Sulfate Inhibits Fibril Formation of \( \beta_2 \)-Microglobulin \textit{in vitro} \\

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Beta-2-microglobulin (\( \beta_2m \)) is a small MHC-I associated protein that undergoes aggregation and accumulates as amyloid deposits in human tissues as a consequence of long-term hemodialysis. Conditions that lead to fibril formation of \( \beta_2m \) remain a largely unknown territory. Predisposing factors that will cause \( \beta_2m \) to change from a soluble protein to an aggregate has been a topic of debate up to now. In this study, the effect of sulfate on \( \beta_2m \) fibril formation was monitored through fluorescence spectroscopy employing the Thioflavin T assay. Sulfate was found to stabilize the native monomeric state of \( \beta_2m \) at a 200-fold sulfate to protein ratio. Circular dichroism of \( \beta_2m \) in the presence of sulfate indicated a spectrum characteristic of the natively folded protein rather than the amyloidogenic state. Electron microscopy analysis showed no needle-like fibrils formed in the presence of sulfate.

\textbf{Key words:} hemodialysis, amyloidosis, circular dichroism, fluorescence

INTRODUCTION

A growing number of proteins with the propensity to misfold and form amyloid fibrils, under appropriate conditions, have been recognized to be associated with the pathology of some important human diseases. One such disease is dialysis-related amyloidosis (DRA), a debilitating complication acquired by patients undergoing long-term hemodialysis characterized by the deposition of beta-2-microglobulin (\( \beta_2m \)) fibrils in joints, bone marrow and kidneys. \( \beta_2m \) consists of a polypeptide chain of 99 residues (molar mass of 11,800 Da) with a disulfide link between Cys 25 and Cys 80 which stabilizes the \( \beta \)-sandwich structure (Gillmore et al. 1997; Brange et al. 1997; Kelly 1998). It is a normal constituent of plasma where its concentration in adults is 1.11 - 2.7 mg/mL. However, the concentration of \( \beta_2m \) is increased in patients with renal failure (Guijarro et al. 1998; Ohnishi et al. 2000; Fezoui et al. 2001; Chien and Weismann 2001).

The formation of \( \beta_2m \) fibrils \textit{in vivo} has been extensively studied (Guijarro et al. 1998; Ohnishi et al. 2000; Fezoui et al. 2001). These fibrils are characterized by a cross-\( \beta \) structure, which is a common characteristic shared by amyloidogenic proteins such as A\( \beta \) protein of Alzheimer’s disease, and the prion protein associated with transmissible spongiform encephalopathy, and Creutzfeld-Jacob disease. Several studies tried to offer an explanation on how these fibrils are formed in the molecular level. A partially folded conformation of \( \beta_2m \), with a higher propensity to aggregate compared to the native protein in an environment close to physiological conditions
has been isolated. β2m lacking six residues in the N-terminus has been shown to increase amyloidogenicity and has been found ex vivo together with other truncated species (Ohnishi et al. 2000). Under acidic conditions, reduction of the single disulfide bond in β2m and a high ionic strength has been shown to form immature fibrils that are short and curvilinear (Fezoui et al. 2001). A peptide fragment (residues 20-41) most responsible for the amyloidogenic property of β2m has also been identified (Kozhukh et al. 2002).

Most of the studies on β2m focused on the factors that destabilize the native state of this protein. There has been no study on the factors that stabilize β2m in order to slow down or inhibit fibril formation. The effect of sulfate on the fibril formation of β2m in vitro is examined in this paper. Fibril formation was monitored using circular dichroism, electron microscopy and the fluorescence-based Thioflavin T assay.

MATERIALS AND METHOD

Purification of recombinant β2m

β2m was obtained from a yeast (Pichia pastoris) expression system that over expresses β2m with an additional four residues (Glu-Ala-Tyr-Val) at the N-terminus. Induction of the protein was made using methanol after 3 d of culture. The supernatant which contains the protein was desalted using Sephadex G-50. The sample was then added to a Sephacryl 200 column and protein was eluted using 10 mM phosphate buffer with pH 7.5. Active fractions were then pooled and applied to a DEAE-Sephrose equilibrated with a 20 mM Tris-HCl at pH 8.5 and elution of β2m followed by a NaCl gradient. Purity of the protein was checked with SDS-PAGE.

Fibril Formation and Thioflavin T Assay

Samples of 25 μM monomeric β2m in 50 mM citrate-NaOH buffer (pH 2.5) and 100 mM KCl were prepared containing copper, sodium, ammonium and potassium sulfate (50 mM) in the first set of experiment. In another set of experiments, varying concentrations of (NH4)2SO4 were added to the sample. β2m seed fibrils were added to the solution to a final concentration of 5 μg/mL. The samples were then incubated at 37°C. Aliquots of 7.5 μL were taken from each reaction mixture and mixed with 1.5 mL of 5 μM ThT in 50 mM glycine-NaOH buffer (pH 8.5). Fluorescence of Thioflavin T (ThT) was monitored at 485 nm with excitation at 445 nm. Fibril formation was followed as a function of time.

Circular Dichroism (CD)

CD measurements were carried out in a Jasco J-715 spectropolarimeter at room temperature. Quartz cells with a 1 mm path length were used. The protein concentration used for all the CD measurement was 25 μM. Measurements were carried out at 25°C.

Electron Microscopy

Reaction mixtures (2.5 μL) were diluted with 25 μL of distilled water. An aliquot of the dilute sample was spread on carbon-coded grids and the solution allowed to stand for 1-2 min before excess solution was removed by filter paper after the residual solution has dried up. The grids were negatively stained with 1% phosphotungstic acid (pH 7.0). Again, the solution on the grids was removed with filter paper and dried. These samples were examined under Hitachi H-700 electron microscope with an acceleration voltage of 75 kV.

RESULTS AND DISCUSSION

Salts affect protein stability in different ways. Sulfate, for instance, is commonly used as a protein stabilizer. From the Hofmeister or chaotropic series, sulfate ranks among the chief structure stabilizers. Thus, the effect of sulfate on the conformational stability of recombinant β2m was determined by trying out different salts of sulfates with a concentration of 50 mM. Formation of fibrils was assessed by the Thioflavin-T assay developed by Naiki et al. (1989). Thioflavin T is a dye that is specific for protein fibrils. This assay has been used to characterize not only β2m, but other amyloidogenic proteins like prion, transthyretin, synuclein, and Aβ-amyloid.

Sulfate, at the indicated concentration, was found to stabilize the native state of β2m that precludes it from forming fibrils at pH 2.5. Regardless of the counterion, β2m does not form fibrils in the presence of sulfate. This finding is clearly delineated in Fig. 1. Even with the counterion Cu(II), 50 mM sulfate still holds the native conformation intact. Cu(II) is known to destabilize the conformation of β2m leading to fibril formation (Villanueva et al. 2004) but Cu(II) here does not exhibit its effect in the presence of sulfate.

Since ammonium sulfate has been found to be a good protein stabilizer at a high concentration (~1 M) among the salts of sulfate tested, we decided to use ammonium sulfate in all the succeeding experiments. The stabilizing effect
Secondary structures of the monomeric β2m at pH 7.0, fibrils formed in vitro at pH 2.5 and monomer in random conformation at pH 2.5 were determined by far-UV CD spectroscopy.

The CD spectrum of native β2m at pH 7.0 showed a small negative peak at 220 nm, characteristic of a β-structure. After acidification and incubation at 37°C, β2m showed the characteristic CD spectrum of a β-structure with a minimum at 220 nm and an intensity greater than the native protein, indicating the presence of fibrils. In contrast, the CD spectrum of β2m with (NH4)2SO4 under the same condition showed only a shallow peak characteristic of the native protein (Fig. 3). From the CD spectra obtained, it is implied that addition of (NH4)2SO4 inhibited fibril formation. To further validate this observation, electron microscopy was performed. Normal β2m fibrils under acidic conditions, without (NH4)2SO4, exhibited needle-like fibrils as seen from the electron micrograph on Fig. 4A. Upon addition of 50 mM (NH4)2SO4, the occurrence of these type of fibrils were not apparent (Fig. 4B). This observation supports the results made in the CD spectra of β2m, which shows the inhibitory property of (NH4)2SO4.

The results obtained on sulfate are very promising. To our knowledge, this is the first study to focus on a factor that stabilizes the native state of β2m. Sulfate offers a method for analyzing the soluble form of β2m at low pH. Preserving β2m at its monomeric state is a novel approach in elucidating the mechanism of protein fibrillogenesis. The stabilizing effect of sulfate on β2m may potentially be utilized to analyze other protein fibrils like the Aβ in Alzheimer’s disease and the prion protein in transmissible spongiform encephalopathy.
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