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# A Generic Mechanism of $\beta_2$-Microglobulin Amyloid Assembly at Neutral pH Involving a Specific Proline Switch

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Although numerous measurements of amyloid assembly of different proteins under distinct conditions *in vitro* have been performed, the molecular mechanisms underlying the specific self-association of proteins into amyloid fibrils remain obscure. Elucidating the nature of the events that initiate amyloid formation remains a particularly difficult challenge because of the heterogeneity and transient nature of the species involved. Here, we have used site-directed mutagenesis to create five proline to glycine variants in the naturally amyloidogenic protein $\beta_2$-microglobulin ($\beta_2$m). One of these variants, P5G, allowed us to isolate and characterise an intermediate containing a non-native *trans* Pro32 backbone conformation, a feature that is known to be required for amyloid elongation at neutral pH. By analysing oligomerisation and amyloid formation using analytical size-exclusion chromatography, multi-angle static light-scattering, analytical ultracentrifugation, circular dichroism and thioflavin T fluorescence we reveal a pathway for $\beta_2$m amyloid assembly at pH 7.5 that does not require the addition of metal ions, detergents, co-solvents or other co-factors that have been used to facilitate amyloid formation at physiological pH and temperature. Assembly is shown to involve the transient formation of a non-native monomer containing a *trans* P32 backbone conformation. This is followed by the formation of dimeric species and higher molecular mass oligomers that accumulate before the development of amyloid fibrils. On the basis of these results, we propose a generic mechanism for $\beta_2$m fibrillogenesis at neutral pH that is consistent with the wide range of published studies of this protein. In this mechanism, amyloid formation is initiated by a specific *cis* to *trans* proline switch, the rate of which we show to be controlled by the amino acid sequence proximal to P32 and to the applied solution conditions.



*Keywords:* dimer; aggregation kinetics; proline isomerisation; *de novo* amyloid assembly; partially unfolded state

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## Introduction

A number of human diseases are known to involve protein misfolding events that ultimately result in the malfunctioning of the cellular machinery.[1,2] One such class of diseases is amyloidosis, which involves a range of disorders in which normally soluble proteins self-associate to form insoluble fibrillar aggregates and plaques known as amyloid.[3,4] Earlier studies suggested that equilibration between the native protein and one or more partially, or more highly unfolded, non-native species is a generic initiating event of amyloidosis.[4] Protein sequences are designed, therefore, to avoid the formation of such precursor species so as to minimise the risk of amyloid formation during an organism's lifetime. Thus, native globular proteins, in general, display co-operative unfolding transitions in which the population of partially unfolded species is rare[5] and contain protective features that disfavour intermolecular interactions,

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Abbreviations used: $\beta_2$m, $\beta_2$-microglobulin; ThT, thioflavin-T; SEC, size-exclusion chromatography; MASLS, multi-angle static light-scattering.

especially for native proteins rich in β-sheets in which edge strands provide particularly aggregation-prone surfaces.[6] In addition, the judicious placement of certain amino acid residues in proteins acts as "gatekeepers", which prevent oligomerisation and, therefore, disfavour aggregation.[7] For example, the incorporation of charged amino acids,[8] proline residues[9] and the avoidance of sequences with regular hydrophobic/hydrophilic patterning or a high amyloid potential[10,11] all reduce the probability of amyloid formation.

Crucial to the elucidation of the molecular mechanisms of amyloidosis and the origins of the cytotoxicity often associated with amyloid formation, is the identification and structural characterisation of oligomeric species formed during assembly.[12] Structural elucidation of early aggregation-prone species is hampered, however, by their transient nature, heterogeneity and instability.[13] Nonetheless, recent insights into these species have revealed the generation of different-sized aggregates/oligomers during the formation of amyloid-like fibrils for acylphosphatase,[14] amyloid-β,[15] human islet amyloid polypeptide,[16] the prion proteins,[17] $\beta_2$-microglobulin ($\beta_2$m),[18] exon 1 of the gene huntingtin,[19] and other proteins and peptides.[20,21] In some cases, these species have been shown to increase in stability as their size increases, suggesting both intra- and intermolecular protein reorganisation during the lag phase of amyloid assembly that leads to continually stronger and denser protein packing,[22,23] Oligomeric species that already contain well-ordered β-sheet structures have also been suggested to have a key role in pre-defining the final amyloid architecture.[24]

Here, we utilised $\beta_2$m, a 99 residue protein with a classical immunoglobulin fold,[25] as a model system to study the nature of events occurring early during its assembly into amyloid fibrils. $\beta_2$m is known to be the major component of fibrillar deposits in patients with dialysis-related amyloidosis, a disorder that arises in all patients with chronic renal failure.[26] While the concentration of monomeric $\beta_2$m is a key risk factor for amyloid deposition in dialysis-related amyloidosis, a wide range of *in vitro* studies have demonstrated that the native monomer itself is not able to assemble into amyloid fibrils spontaneously at neutral pH in the absence of additional factors.[27,28] Biological components, therefore, including glycosaminoglycans, proteoglycans, collagen and $Cu^{2+}$ in the presence of urea have all been used to promote and/or induce fibril formation of $\beta_2$m at neutral pH *in vitro*.[29–32] In addition, co-solvents such as TFE[33] or SDS,[34] alteration of the physical environment by ultrasonication,[35] elevated temperatures[36] or stirring at high concentrations of salt[37] at neutral pH, or incubation under mild acidic conditions,[38,39] have been shown to result in an increased propensity for $\beta_2$m to self-assemble into amyloid fibrils. How these agents and conditions influence the amyloid assembly mechanism, and whether the different conditions result in an acceleration of one common mechanism, or distinct routes to amyloid assembly, however, remain obscure.

To elucidate the mechanism of $\beta_2$m assembly into amyloid fibrils at pH 7.5, we adopted a systematic approach commencing with the natively folded protein. By substitution of each of the five proline residues individually with glycine, we identified a variant (P5G) that populates the native state and a non-native species containing a *trans* P32 backbone conformation in equal proportion at pH 7.5 at equilibrium. By exploiting the slow interconversion of these species and varying the concentration of protein and temperature, we show that the non-native species is able to assemble into amyloid fibrils spontaneously at neutral pH in the absence of seeds. The assembly mechanism involves the formation of a dimeric species as the first assembled oligomer. Incubation at 37 °C leads, additionally, to the formation of higher oligomeric species that exhibit the amyloid-specific dye thioflavin T (ThT)-binding property of amyloid. $\beta_2$m amyloid fibrils form after an extended period of incubation at 37 °C with shaking. Comparison of these results with amyloid formation of the wild-type protein in the presence of $Cu^{2+}$ and of the species ΔN6, a variant of $\beta_2$m found in *ex vivo* amyloid deposits[40] and known to have enhanced amyloid potential,[30,41] using similar approaches leads us to propose a generic mechanism for $\beta_2$m amyloid assembly consistent with the wide range of published studies of this protein.[27,29–32,34,36,37,41–48] Crucially, all of these studies are linked by the switch of the native *cis* P32 to a *trans* isomer, forming a non-native and aggregation-prone monomeric intermediate, the population of which is controlled by the amino acid sequence proximal to P32 and the solution conditions.

## Results

### The rate of amyloid nucleation of $\beta_2$m is correlated with the concentration of a slow folding intermediate

Earlier studies by our own[27] and other laboratories[32,48] have suggested a role of *cis/trans* isomerisation of P32 as a key initiating event in amyloid formation of $\beta_2$m at neutral pH. To investigate the potential role of other proline residues in amyloid formation, all of which are highly conserved in $\beta_2$m between organisms,[49] the single *cis* P32 and the four *trans* proline residues (P5, P14, P72 and P90 (Fig. 1)) were individually substituted with glycine. While the far-UV CD spectra of P5G and P32G show significant differences from each other and from the spectrum of wild-type $\beta_2$m, the CD spectra of P14G, P72G and P90G were found to be similar to that of the wild-type protein (Fig. 2a and data not shown). The data suggest that P5G and P32G may form distinct, structurally altered conformations compared with that of the wild-type protein. Alternatively, they may populate significant concentrations of non-native species at equilibrium with the native state, as suggested by previous studies that reported