Critical Balance of Electrostatic and Hydrophobic Interactions Is Required for β_2-Microglobulin Amyloid Fibril Growth and Stability†

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ABSTRACT: Investigation of factors that modulate amyloid formation of proteins is important to understand and mitigate amyloid-related diseases. To understand the role of electrostatic interactions and the effect of ionic cosolutes, especially anions, on amyloid formation, we have investigated the effect of salts such as NaCl, NaI, $NaClO_4$, and Na_2SO_4 on the amyloid fibril growth of β_2-microglobulin, the protein involved in dialysis-related amyloidosis. Under acidic conditions, these salts exhibit characteristic optimal concentrations where the fibril growth is favored. The presence of salts leads to an increase in hydrophobicity of the protein as reported by 8-anilinonaphthalene-1-sulfonic acid, indicating that the anion interaction leads to the necessary electrostatic and hydrophobic balance critical for amyloid formation. However, high concentrations of salts tilt the balance to high hydrophobicity, leading to partitioning of the protein to amorphous aggregates. Such amorphous aggregates are not competent for fibril growth. The order of anions based on the lowest concentration at which fibril formation is favored is $SO_4^{2-} > ClO_4^- > I^- > Cl^-$, consistent with the order of their electroselectivity series, suggesting that preferential anion binding, rather than general ionic strength effect, plays an important role in the amyloid fibril growth. Anion binding is also found to stabilize the amyloid fibrils under acidic condition. Interestingly, sulfate promotes amyloid growth of β_2-microglobulin at pH between 5 and 6, closer to its isoelectric point. Considering the earlier studies on the role of glycosaminoglycans and proteoglycans (i.e., sulfated polyanions) on amyloid formation, our study suggests that preferential interaction of sulfate ions with amyloidogenic proteins may have biological significance.

Aggregation of proteins is a manifestation of their stability alterations or kinetic trapping to such misfolded state(s) due to either environmental factors or mutations. A growing number of diseases that appear to be caused by protein aggregation or folding disorders (*1*−*5*) emphasize the importance of studying the protein aggregation process. Understanding the molecular mechanism underlying such a deleterious fate of a protein should help in designing strategies to mitigate the problem. Aggregation of proteins can be classified into two types: (i) the well-ordered amyloid fibril formation with intermolecular β-sheet structure (*6*−*9*) and (ii) irregular or amorphous aggregation. About 20 different proteins are known so far to form amyloid fibrils either as full-length proteins or as proteolytically cleaved peptides, which results in pathological conditions (*4*). Amyloid formation is a generic property of polypeptides as a diverse set of proteins forms amyloid aggregates under suitable conditions where the native structure is perturbed (*3*, *10*−*12*). Whether all denatured state(s) of proteins can form amyloid fibrils is not clear. As has been realized, the population of specific denatured state(s) may be critical in amyloid formation (*13*). The propensity to form amyloid fibrils can be expected to vary from protein to protein under a given set of conditions. Therefore, understanding the factors that govern the amyloidogenic propensity becomes an important issue.

β_2-Microglobulin (β2m),[1] a component of the type I major histocompatibility complex, is found at low concentration in circulating blood, and its turnover depends on its degradation in the kidney. Defective homeostasis of this protein due to failure of the kidney function and its inability to flow through the dialysis membrane leads to accumulation of β2m in the blood (*14*). Due to a still unclear mechanism, β2m has been found to form amyloid deposits in the synovia of the carpal tunnel of patients undergoing long-term hemodialysis, leading to pathological conditions called dialysis-related amyloidosis (*15*). The monomeric β2m undergoes

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[1] Abbreviations: β2m, β_2-microglobulin; ThT, thioflavin T; ANS, 8-anilinonaphthalene-1-sulfonic acid ammonium salt; CD, circular dichroism; TIRFM, total internal reflection fluorescence microscopy.

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in vitro amyloid formation under acidic conditions below pH 4 (*16*, *17*). However, the fibrils are found to be unstable at neutral pH (*18*).

The fact that β2m is found in the amyloid fibrils in dialysis-related amyloidosis triggers investigations to explore the various external factors or conditions which influence amyloid formation of the protein close to neutral pH conditions. Copper ion has been implicated as one of the risk factors in dialysis-related amyloidosis, as its binding to monomeric β2m leads to conformational changes and increased amyloidogenicity (*19*, *20*). Copper ion binding can increase the conformational flexibility of β2m as shown by nuclear magnetic resonance spectroscopic studies (*21*), suggesting that factors that affect the dynamic behavior of a protein can influence its amyloidogenic propensities probably by perturbing the equilibrium between the states differing in their amyloidogenic propensities. It has been suggested that the partially structured species found under equilibrium at physiological pH has propensity to form amyloid fibrils (*22*). It appears that both the amyloidogenic propensity of the monomeric species and the stability of the fibril may determine the fibril formation of β2m. Moreover, it has been shown that glycosaminoglycans, heparin, for example, can stabilize the β2m fibrils at neutral pH and enhance the trifluoroethanol-induced fibril growth of β2m (*23*, *24*). It is, therefore, important to understand the factors that govern fibril stability and amyloidogenic propensity of β2m. Such an understanding would also contribute in broad perspective to the amyloid formation of proteins in general.

Both hydrophobic and electrostatic interactions play a role in amyloid formation (*25*−*30*). However, our understanding on the role of electrostatic interactions in amyloid formation is far from complete. It has been known that the interaction of solute anions leads to generation of partially folded states of proteins in acidic conditions (*31*, *32*). How anionic interactions affect the amyloid formation of β2m is not understood so far. To gain insight into the role of charge interactions in amyloid fibril growth, we have investigated the effects of some selected salts on the β2m fibril growth. Our study shows that there is an optimal concentration of salt required for promotion of the fibril growth. Preferential anion interaction (i.e., binding) is important for the necessary electrostatic and hydrophobic balance critical in amyloid formation. Anion binding appears to be an important factor in stabilizing the fibrils as well as their growth at pH 2.5. In addition, our study shows that sulfate can promote amyloid fibril growth of β2m at pH between 5 and 6, suggesting that, among other possible factors, environmental anion interactions (for example, sulfate either free or in the form of sulfated polysaccharides) and local fluctuation of pH may have implication in vivo in dialysis-related amyloidosis.

EXPERIMENTAL PROCEDURES

Preparation of the Recombinant Monomeric β2m. β2m was expressed in *Escherichia coli* BL21(DE3) pLysS (Novagen, Inc., Madison, WI) and purified from its inclusion bodies as described earlier (*33*). Briefly, the inclusion bodies were dissolved in 20 mM Tris-HCl buffer, pH 8.0, containing 8 M urea and air-oxidized for 2−3 days at 4 °C to form the intrachain disulfide bond as confirmed by reverse-phase high-performance liquid chromatography (*34*). The oxidized sample in urea was dialyzed against 20 mM Tris-HCl buffer, pH 8.0, to refold the protein. The refolded protein sample was subjected to ion-exchange chromatography on a DEAE-Sepharose CL-4B (Amersham Biosciences) column equilibrated with the same buffer. The bound protein was eluted with a linear gradient of 0−200 mM NaCl. The major peak fractions corresponding to the monomeric β2m were found to be homogeneous on SDS−PAGE and were pooled, dialyzed against deionized water, and lyophilized. The molecular mass of the purified monomeric β2m was confirmed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (Applied Biosystems, Foster City, CA). The concentration of β2m was determined using its absorption coefficient at 280 nm of 19300 cm^{-1} M^{-1}, calculated on the basis of its amino acid sequence (*35*).

Effect of Salts on β2m Fibril Growth. The seed-dependent elongation of β2m fibrils was performed essentially following the method described earlier (*16*). Monomeric β2m (25 μM) in 50 mM sodium citrate buffer (referred to as citrate buffer henceforth), pH 2.5, either in the absence or presence of different concentrations of salts such as NaCl, NaI, $NaClO_4$, and Na_2SO_4, was incubated along with 5 μg/mL sonicated preformed fibrils (referred to as fibril seed henceforth) at 37 °C. Aliquots of the sample (10 μL) were withdrawn at different time intervals and added to 1 mL of 5 μM thioflavin T (ThT) in 50 mM glycine−NaOH buffer, pH 8.5 (referred to as 5 μM ThT solution henceforth). Fluorescence intensity of the sample at 485 nm, which is proportional to the extent of amyloid fibril-bound ThT (*36*), was measured using a Hitachi F-4500 fluorescence spectrophotometer with the excitation wavelength set at 445 nm. The role of salts on the fibril growth at different pHs was examined similarly, except that 25 mM phosphate−citric acid buffers with pH ranging from 4 to 7 were used at a fibril seed concentration of 10 μg/mL. The results were found to be reproducible. Representative data are shown in the figures.

Effect of Salts on the Stability of β2m Amyloid Fibrils. The stability of the β2m fibrils was studied under two different conditions. First, the fibrils were incubated with the indicated salts (100 mM) in 50 mM citrate buffer, pH 2.5, at 37 °C for 1 h. Then, 10 μL of the sample was withdrawn and added to 1 mL of 5 μM ThT solution, and fluorescence was measured as described above. This experiment was performed to find out whether the inhibition of the fibril elongation reaction observed in the presence of 100 mM sulfate or perchlorate is due to their effect on the fibril stability under similar conditions.

Second, we have systematically studied the role of different anions on the fibril stability, as we found that they are unstable upon incubation of the fibrils in the absence of salt even though the medium pH was maintained at 2.5 by HCl. Amyloid fibrils of β2m prepared at pH 2.5 by the seed-dependent method were collected by centrifugation and resuspended at a concentration of 0.8 mg/mL in ~3.2 mM HCl solution, pH 2.5, containing 0.1 M NaCl, which was used as a stock solution. Salt effect was studied by incubating fibrils at a concentration of 20 μg/mL in the ~3.2 mM HCl solution, pH 2.5, containing different concentrations of various salts overnight (~20 h) at 37 °C. An aliquot (5 μL) of the sample was added to 1 mL of 5 μM ThT solution, and the fluorescence intensity at 485 nm was measured.

Salt-Induced Changes in the Hydrophobicity of β2m. Samples of β2m (0.1 or 0.2 mg/mL) in the required buffer were incubated with 100 μM 8-anilinonaphthalene-1-sulfonic acid ammonium salt (ANS), purchased from Nacalai Tesque, Kyoto, Japan, at 37 °C in the thermostated cuvette holder of a Hitachi F-4500 fluorescence spectrophotometer for 5 min. The samples were titrated with increasing concentrations of the salts, and the fluorescence spectra were recorded with the excitation wavelength of 365 nm.

Intrinsic Tryptophan Fluorescence. To study the effect of salts on the intrinsic tryptophan fluorescence of the monomeric β2m, 0.1 mg/mL β2m in 50 mM citrate buffer, pH 2.5, was incubated at 37 °C in the thermostated cuvette holder of a Hitachi F-4500 fluorescence spectrophotometer for 5 min. The fluorescence spectrum was recorded with the excitation wavelength of 295 nm. The sample was titrated with increasing concentrations of the selected salts, and fluorescence spectra were recorded after 5 min incubation at 37 °C for every addition of salt.

Circular Dichroism (CD). The far-UV CD spectra of the native (in 50 mM sodium phosphate buffer, pH 7.4) and the acid-denatured (in 50 mM sodium citrate buffer, pH 2.5) β2m in the absence and presence of salts were recorded at 37 °C using a Jasco-600 spectropolarimeter with a thermostated cell holder. A quartz cuvette with 0.1 cm path length was used. The samples were incubated in the required media at 37 °C for at least 15 min before the spectra were recorded. The fibril β2m samples in the presence of the indicated concentrations of salts were prepared as described above and diluted 1:1 (v/v) with respective media before the spectra were recorded. The results shown are expressed as the mean residue ellipticity [θ].

Sedimentation Velocity. Sedimentation velocity measurements were performed using an Optima XL-I analytical ultracentrifuge (Beckman Coulter, Fullerton, CA) with An-60 rotor and two-channel charcoal-filled Epon cells. Samples (0.3 mL) of 50 μM β2m in 50 mM citrate buffer, pH 2.5, in the absence and presence of required concentrations of salts were incubated at 37 °C for 15 min, and then the centrifugation experiment was performed. The data were analyzed using the software Ultrascan 6.01 (SciScan LLC, Missoula, MT).

Total Internal Reflection Fluorescence Microscopy (TIRFM). The sample (10 μL) was mixed with 10 μL of 10 μM ThT in 100 mM glycine−NaOH buffer, pH 8.5, and the mixture was placed on a glass slide. The TIRFM system to observe ThT-bound amyloid fibrils was developed on the basis of an inverted microscope (IX70; Olympus, Tokyo, Japan) as described earlier (*37*). ThT was excited by an argon laser (Model 185F02-ADM; Spectra Physics, Mountain View, CA). The fluorescent image was filtered by a band-pass filter (D490/30; Omega Optical, Brattleboro, VT) and visualized using a digital camera (DP70; Olympus, Tokyo, Japan). This system has also been demonstrated to be useful in monitoring growth of amyloid fibrils (*38*).

Electron Microscopy. The amyloid fibril sample of β2m was diluted 20-fold by deionized water and immediately placed on a carbon-coated grid. The excess solution was removed with filter paper after the sample was allowed to stand for 2−3 min. The grid was air-dried. The fibrils adsorbed on the grid were negatively stained with 1% phosphotungstic acid, pH 7.0, and examined under an H-7000 electron microscope (Hitachi) with an acceleration voltage of 75 kV.

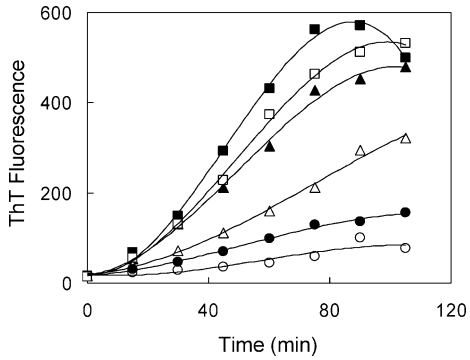


FIGURE 1: Effect of NaCl on the β2m amyloid fibril growth at 37 °C. Fibril growth as a function of time in 50 mM citrate buffer, pH 2.5, alone (○) and the buffer containing 25 mM (●), 50 mM (△), 100 mM (▲), 150 mM (□), and 200 mM (■) NaCl. 5 μg/mL fibril seeds and 25 μM β2m monomers were used. Fibril growth was monitored by ThT fluorescence intensity at 485 nm (shown in arbitrary units).

RESULTS

Effect of NaCl on the Amyloid Fibril Growth at pH 2.5. Figure 1 shows the fibril elongation reaction of β2m at pH 2.5 in the absence and presence of different concentrations of NaCl at 37 °C as monitored by the binding of ThT. When 25 μM β2m and 5 μg/mL fibril seed were incubated in buffer alone in the absence of salt, no significant increase in the ThT fluorescence was observed during the experimental period of 105 min, showing that significant fibril growth does not occur in the absence of salt even though the reaction is seeded with preformed fibrils. In the presence of NaCl, the ThT fluorescence intensity increased markedly as a function of time, indicating the growth of the β2m fibrils. The extent and the rate of increase in the fibril growth depended on the salt concentration, the maximum being at 200 mM NaCl (Figures 1 and 2A). Further increase in concentration of NaCl, however, leads to decrease in the fibril growth. The value of ThT fluorescence intensity at 60 min incubation showed a bell-shape profile revealing an optimal concentration of the salt required for efficient fibril growth of β2m (Figure 2A).

Role of Different Anions on the Amyloid Fibril Growth. To understand the role of different anions, we have compared the effect of various salts, such as Na_2SO_4, $NaClO_4$, NaI, and NaCl (composed of different anions and the same countercation, Na^+), on the fibril growth of β2m. Similar to the observation made for NaCl, we also found a concentration-dependent change in the fibril growth for other salts (Figure 2A). Intriguingly, the optimal concentrations of the salts differed drastically depending on the anionic species of the salts. The optimal concentration under which fibril elongation is favored was around 200, 50, 25, and 3 mM for NaCl, NaI, $NaClO_4$, and Na_2SO_4, respectively. Thus, the order of minimum concentration at which fibril growth is favored is $SO_4^{2-} > ClO_4^- > I^- > Cl^-$. This order also holds for higher concentrations of the salt at which the fibril growth is inhibited.

TIRFM Image of Amyloid Fibrils. β2m fibrils formed under acidic conditions depolymerize (i.e., unfold) upon

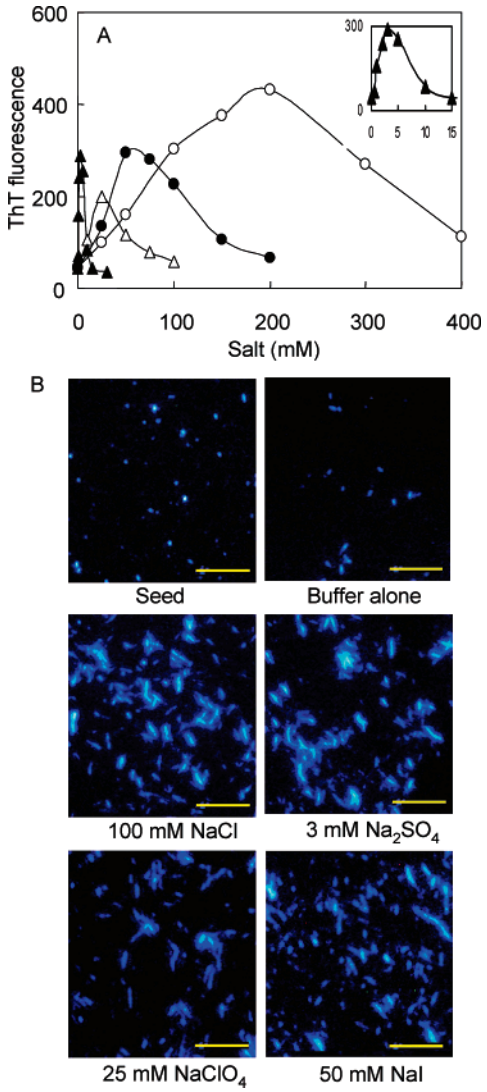


FIGURE 2: Modulation of β2m amyloid fibril growth at pH 2.5 by different anions. (A) Effect of different salts on the β2m (25 μM) amyloid fibril growth at 37 °C: NaCl (○), NaI (●), $NaClO_4$ (△), and Na_2SO_4 (▲). ThT fluorescence intensity at 485 nm of the samples after 60 min incubation is shown in arbitrary units. 5 μg/mL fibril seed was used. The inset shows the effect of Na_2SO_4 in the expanded scale for clarity. (B) TIRFM image of the ThT-bound fibril seeds and the fibrils formed in the absence and presence of indicated concentrations of the salts. 10 μL of the sample (incubated for 60 min at 37 °C) was mixed with 10 μL of 10 μM ThT in 100 mM glycine−NaOH buffer, pH 8.5, before being used for fluorescence imaging. Bars (yellow) represent 5 μm.

shifting to neutral pH (*18*). We found that the binding of ThT inhibits the fibril depolymerization significantly (data not shown), thus allowing us to visualize the ThT-bound fibrils under TIRFM. Figure 2B shows the fluorescence microscopy image of the ThT-bound seed fibrils and the fibrils formed in the presence of various salts. As evident from the figure, the different salts promoted the growth of β2m fibrils with rodlike morphology, which are indistinguishable by fluorescence microscopy.

Induction of β-Sheet Structure upon Amyloid Fibril Growth. Association-induced β-sheet formation appears to be a common property among various amyloid fibrils. We, therefore, investigated the generation of β-structure upon fibril growth promoted by various anions. Native monomeric β2m exhibits unique chiral structure as seen in its far-UV

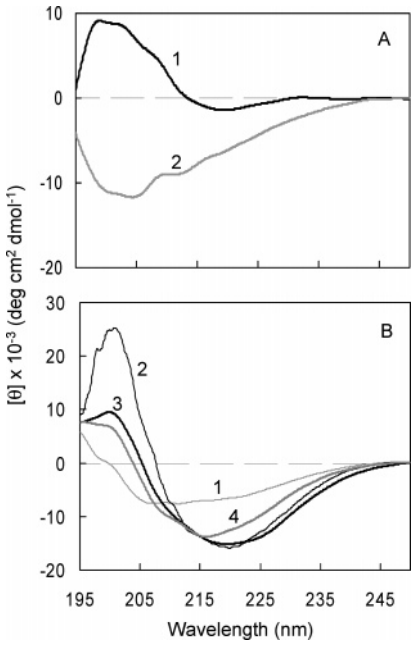


FIGURE 3: Far-UV CD spectra of native, acid-denatured, and amyloid states of β2m. (A) Spectra of the monomeric, native β2m in 50 mM sodium phosphate buffer, pH 7.4 (curve 1), and the acid-denatured β2m in 50 mM citrate buffer, pH 2.5 (curve 2). (B) Spectra of β2m amyloid fibrils. 25 μM β2m monomers in 50 mM citrate buffer, pH 2.5, incubated with 5 μg/mL fibril seeds at 37 °C for 80 min in the absence and presence of salts. Spectra shown represent that of the samples in the absence of salts (curve 1) and in the presence of 100 mM NaCl (curve 2), 3 mM Na_2SO_4 (curve 3), and 25 mM $NaClO_4$ (curve 4). The CD spectrum of the sample in the presence of NaI could not be recorded due to high absorbance.

CD spectrum (Figure 3A). Under acidic conditions (pH 2.5), it exhibits almost completely random coil structure (Figure 3A). The far-UV CD spectrum of the sample of β2m at pH 2.5, incubated with 5 μM fibril seeds in the absence of salt for 80 min at 37 °C, does not appear to differ significantly, especially between 250 and 200 nm region, from that of the acid-denatured state of β2m. These results corroborate well with the conclusion that no fibril growth occurs in the absence of salts (Figure 1).

The far-UV CD spectrum of the sample in the presence of 100 mM NaCl indicates an induction of β-sheet structure upon fibril growth (curve 2 of Figure 3B). The conformational state of the protein molecule in the fibrillar state is distinct from that of the native state as seen from their far-UV CD spectra. A hydrogen/deuterium exchange study using nuclear magnetic resonance spectroscopy revealed marked differences in the residues involved in the core region of the fibrils, which are protected from exchange, from that of the β-sheets in the native protein (*39*). In addition, comparison of immature and mature β2m fibrils and the fibrils formed with an amyloidogenic fragment of β2m showed that they exhibit a distinct core region (*40*). Similar to the observation of structure induction upon fibril growth in the presence of NaCl, structural induction is also observed for β2m fibrils promoted by sulfate and perchlorate (curves 3 and 4 in Figure 3B, respectively).

Anions Stabilize the Amyloid Fibrils under Acidic Conditions. Although salts are required, higher concentrations of salts inhibit the fibril growth. There are two possible reasons for this observation: (i) High concentrations of salts destabilize the fibril. (ii) They modify the monomeric species of

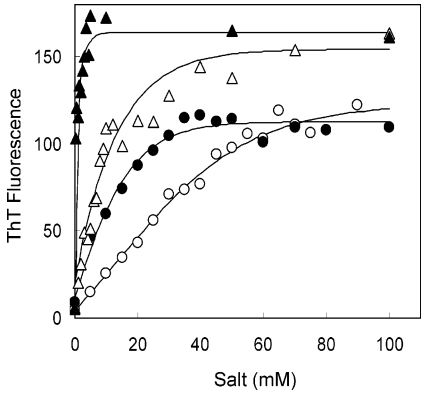


FIGURE 4: Stabilization of β2m amyloid fibrils by anions. Preformed fibrils were incubated in ~3.2 mM HCl solution (pH 2.5) in the presence of different salts at 37 °C for about 20 h. The amount of fibrils in the samples was measured by the ThT binding. Different salts used are NaCl (○), NaI (●), $NaClO_4$ (△), and Na_2SO_4 (▲).

β2m, causing conformational changes that are not appropriate for fibril growth or promoting nonamyloidogenic aggregates that do not bind ThT. We, therefore, set out to investigate fibril stability under various conditions to gain insight into whether the anion-dependent fibril stability is one of the important factors in amyloid growth.

When the preformed fibrils were incubated, for example, in the presence of 100 mM sulfate or perchlorate, the fibrils were found to be stable as measured by ThT fluorescence (data not shown). Thus, the observed inhibition of fibril elongation at high concentrations of salts is not due to the fibril instability. Rather interestingly, we found that salt is required for the stability of the fibrils under certain conditions.

We observed that the β2m fibrils become unstable when placed in water (i.e., in the absence of salts) even though the pH was maintained at 2.5 by a small amount of HCl (~3.2 mM). This system allowed us to investigate the influence of different anions, if any, toward fibril stability. Preformed fibrils were incubated in the presence of indicated concentrations of different salts in ~3.2 mM HCl solution, pH 2.5, for approximately 20 h at 37 °C, and the samples were tested for their ability to bind and enhance the fluorescence of ThT. In the absence of salt, no significant amount of fibril is present as judged by the ThT assay (Figure 4), indicating that the fibrils depolymerize in the absence of salts. The ThT fluorescence intensity increased as a function of salt concentration depending on the nature of the salt used. This result indicates that (i) salts stabilize the amyloid fibrils of β2m and (ii) the stability of the β2m fibril varies depending on the nature of the anionic species of salts. It is important to note that, even at very low concentrations (as low as 0.2 mM), sulfate stabilizes the fibrils to a significant extent while chloride exhibits a relatively negligible stabilizing effect at this concentration. The order of minimum concentration of the anions required to stabilize the fibril is again $SO_4^{2-} > ClO_4^- > I^- > Cl^-$, the same order as observed for promoting fibril growth. Moreover, the concentration ranges of salts required for stabilizing the fibrils are lower than those for the fibril growth (Figure 2A), indicating that low concentrations of salts are enough to maintain the preformed fibrils. Thus, preferential anion binding plays a role in fibril stability as well, in addition to its effect on the β2m fibril growth.

Anion-Induced Changes in the Hydrophobic Surfaces of the Acid-Denatured β2m. Anions appear to preferentially interact and modulate amyloidogenicity of β2m and stabilize the amyloid fibrils under acidic condition. Since hydrophobic interactions also mediate amyloid formation, we have investigated whether salt-induced modulation of charge interaction can influence the hydrophobicity of the protein molecule. We have probed the hydrophobic surfaces of the acid-denatured state of β2m using a hydrophobic probe, ANS (*41*−*43*). Upon binding to the hydrophobic surfaces, the fluorescence intensity of the probe increases accompanying a blue shift in the wavelength of the emission maximum depending on the extent of the apolar nature of its microenvironment.

Addition of Na_2SO_4 to the sample of acid-denatured β2m leads to an increase in the ANS fluorescence accompanying a blue shift in the emission wavelength (Figure 5A). The order of effectiveness of various anions is $SO_4^{2-} > ClO_4^- > I^- > Cl^-$, the same as the order promoting the amyloidogenicity (Figure 5B,C). Although iodide is a known fluorescence quencher (*44*), a net increase in the fluorescence intensity is observed upon addition of iodide, suggesting that the ion-induced effects dominate over any quenching effect of the iodide at its concentration range studied. It is also to be noted that electrostatic interactions of the probe, ANS, can induce partially folded states in acid-unfolded cytochrome *c* (*45*) and pectate lyase *c* (*46*). These examples suggest that the electrostatic interaction of the probe may sometimes complicate the interpretation of the results. However, we believe that the possible electrostatic interaction of the probe with β2m may not complicate our results due to the following reasons. First, we have investigated the effect of ANS on the far-UV CD spectrum of unfolded β2m at pH 2.5 and found that, at the probe concentration used to probe the hydrophobic surface (100 μM), the far-UV CD spectrum of the unfolded state of β2m is not affected significantly (data not shown), indicating that ANS-induced structural induction does not occur in the case of β2m. Second, our study deals with the effect of various concentrations of salts on ANS binding to the protein. If electrostatic interaction of ANS is significantly responsible for the observed enhancement of the fluorescence intensity and the blue shift in the emission maximum, we should expect screening of charges upon addition of salt leading to decrease in the fluorescence intensity and red shift in the emission maximum. Contrary to this expectation, our results show an increase in the ANS fluorescence accompanying blue shift in the emission maximum upon addition of salts. Therefore, the possible electrostatic interactions of ANS with the protein molecule do not significantly influence the observed results of salt-induced changes in the hydrophobicity of the β2m molecule.

Thus, it is evident from our results that modulation of electrostatic interactions by preferential anion interactions can, indeed, lead to increase in the hydrophobicity of β2m. It is to be noted that, although the hydrophobicity of the protein molecule continues to increase as a function of salt concentrations (Figure 5), amyloid fibril formation is favored only in a certain concentration range of the salts (Figure 2). This comparison, therefore, suggests that a critical balance of electrostatic and hydrophobic interactions is a determining

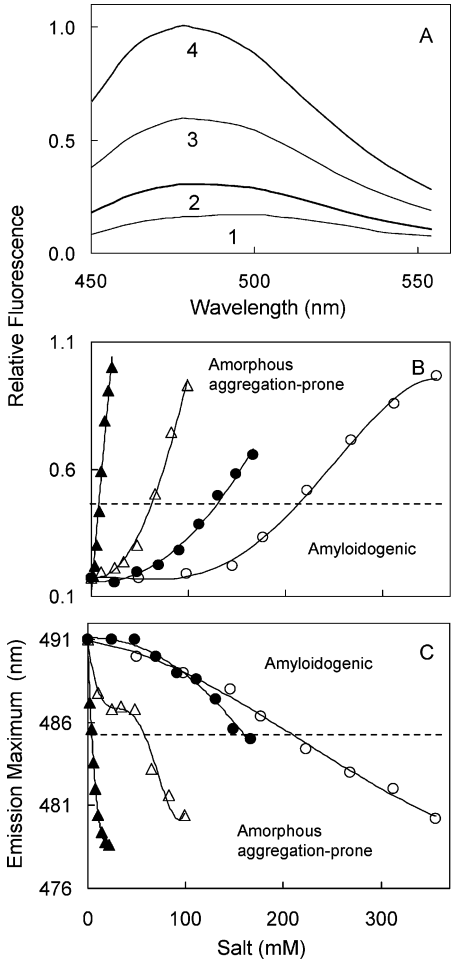


FIGURE 5: Salt-induced increase in the hydrophobicity of β2m (0.2 mg/mL) in 50 mM citrate buffer, pH 2.5. (A) Fluorescence spectrum of ANS bound to the acid-denatured β2m in the absence (curve 1) and presence of 6 mM (curve 2), 10 mM (curve 3), and 22 mM (curve 4) Na_2SO_4. (B) Changes in the relative fluorescence intensity and (C) changes in the wavelength of emission maximum of the protein-bound ANS as a function of salt concentration: NaCl (○), NaI (●), $NaClO_4$ (△), and Na_2SO_4 (▲). It is to be noted that although iodide is a fluorescence quencher (*44*), a net increase in the fluorescence intensity is observed under the condition. Dashed lines approximately divide the plots to indicate conditions and the species that relatively favor amyloid fibril growth and amorphous aggregation.

factor in amyloid formation and the protein−anion interactions can modulate the necessary balance between the two forces.

Effect of Salts on the Conformation of the Acid-Denatured β2m. We have investigated the effect of anions on the conformation of the monomeric β2m at pH 2.5. Figure 6A shows the tryptophan fluorescence spectra of β2m in the absence and the presence of 100 mM NaCl and Na_2SO_4 at pH 2.5. In the absence of salts, β2m exhibits an emission maximum around 345 nm. In the presence of 100 mM NaCl, a minor shift toward lower wavelength is observed. On the other hand, in the presence of 100 mM Na_2SO_4, a clear blue shift of about 6 nm in the emission maximum is observed, suggesting that the tryptophan residues of β2m becomes less solvent accessible in the presence of 100 mM sulfate. Figure 6B compares the changes in the wavelength of the emission maximum of β2m in the presence of different concentrations of the selected salts, revealing that sulfate is more potent than other salts such as perchlorate, iodide, and chloride.

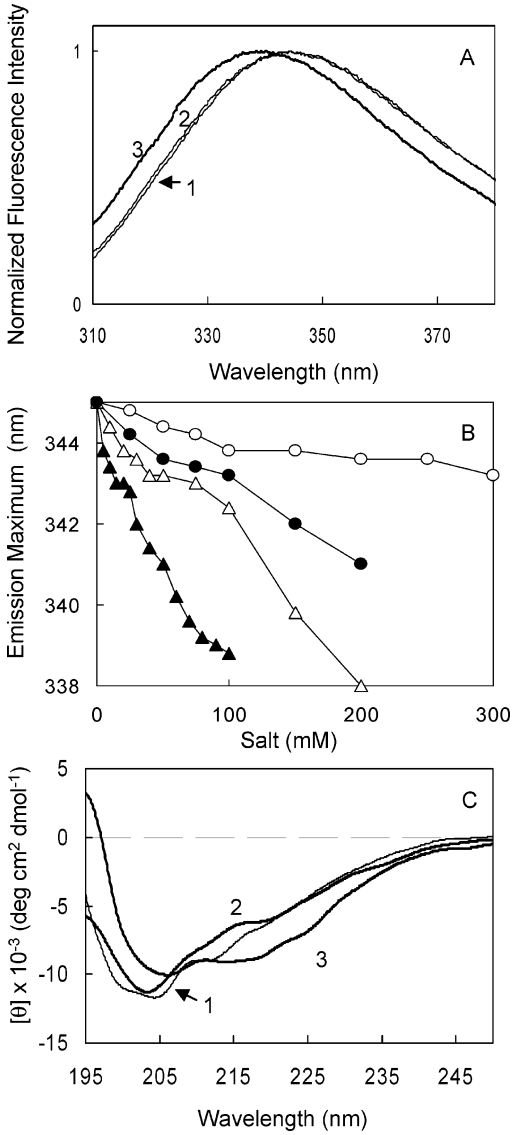


FIGURE 6: Salt-induced conformational change in the acid-denatured state of β2m. (A) Intrinsic tryptophan fluorescence spectra of β2m at 37 °C in 50 mM citrate buffer, pH 2.5, alone (curve 1) and in the presence of 100 mM NaCl (curve 2) and 100 mM Na_2SO_4 (curve 3). (B) Shift in the wavelength of the emission maximum of the tryptophan fluorescence of β2m induced as a function of concentration of NaCl (○), NaI (●), $NaClO_4$ (△), and Na_2SO_4 (▲). (C) Effect of Na_2SO_4 on the far-UV CD spectrum of the acid-denatured state of β2m in 50 mM citrate buffer, pH 2.5, at 37 °C: in buffer alone (curve 1) and in the presence of 3 mM (curve 2) and 30 mM (curve 3) Na_2SO_4.

The far-UV CD spectrum (Figure 6C) shows a change in secondary structure induced by high concentration (30 mM, for example) of sulfate. These results indicate that a change in the conformational state of β2m in acidic pH induced by salt depends on the nature and the concentration of anions.

We have also investigated whether such a conformational change reflected by fluorescence and CD studies also coupled with changes in the association state of β2m at pH 2.5. Figure 7 shows the distribution of the sedimentation coefficient ($s_{20,w}$) of β2m at pH 2.5 in the absence and the presence of NaCl and Na_2SO_4. In the absence of the tested salts, the $s_{20,w}$ value is around 1.3, indicating that the protein exists predominantly in the monomeric state (Figure 7A). In the presence of 100 mM NaCl or 2 mM Na_2SO_4, the concentrations of the salts at which fibril growth is favored, the $s_{20,w}$

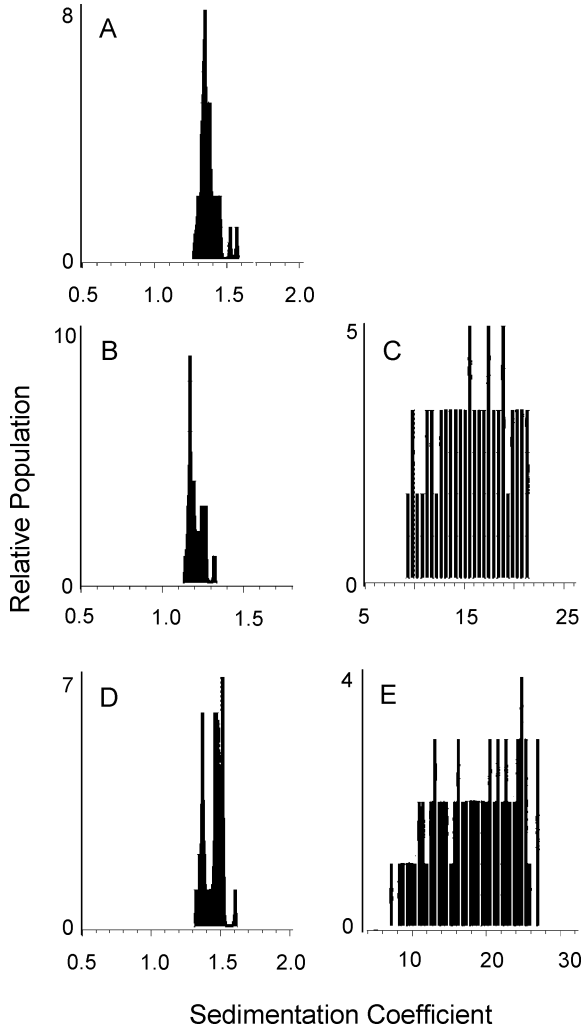


FIGURE 7: Effect of salts on the distribution of sedimentation coefficients ($s_{20,w}$) of β2m species at pH 2.5 in 50 mM citrate buffer, pH 2.5, alone (A), in the presence of 100 mM (B) and 400 mM (C) NaCl, and in the presence of 3 mM (D) and 30 mM (E) Na_2SO_4.

values did not differ significantly (Figure 7B,D). However, at high concentrations of NaCl (400 mM) and Na_2SO_4 (30 mM), where the fibril growth is inhibited, high and heterogeneous distribution of the $s_{20,w}$ value is observed (Figure 7C,E).

Thus, all of these results argue that moderate concentration of the anions is required for the critical balance of charge repulsion and hydrophobic interactions required for fibril growth, while high concentration of the anions leads to partitioning of the protein molecule into heterogeneous less ordered associated state(s), which are not competent for amyloid growth. Such less ordered associated states do not bind ThT. However, our results show that the amyloid fibrils once formed are stable when treated with even high concentrations of the anions.

Effect of Salts at Physiologically Relevant pH. Having found a marked effect of anions on the fibril growth of β2m at pH 2.5, it is pertinent to investigate their effect, if any, at near neutral pH conditions. We have, therefore, incubated 25 μM monomeric β2m in the presence of 10 μg/mL fibril seeds in the absence and presence of 100 mM NaCl and Na_2SO_4 at different pH conditions and carried out the ThT assay. Interestingly, the ThT fluorescence increases markedly in the presence of 100 mM Na_2SO_4 at pH between 5 and 6

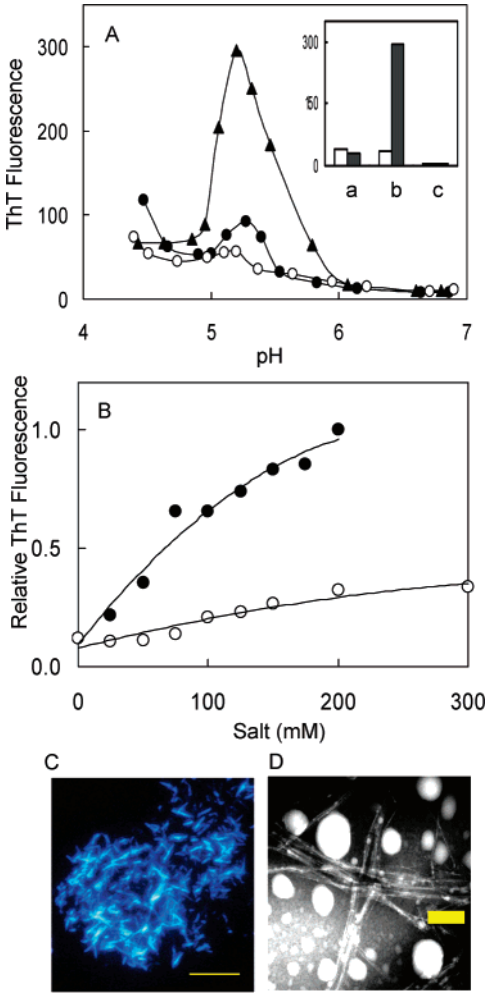


FIGURE 8: Effect of pH on the salt-induced promotion of the β2m amyloid fibril growth. (A) β2m fibril growth at 37 °C in 25 mM sodiun phosphate−citric acid buffer alone at various pHs (○) and in the presence of 100 mM NaCl (●) and 100 mM Na_2SO_4 (▲). 10 μg/mL fibril seed was used. ThT fluorescence intensity (in arbitrary units) at 485 nm was measured after 48 h of incubation at 37 °C. Inset: (a) 10 μg/mL fibril seed alone; (b) 10 μg/mL fibril seed plus 25 μM monomeric β2m; (c) 25 μM monomeric β2m alone. Open and solid bars represent 0 and 48 h incubation periods. (B) Effect of NaCl (○) and Na_2SO_4 (●) on β2m fibril growth at pH ~5.3 as a function of salt concentration. The relative ThT fluorescence intensity at 485 nm is shown. (C) TIRFM image of ThT-bound β2m amyloid fibril formed in the presence of 100 mM Na_2SO_4 at pH 5.3. 10 μL of the sample (incubated for 45 h at 37 °C) was mixed with 10 μL of 10 μM ThT in 100 mM glycine−NaOH buffer, pH 8.5, before being used for fluorescence imaging. The bar (yellow) represents 5 μM. (D) Electron microscopy image of negatively stained β2m amyloid fibril formed in the presence of 100 mM Na_2SO_4 at pH 5.3. The bar (yellow) represents 200 nm.

(Figure 8A). On the other hand, relatively less increase in ThT fluorescence is observed in the presence of 100 mM NaCl. Such an increase in ThT fluorescence of the samples incubated at pH 5.3 is dependent on the concentration of the anions, the effect being more pronounced in the case of sulfate than chloride (Figure 8B). Fluorescence microscopic examination of the ThT-bound state formed in the presence 100 mM Na_2SO_4 at pH 5.3 shows rodlike structures of varying lengths (Figure 8C) consistent with the presence of amyloid fibrils in the electron microscopy image (Figure 8D).

We have investigated the effect of sulfate on the hydrophobic surfaces of the protein at pH 5.1 where the fibril growth is favorable. Sulfate is more effective than chloride

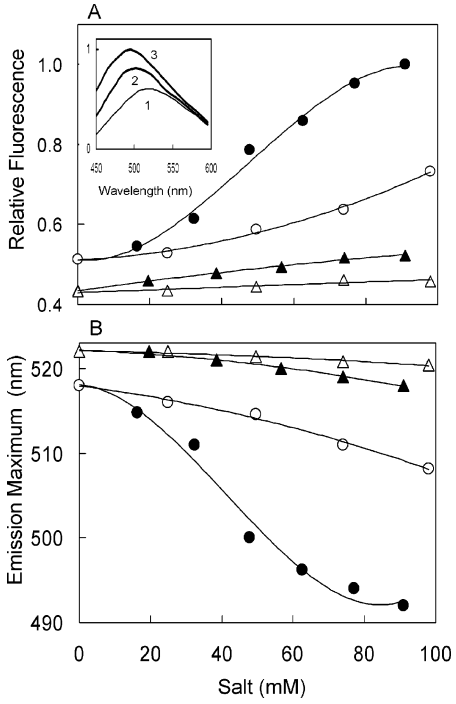


FIGURE 9: Effect of NaCl (open symbols) and Na_2SO_4 (closed symbols) on the hydrophobicity of β2m (0.1 mg/mL) in 25 mM sodium phosphate−citric acid buffer at pH 5.1 (○, ●) and pH 6.8 (△, ▲). Changes in the relative fluorescence intensity (A) and the wavelength of the emission maximum (B) of the protein-bound ANS as a function of salt concentration. The inset in panel A shows the fluorescence spectra of the ANS bound to the protein in the absence (curve 1) and in the presence of 48 mM (curve 2) and 91 mM (curve 3) Na_2SO_4.

in increasing the hydrophobicity of the protein (Figure 9). The increase in the hydrophobicity of the protein induced by sulfate seems to be moderate in this condition (pH 5.1) as compared to the extremely acidic condition (pH 2.5) as judged by the extent of increase in ANS fluorescence intensity and the shift in the wavelength of the emission maximum (compare Figures 5 and 9). This observation is consistent with our inference that moderate hydrophobicity is favorable for amyloid fibril growth. The effects of perchlorate (100 mM) in promoting fibril growth at pH around 5, however, did not differ significantly from the effects of 100 mM NaCl (data not shown). Our result of anionic interactions, especially the effect of Na_2SO_4, on promoting the fibril growth is striking. Such anion interactions might play a crucial role in vivo fibril formation as glycosaminoglycans and proteoglycans (can be considered as sulfated polyanions) are known to be involved in amyloid formation (*23*, *24*, *47*−*54*).

DISCUSSION

Role of Anions on the Amyloid Fibril Growth of β2m under Acidic Conditions. The role of anionic interactions in the amyloid aggregation is not clearly understood. Anion binding is found to populate the partially folded state in acid-induced unfolding of many proteins such as apomyoglobin, cytochrome *c*, β-lactamase, and staphylococcal nuclease (*31*, *32*, *55*, *56*). In this context, it is important to investigate whether anions influence amyloid formation. Interestingly, our results show that there is an optimal concentration of salts required for efficient fibril growth, suggesting that counterion interaction is crucial in amyloid formation. A critical balance in hydrophobic and hydrophilic interactions may be necessary in selecting out specific populations of denatured state(s) that are amyloidogenic in nature. Our finding shows that different anions exhibit different optimal concentrations for promoting fibril growth of β2m at acidic pH (2.5). The order of anions, based on the lowest concentration at which the fibril elongation favored, was found to be $SO_4^{2-} > ClO_4^- > I^- > Cl^-$. Thus, anions differ in modulating the necessary hydrophobic and electrostatic balance critical for amyloid aggregation.

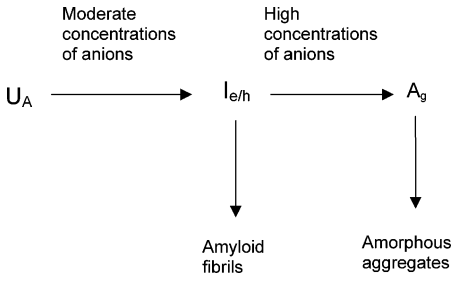
Mechanism of the Anion Effects under Acidic Conditions. Salts may affect the hydrophilic and hydrophobic balance in protein molecules by charge screening effects, which disrupt either repulsive or attractive intra- and intermolecular electrostatic interactions. They may also indirectly affect the balance through perturbing water structure that affects hydration of the protein molecule (*57*). The mechanisms of salt-induced electrostatic effects in protein molecules may involve (i) the Debye−Huckel screening and (ii) ion pairing (or binding) (*31*, *32*). Which mechanism predominantly operates in a given system may depend on various factors including nature of the protein, pH, salt concentration, etc.

If salts affect the system through perturbing water structure, then they should follow Hofmeister's series (*58*), which in this case is $SO_4^{2-} > Cl^- > I^- > ClO_4^-$, as perchlorate is a chaotropic ion while sulfate is a kosmotropic ion. However, our results show that the order of these ions in promoting fibril growth of β2m is $SO_4^{2-} > ClO_4^- > I^- > Cl^-$, thus ruling out water structure perturbation as being a predominant factor at pH 2.5. In electrostatic interactions, if the Debye−Huckel screening effect is alone a predominant factor, then it should depend on the ionic strength regardless of the nature of ions. While β2m fibril growth is favored by 3 mM sodium sulfate, it is found to be far less at the equivalent ionic strength contributed by 9 mM sodium chloride (inferred from Figure 2A), thus suggesting that ionic strength alone cannot explain the observed behavior of these salts toward the β2m fibril growth at pH 2.5. On the other hand, our results (the order of the anions) are consistent with the electroselectivity series of anions (*59*, *60*), suggesting that preferential interaction (or binding) of anions with the positive charges on the protein can modulate the amyloid formation of β2m. Although investigating detailed thermodynamic aspects of anion bindings is intriguing, such anion bindings are relatively weak, so that the detailed study of the binding thermodynamics would be fairly difficult at this moment.

Recent studies have shown that anions can affect the fibril formation of α-synuclein and histones (*61*, *62*). Interestingly, in the case of α-synuclein, the relative ability of various anions in promoting fibril formation was found to follow Hofmeister's series at salt concentrations above 10 mM while below the concentration of the salts electrostatic interactions dominated in the mechanism (*61*). Our study shows that, in the case of β2m, preferential anion interactions play a predominant role in modulating the amyloid formation.

Hydrophobic interactions should be considered in conjunction with electrostatic interactions as the balance appears to be critical in amyloid formation. Our results also show that preferential anion interactions can lead to significant increase in the hydrophobicity of the molecule (Figure 5). However, only a certain concentration range, depending on the nature

Scheme 1



of anions, is favorable for amyloid fibril growth where the salt-induced hydrophobicity increase is moderate. High concentrations of anions lead to drastic change in the conformation as revealed by tryptophan fluorescence and CD ultimately producing amorphous aggregation.

On the basis of our results, we propose a model for the role of anion interactions in the amyloid fibril growth of β2m from its acid-unfolded state at pH 2.5 as shown in Scheme 1. The electrostatic repulsions in the highly positively charged unfolded state (U_A) are not favorable for the amyloid fibril formation. Preferential binding of the anions, at low concentrations, moderates the charge repulsions, accumulating an intermediate state(s) ($I_{e/h}$) with appropriate electrostatic and hydrophobic surfaces for the critical interactions leading to amyloid fibril growth. This state seems to differ marginally from U_A but is still highly disordered. Decreasing the effective charge on the protein molecule further, by higher concentrations of the anions, leads to induction of secondary structures, burial of aromatic residues, and clustering of exposed hydrophobic surfaces. Such an unbalanced collapsed state (A_g) with exposed hydrophobic surface partitions quickly to amorphous aggregation. The amorphous aggregates are not competent for amyloid fibril growth. Similar compact states favored by certain anions, which rapidly form amorphous aggregates, were also observed for the SH3 domain under acidic conditions (*28*), suggesting that this model may find general applicability, especially under acidic conditions. It has also been proposed that the amyloidogenic conformation is relatively unfolded and shares structural properties with the pre molten globule state whose inherent flexibility is essential in allowing conformational rearrangements necessary to form the core cross-β structure of amyloid fibrils (*5*). The largely unfolded nature of the amyloidogenic species of β2m ($I_{e/h}$) at low concentrations of anions is consistent with the above-mentioned proposal.

Role of Anions in β2m Amyloid Fibril Stability under Acidic Condition. Our study also shows that moderating charge balances is also important in β2m fibril stability at pH 2.5. Anions in the order $SO_4^{2-} > ClO_4^- > I^- > Cl^-$ stabilize the fibril state of β2m under the acidic conditions, suggesting that preferential interaction with anions may influence not only fibril growth but also fibril stability. It appears that the factors important for fibril stability are also important in amyloid formation. This suggestion corroborates well with the earlier mutation studies from this laboratory (*33*): Though proline mutations at specific locations destabilize the native state of β2m to a significant extent, the net extent of amyloid formation was found to correlate with the stability of the fibril state of the mutants (*33*). Thus, preferential interactions of sulfate ions seem to favor both fibril stability and fibril growth.

Sulfate Promotes the Amyloid Fibril Growth of β2m at pH near pI. Earlier in vitro studies have shown that monomeric wild-type β2m readily forms fibrils at pH below 4 (*13*, *16*, *17*), while mutations or truncation of N- or C-terminal residues is required to facilitate the fibril formation around neutral pH (*63*−*66*). On the other hand, the full-length β2m is found in the amyloid deposits from patients of dialysis-related amyloidosis.

One of the hypotheses to explain this puzzle would be that "as of now unknown serum factors are required in the formation of amyloid aggregates in vivo". Other schools of thought include the following: (i) the intrinsic crystal structural difference of the monomeric β2m compared to its form in the major histocompatibility complex class 1 (*67*) and the dynamic nature of the N-terminal and the C-terminal ends of the molecule as revealed by nuclear magnetic resonance spectroscopic studies (*68*) make the molecule vulnerable for the amyloid association and (ii) an equilibrium population detected in capillary electrophoresis under neutral pH condition, though found to be minor, yet forms amyloid fibrils, suggesting that this equilibrium population may be important under physiological condition (*22*). Some peptides derived from the β2m sequence exhibit intrinsic propensities to form amyloid fibrils at neutral pH (*69*, *70*), showing that exposure of such amyloidogenic regions are crucial in the formation of amyloid aggregation of the full-length protein. In fact, either deletion or perturbing the N- and C-terminal strands facilitates the fibril formation of β2m near neutral pH conditions (*64*). Despite these advancements in this area, factors involved in amyloid aggregation of β2m in vivo leading to dialysis-related amyloidosis are not completely understood.

Whether in Hofmeister's series of anions (involving water structure effects) or in electroselectivity series of anions (involving electrostatic interactions), sulfate occupies high in the order, suggesting that sulfate may exhibit a unique property. We have investigated the β2m fibril growth in the presence of salts at pH between 4 and 7. Interestingly, we found that sulfate can promote β2m fibril growth significantly at pH between 5 and 6, while chloride is less effective under the same conditions. Sulfate-induced promotion of fibril elongation occurs through a rather narrow range of pH values below the theoretical p*I* value of 6.1 for β2m. The net charge on the protein appears to contribute to a significant extent in amyloid formation (*25*−*28*): a marked correlation has been observed between the net charge and aggregation rate among two homologous proteins, the N-terminal domain of *E. coli* HypF and human muscle acylphosphatase and their mutants, indicating that a decrease in the positive net charge of the proteins leads to an increase in the aggregation rate (*25*). Interestingly, 14 out of 16 mutations involved in pathological conditions were found to decrease the net charge, suggesting that decrease in net charge is an important predisposition factor in some forms of protein aggregation diseases (*26*). It has been proposed that the pH at maximal fibril formation for some proteins is near their p*I*, suggesting that solubility of the protein is important in amyloid formation (*27*). Cosolute anion interaction is one of the important factors that can reduce effective charge on a protein molecule, thereby facilitating intermolecular association of β2m. However, fibril formation of β2m is relatively less pronounced around its p*I* value under normal experimental

conditions. Our present study shows that sulfate can potentiate β2m fibril growth over the pH range between 5 and 6, which is close to its p*I* value. As a moderately low pH value can occur physiologically under certain inflammatory conditions, circulating β2m may have the probability of encountering such low pH condition (*71*).

The exact mechanism involved in the sulfate-induced amyloid fibril growth of β2m at pH between 5 and 6 is not clear. However, we found sulfate to be more effective than chloride in increasing the hydrophobicity of the protein under the condition (pH 5.1), similar to the observation made under the extremely acidic condition (pH 2.5). On the other hand, the salt-induced changes in hydrophobicity of the protein near neutral pH (pH 6.8) are found to be relatively less significant (Figure 9). One reason could be that sulfate promotes association as usually involved in ammonium sulfate precipitation of proteins. However, our sedimentation velocity measurement under the condition (100 mM Na_2SO_4, pH 5.3) showed that the protein predominantly exists as a monomer (data not shown). We found that perchlorate, the anion high in the order of electroselectivity series compared to chloride, was only as effective as chloride at pH between 5 and 6. Thus, in addition to the preferential anion interaction, other relevant factors such as the kosmotropic nature of the sulfate affecting the system through perturbing water structure and the possible involvement of protonation of histidine residues (pK_a around 6) cannot be ruled out. It is also possible that sulfate, a stabilizing agent, can stabilize amyloidogenic intermediate state(s), such as those observed in minor quantity (*22*), thus promoting the amyloid fibril growth. Considering the surface exposed, intramolecular salt bridges in β2m (*68*), perturbing such intramolecular electrostatic interactions by ionic cosolute, for example, sulfate, can also affect the equilibrium between the nativelike and amyloidogenic species.

Role of Polyanions in Amyloid-Related Diseases. Glycosaminoglycans and proteoglycans are known to be involved in amyloid deposition of several amyloid-related diseases (for review see refs *47* and *48*). These molecules are extensively sulfated and sulfonated, imparting a large negative charge to the molecule, and can thus be considered as polyanions. Glycosaminoglycans have been shown to promote the paired helical filaments, the major component of the neurofibrillary deposits in Alzheimer's disease, of tau protein even in its unphosphorylated form in vitro (*49*). Heparin but not the desulfonated heparin can promote Aβ amyloid fibril formation in vitro (*50*). One of the risk factors in sporadic Alzheimer's disease, apolipoprotein E, has been shown to stimulate sulfation of glycosaminoglycans when externally added to neuroblastoma cells in culture (*51*). The sulfate content and specific glycosaminoglycans backbone of perlecan are critical for its enhancement of the fibril formation of islet amyloid peptide, amylin (*52*). Heparin and other glycosaminoglycans are known to stimulate the formation of amyloid fibrils of the protein involved in Parkinson's disease, α-synuclein, in vitro (*53*).

β2m-related amyloid deposits from patients are found to contain glycosaminoglycans and proteoglycans (*54*). Glycosaminoglycans and proteoglycans are found to inhibit the β2m fibril depolymerization in vitro (*23*). Glycosaminoglycans, especially heparin, have been reported to enhance the trifluoroethanol-induced β2m fibril extension at neutral pH (*24*). Our present study shows that anion interactions, notably sulfate interaction, can favor the amyloid fibril growth of β2m. The phenomenon of anion interactions as observed in our study is likely to be involved in the role of glycosaminoglycans and proteoglycans in amyloidogensis. Although the inorganic sulfate used in our study is dianionic in nature and the sulfate moiety present as an ester in glycosaminoglycans and proteoglycans bears a single negative charge, the large charge density per molecule in these polyanions might impart greater potential for charge interactions to these molecules. A recent study (*72*) provides an experimental analysis of the extent to which proteins interact with polyanions inside cells and argues for the importance of the "polyanion world" to be taken into account in future understanding of the proteome. Thus, we believe our study, which demonstrates anion binding modulating the amyloid fibril formation of β2m, is one such study that should prove to be useful in understanding the polyanion world in amyloid-related diseases.

Conclusions. We conclude that cosolute anion interaction can modulate amyloid fibril growth of β2m as well as the stability of the amyloid fibrils. Critical balance in electrostatic interactions and hence its influence on the hydrophobic interactions are important in amyloid formation of β2m. Such an influence of cosolute anions may also be applicable to other amyloid systems. Taken together with the earlier reports on the role of glycosaminoglycans and proteoglycans on promotion of amyloid formation of several polypeptides (*23*, *24*, *47*−*54*), our study shows that anion interactions may be one of the critical factors in amyloid formation and its stability. We, therefore, speculate that sulfotransferases and sulfatases, the enzymes regulating biological sulfation processes, may be potential targets for mitigating amyloid-related diseases.

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