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Fluorescence studies on the interaction of β2-microglobulin and metallothionein

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β2-microglobulin (β2m) is a small, major histocompatibility complex class I-associated protein that undergoes aggregation and accumulates as amyloid deposits in human tissues as a consequence of long-term hemodialysis. The exact mechanism of its aggregation to form insoluble fibrils is unknown. Determining the factors which may lead to β2m fibril formation will be of great help in elucidating the mechanism of β2m aggregation. This study was undertaken to determine the interaction of β2m with the stress protein metallothionein (MT) using fluorescence spectroscopy. All spectroscopic probes gave proofs on the interaction of β2m and MT. Fluorescence quenching of the tryptophan residues of β2m with and without MT were measured at different pH values. At pH 4.0, 7.0, and 8.0 quenching of β2m fluorescence in the presence of MT were observed suggesting the possibility of binding of the two proteins. Fluorescence quenching experiments were also performed using acrylamide and KI as quenchers. Quenching of β2m in the presence of MT gave nonlinear Stern-Volmer plots showing a dynamic quenching mechanism. In addition, the larger quenching constants when MT was present indicated that the interaction of the two proteins resulted in the exposure of the tryptophan residues of β2m. To date, this is the first report on the interaction of the two proteins. The interaction between β2m and MT may give insights in further understanding the mechanism of β2m fibril formation.

Keywords: metallothionein; β2-microglobulin; amyloidosis; fluorescence spectroscopy

INTRODUCTION

Dialysis related amyloidosis (DRA) is defined as an accumulation and deposition of β2-microglobulin (β2m) derived fibrils, predominantly in the bones and joints, due to insufficient elimination of this protein by renal replacement therapy [1]. This disease becomes apparent only after more than 5–7 years of renal replacement therapy [2, 3].

β2m, the protein implicated in DRA, is the nonpolymorphic glycoprotein moiety of HLA class I molecules present on the surface of most nucleated cells [4]. In normal kidneys, 95% of the β2m is filtered, reabsorbed and catabolized in the proximal tubule [1]. When the kidneys do not work properly, as in patients receiving long term hemodialysis, β2m is deposited in nonbranching, curved, or linear fibrils arranged in bundles or nodules [5].

β2m was isolated from nucleated cells in 1968 [6]. It is a relatively small protein containing 99 amino acid residues with a single disulfide bridge between the two Cys residues of the

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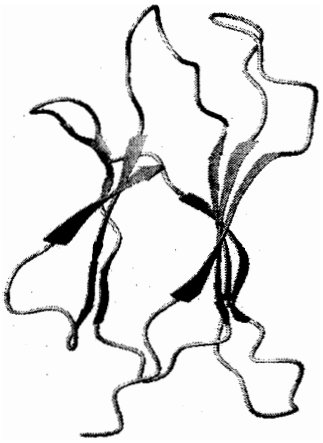


Fig. 1. Ribbon diagram of the X-ray crystal structure of human β2m obtained using MOLMOL.

sequence at positions 25 and 80 and has an approximate molecular weight of 12,000 daltons [6]. Five proline residues are present in β2m, one of which (Pro 32) adopts a cis configuration in the native state. The seven β-pleated strands of β2m folds into the classical β-sandwich motif of the immunoglobulin superfamily, as shown by the crystal structure of MHC-I [7]. A similar β-sandwich topology is also exhibited by transthyretin monomers and the variable domain of immunoglobulin light chain, two proteins associated with two forms of systemic amyloidosis [8, 9]. Figure 1 shows the structure of β2m.

The amino acid sequence and structure of proteins involved in amyloid diseases are unrelated but amyloid fibrils share a common feature [10]. Their structures involved the ordering of β-strands perpendicular to the fiber long axis into an array commonly known as a cross-β structure. Fibrils are typically ~10 nm in diameter, long, and unbranching and are formed from simpler units known as protofilaments and filaments that intertwine to form the rope-like structure typical of amyloid fibrils [10]. Fibrils can be characterized by their distinct x-ray diffraction pattern and their unique ability to bind Congo red, producing a characteristic shift in its absorbance spectrum and a green birefringence when viewed through cross-polarizers [11]. Fibrils also bind the dye thioflavin-T resulting in a characteristic fluorescence emission at 480 nm [12].

The mechanism of the deposition of β2m fibrils is not yet well understood. Various hypotheses have been formulated as possible mechanisms for β2m fibrillogenesis. These include destabilization of a fraction of β2m molecules by proteolytic cleavage of the six N-terminal residues [13], uptake into lysosomes of macrophages with consequent exposure of the protein to acidic pH values at which aggregation is more favored [10], and destabilization by Cu^{2+} [14]. These mechanisms all point out that a partially unfolded intermediate of β2m as key precursor for amyloid deposition. One example of these mechanisms is the one that Chiti et al. proposed [15].

According to the authors the fully native state (N) of β2m is in a reversible equilibrium with a partially folded conformation (I_2). Preformed fibrils interact preferentially with I_2, allowing the aggregates to grow. The equilibrium between N and I_2 is fast compared with the process of amyloid growth. The same fraction of monomeric β2m is therefore available, in the form of I_2, as a substrate for the aggregation process.

Metallothioneins (MTs), on the other hand, are metal-binding proteins containing 60–68 amino acid residues, among them 20 Cys, and binding a total of seven equivalents of bivalent metal ions [16]. These proteins do not usually contain aromatic amino acids. The most conspicuous biological feature of the MTs is their inducibility by a variety of agents and conditions. Thus, the biosynthesis of many MTs is greatly enhanced both in vivo and in cultured cells by transition and d^{10} metal ions and by certain hormones, cytokines, growth factors, tumor promoters and many other chemicals [17]. A massive accretion of MT is also observed in the livers of animals submitted to physical stress [17]. MTs are thought to play roles both in the intracellular fixation of the zinc and copper, in neutralizing the harmful influences of exposure to toxic elements such as cadmium and mercury and in the protection from a variety of stress conditions [18].

This study determined the interaction of β2m with a stress protein, MT using fluorescence spectroscopy. The results of this study may serve as references to further understand the mechanism of β2m fibril formation.

EXPERIMENTAL

Sample preparation. 1.0 mM β2m solutions were prepared by dissolving lyophilized β2m in appropriate buffers. Phosphate buffer was used to obtain a solution of β2m at pH 7.0, acetate buffer for pH 4.0 and Tris-HCl buffer for pH 8.0. In all solutions the total buffer concentration was 50 mM. Potassium chloride solution was also added to each solution to adjust the ionic concentration to 100 mM. Another set of β2m solutions at pH 4.0, 7.0, and 8.0 were also prepared and to these were added MT. The β2m–MT concentration is 1:1 for all solutions.

Fluorescence measurement. The fluorescence of the β2m solutions with and without MT and solutions containing MT only at pH 4.0, 7.0, and 8.0 were measured using a Shimadzu Spectrofluorometer set at 290 nm (excitation) and 350 nm (emission). Emission signals were measured from 280 nm to 500 nm. Appropriate blank solutions were also used during all measurements. Emission signals were plotted as a function of wavelength.

Fluorescence quenching. Quenching of the tryptophan residues of β2m were done using acrylamide and KI as quenchers. For the solution of β2m at pH 4.0, 7.0, and 8.0, 100-mL increments of the quencher were added and the emission signals at 350 nm were measured after the addition of increasing amounts

of the quencher. Titration of the solution and measurement of its fluorescence were carried out until the concentration of the quencher reached 0.25 M. The whole process was repeated for the solution containing β2m + MT at pH 4.0, 7.0, and 8.0. Stern-Volmer plots were constructed using the corrected fluorescence data taking into account the effect of dilution.

RESULTS AND DISCUSSION

Fluorescence analysis of β2m with and without MT. Fluorescence studies were undertaken to investigate the interaction between β2m and MT. Fluorescence spectroscopy is an appropriate technique because β2m contains two tryptophan residues at positions 60 and 95 which are partially exposed to the solvent. These trp residues show characteristic emission signals. On the other hand MT does not contain trp as well as other aromatic amino acids. By following the emission signals of β2m with and without MT, the interaction of the two proteins can be assessed.

The fluorescence of β2m with and without MT were measured at various pH values. The spectrum of β2m alone and MT alone were added and the resulting spectrum was compared to the spectrum of β2m in the presence of MT (Fig. 2). All plots showed emission maxima at around 350 nm which is a characteristic of exposed trp residues. The fluorescence intensity of β2m were decreased at all pH values, the largest of which was observed at pH 7.0. These results showed that the fluorescence of Trp in β2m was quenched by MT suggesting the possibility of binding between the two proteins and the involvement of the Trp residues in the binding process. The data at pH 7.0 revealed a more efficient quenching of the fluorophores as compared when the pH was 4.0 or 8.0.

Fluorescence quenching. To further investigate the interaction between β2m and MT, quenching experiments were done using acrylamide and KI as quenchers. Acrylamide is an efficient quencher of Trp fluorescence and can distinguish between buried and exposed side chains. In contrast, KI is highly hydrated, charged chemical species and its quenching ability is limited to surface exposed tryptophans. Quenching by KI is also dependent upon the neighboring charged groups [17, 18]. Stern-Volmer plots were prepared using the equation:

$$F_0/F = 1 + K_{SV}[Q]$$

where F and F_0 are the fluorescence intensities with and without the quencher, respectively, K_{SV} is the quenching constant, and Q is the molar concentration of the quencher.

Figure 3 shows the Stern-Volmer plots of β2m with and without MT at various pH values using KI as quencher. At pH 4.0, quenching of β2m gave a linear plot with K_{SV} of 3.31 but in the presence of MT a plot with an upward curvature was produced. The quenching constant in the presence of MT was approximated to be 65.59 by extrapolation. At pH 7.0 the Stern-Volmer

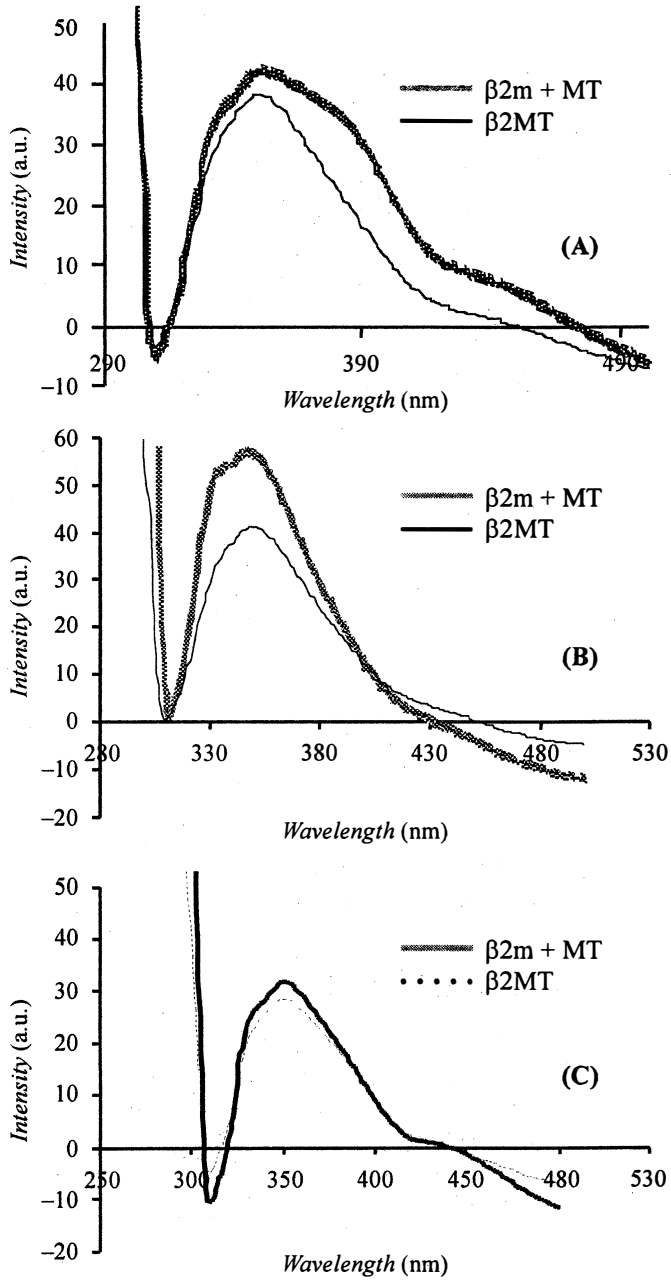
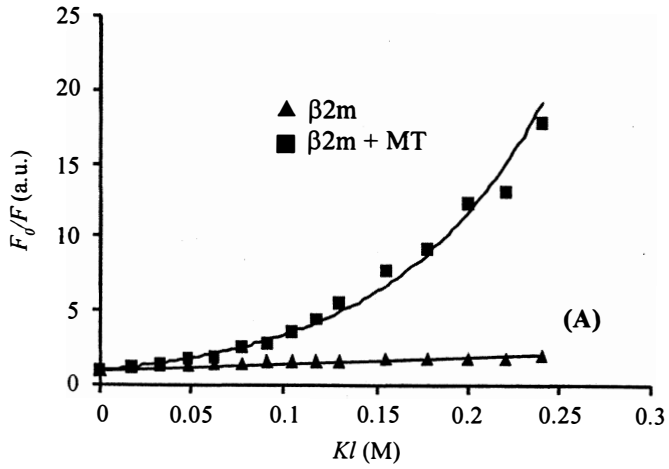
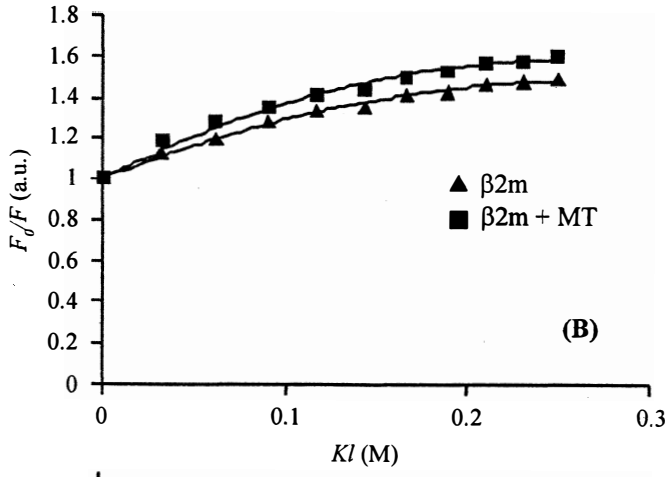


Fig. 2. Fluorescence spectra of β2m with and without MT: (A) *at pH 4.0;* (B) *at pH 7.0;* (C) *pH 8.0. In both sets the concentrations of β2m and MT are 1.0μM. β2m + MT represents the sum of the spectra of β2m alone and MT alone while b2mt represents the spectrum of the solution containing both β2m and MT.*

plots were both nonlinear. From these plots the K_{SV} were approximated to be 1.871 for β2m only and 2.193 for β2m with MT. Nonlinear Stern-Volmer plots were also observed at pH 8.0 with K_{SV} of 0.613 and 3.658 for β2m without MT and with MT, respectively.

The nonlinear Stern-Volmer plots obtained with KI as quencher revealed a dynamic type of quenching. The higher K_{SV} values





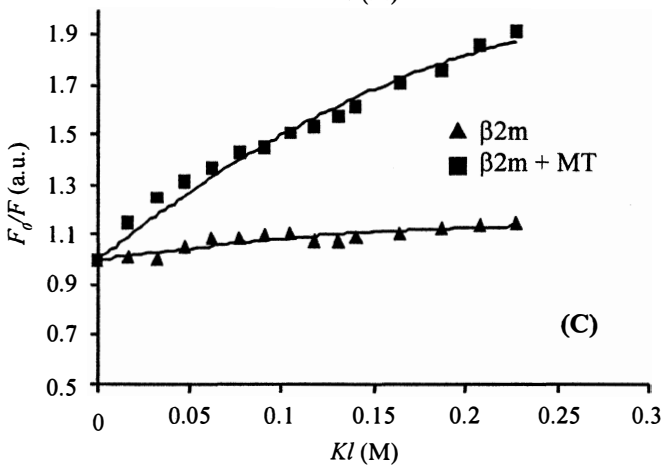
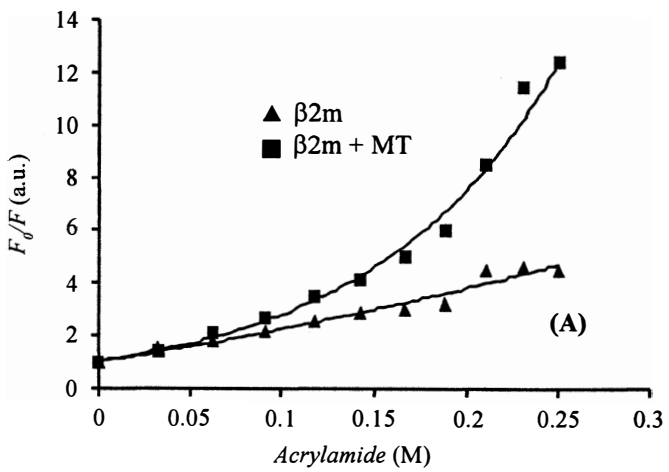
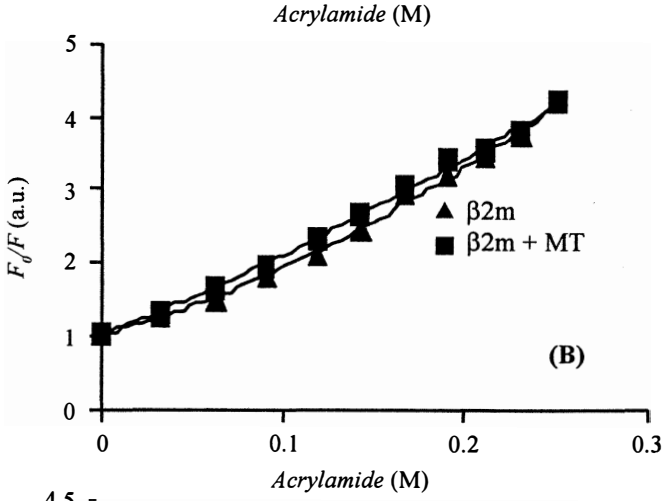


Fig. 3. Fluorescence quenching of β2m with and without MT by KI (A) *at pH 4.0,* (B) *at pH 7.0, and* (C) *pH 8.0. In all sets the concentration of β2m and MT are 1.0 μM.*





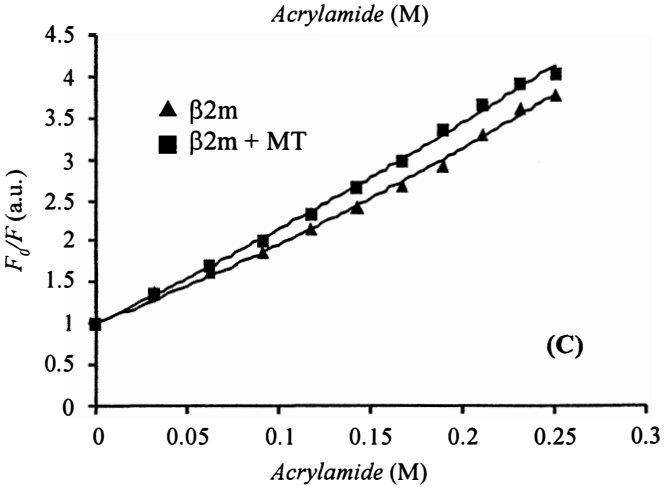


Fig. 4. Fluorescence quenching of β2m with and without MT by acrylamide (A) *at pH 7.0 and* (B) *at pH 8.0. In all sets the concentration of β2m and MT are 1.0 μM.*

in the presence of MT at all pH values indicated the exposure of the fluorophores of β2m. The very large value of K_{sv} at pH 4.0 in the presence of MT showed that KI effectively quenched the Trp residues because of much more exposed fluorophores as compared when the pH was 7.0 or 8.0.

Figure 4 shows the Stern-Volmer plots using acrylamide as quencher. Quenching at pH 4.0 gave nonlinear plots similar to the plots when KI was used as quencher. The K_{sv} values were estimated to be 14.55 in the absence of MT and 44.08 in the presence of MT. At pH 7.0 and 8.0 nonlinear plots were also obtained suggesting a dynamic quenching at these pH values.

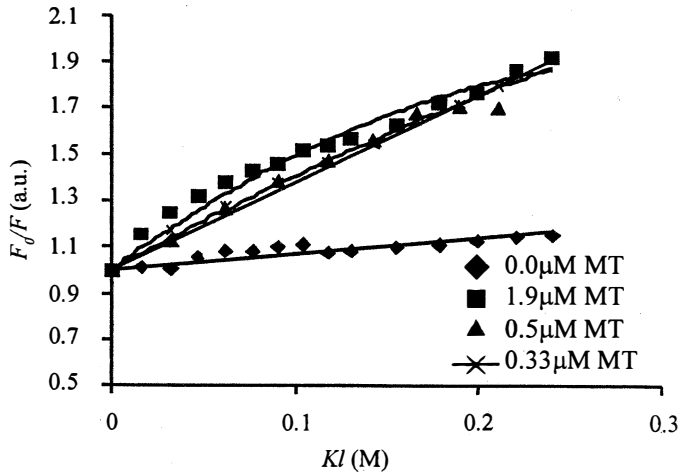


Fig. 5. Fluorescence quenching of β2m with varying concentrations of MT at pH 8.0. In all sets the concentration of β2m is 1.0μM.

The quenching constants with and without MT, 12.90 and 12.82 respectively, were almost the same at pH 7.0 while at pH 8.0 the quenching constants did not differ that much (11.09 for β2m alone and 12.65 for β2m+MT).

Surface accessibility measurement of β2m using DeepView revealed that in the native conformation of β2m, Trp60 is more exposed than Trp95 suggesting the possibility that Trp60 may be involved in β2m binding with MT. The solvent accessibility of the two Trp residues also explained the higher quenching constants obtained when acrylamide was used as quencher. Acrylamide, being a more efficient quencher than KI, can penetrate into the interior portion of the protein thus decreasing not only the fluorescence of Trp60 but also the fluorescence of the slightly buried Trp95. The lower values of K_{sv} when KI was used was due to the fact that the ability of KI as quencher is limited only to surface exposed tryptophans, that is, the fluorescence of the more exposed Trp60 was greatly affected but not Trp95.

Accessibility to iodide is generally taken as an effective indication of exposure of a protein side chain, especially tryptophan, to the solvent [19]. The large increase in K_{sv} values in the presence of MT at pH 4.0 and 8.0 using KI as quencher revealed that the interaction of MT and β2m may result in a conformational change in β2m causing its tryptophan residues to be more exposed to the solvent.

Figure 5 shows quenching of β2m with changing MT concentrations at pH 8.0. The plots showed that binding of MT with β2m can occur even at β2m–MT concentration of 4:1. Increasing the MT concentration did not affect β2m–MT binding as shown by the almost constant slopes of the plots. These results strongly suggested that binding of β2m to MT is 1:1.

CONCLUSION

The change in emission maxima and the decrease in fluorescence intensities at pH 4.0 and 8.0 are all proofs of the interaction of β2-microglobulin and metallothionein. The K_Q values revealed that binding of MT to β2m resulted in a conformational change in β2m causing the exposure of its intrinsic fluorophores to the solvent. Also, the results of the quenching experiments suggested that the tryptophan residues may be involved in the binding of β2m and MT.

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