofluorimeter set at 444 nm (excitation) and 480 nm (emission) using slit widths of 1 nm and 10 nm, respectively. The excitation and emission wavelengths were determined as peak values by performing excitation and emission scans in the presence of preformed β_2 m fibrils. Control experiments showed that fibrils formed at lower pH values were stable at pH 8.5 over the time course of the measurement (10 sec). The fluorescence of thio-T in the absence of protein was also taken and used as a blank. Positive controls were included in all experiments using pre-formed ox- β_2 m fibrils. The data were scaled by taking the thio-T fluorescence value of the sample of ox- β_2 m at pH 8.0 as zero and that of ox- β_2 m at pH 1.0 as unity and scaling all other values accordingly.

In experiments to measure the initial rate of fibrillogenesis, red- β_2 m (0.2 mg/ml in 25 mM Tris HCl, 25 mM sodium acetate, 25 mM glycine, 25 mM MES) was prepared as described above and thio-T (10 μ M final concentration) was added. Fibrillogenesis was then initiated by the addition of 0.4 M NaCl at 10 °C. The fluorescence of thio-T was then measured continuously (using an excitation wavelength of 444 nm and emission at 480 nm). The initial rate of fibrillogenesis was determined from the initial change in thio-T fluorescence with time observed over the first 20 sec of the experiment. The initial rate of growth was reproducible to within 10% over several experiments.

Electron microscopy

Colloidal coated copper EM grids were placed coated side down onto sample drops containing preformed β_2 m fibrils for 20 sec. The grids were then blotted with filter paper to remove excess solvent and the sample was stained with 4% (wt/vol) uranyl acetate for 20 sec. Grids were then blotted again and air-dried before analysis. All images were taken using a Philips CM10 electron microscope operating at 100 keV.

Circular dichroism

CD experiments were performed on a Jasco J715 CD spectropolarimeter. For measurements in the far UV (210–260 nm) the CD signal was recorded at 10°C in a 1-mm path-length cell using a protein concentration of 0.2 mg/ml. For measurement in the near UV (250–350 nm), CD spectra were recorded using a protein

concentration of either 0.4 mg/ml (at pH 7) or 0.6 mg/ml (at pH 3.6 and 1.5) and a 1-cm path-length cuvette. Measurements were recorded with a 1 nm bandwidth, 1 nm resolution, interval speed of 50 nm/min and response times of 8 sec. Six accumulations were taken and the results were averaged. DTT absorbs light strongly below 210 nm. As a consequence, far UV CD spectra could not be acquired below 210 nm.

ANS binding

The fluorescence emission spectra of ANS with and without protein were recorded on a Perkin-Elmer LS50B spectrofluorimeter at 10°C. Protein samples (10 μ M) were prepared as described as above and were diluted 10-fold into buffer containing 25 mM Tris HCl, 25 mM sodium acetate, 25 mM glycine, 25 mM MES and ANS (250 μ M final concentration) at the appropriate pH value. ANS binding was determined immediately using an excitation wavelength of 389 nm and fluorescence emission was collected between 400 and 600 nm using slit widths of 5 nm. The fluorescence emission of ANS in buffer alone was then subtracted from that in the presence of β_2 m.

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