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The Intrachain Disulfide Bond of β_2-Microglobulin Is Not Essential for the Immunoglobulin Fold at Neutral pH, but Is Essential for Amyloid Fibril Formation at Acidic pH[1]

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β_2-Microglobulin (β2M), the light chain of the type I major histocompatibility complex, is a major component of dialysis-related amyloid fibrils. β2M in the native state has a typical immunoglobulin fold with a buried intrachain disulfide bond. The conformation and stability of recombinant β2M in which the intrachain disulfide bond was reduced were studied by CD, tryptophan fluorescence, and one-dimensional NMR. The conformation of the reduced β2M in the absence of denaturant at pH 8.5 was similar to that of the intact protein unless the thiol groups were modified. However, reduction of the disulfide bond decreased the stability as measured by denaturation in guanidine hydrochloride. Intact β2M formed amyloid fibrils at pH 2.5 by extension reaction using sonicated amyloid fibrils as seeds. Under the same conditions, reduced β2M did not form typical amyloid fibrils, although it inhibited fibril extension competitively, suggesting that the conformation defined by the disulfide bond is important for amyloid fibril formation of β2M.

Key words: amyloid, β_2-microglobulin, conformational-disease, disulfide.

β_2-Microglobulin (β2M) amyloidosis is a common and serious complication in patients undergoing long-term hemodialysis (*1–3*). Carpal tunnel syndrome and destructive arthropathy associated with cystic bone lesions are the major clinical manifestations of β2M amyloidosis (*3, 4*). Although β2M has been identified as a major structural component of amyloid fibrils deposited in the synovia of the carpal tunnel, the mechanism of amyloid fibril formation by β2M is still unknown. Naiki and co-workers have studied amyloid fibril formation of β2M as well as other amyloid fibrils including Alzheimer's β-amyloid (*5, 6*) and murine senile fibrils (*7, 8*). They established a kinetic experimental system in which amyloid fibril formation can be analyzed *in vitro*, and in which the extension phase with seed fibrils is quantitatively characterized by the fluorescence of thioflavin T (ThT) (*5, 8–10*). They proposed a first-order kinetic model of fibril extension as a mechanism common to amyloid fibril formation of various proteins, where the amyloid fibrils extend by the consecutive association of precursor proteins onto the ends of existing fibrils.

To clarify the mechanism of amyloid fibril formation by β2M, it is useful to analyze the conformation and stability of the native state and its relation to amyloid fibril formation. Native β2M, comprising 99 amino acid residues, has a typical immunoglobulin fold with an intrachain disulfide bond buried in the interior of the protein molecule (*11, 12*). Goto and co-workers previously studied the conformation of the isolated constant domain of the immunoglobulin light chain (C_L fragment) (*13–15*). They showed that the reduced C_L fragment, in which the disulfide bond is reduced, assumes a conformation similar to that of the intact C_L fragment, although the stability to denaturation is significantly decreased. As the structure of β2M is similar to that of the C_L fragment, we were interested in analyzing the role of the disulfide bond in the conformation and amyloid fibril formation of β2M.

We expressed human β2M in the methyltropic yeast *Pichia pastoris*. The conformations of several forms of disulfide-cleaved β2M were analyzed by CD, fluorescence, and 1D NMR. As was the case with the C_L fragment, the reduced β2M assumed a native-like conformation in the absence of denaturant at pH 8.5. In contrast, reduction of the disulfide bond completely abolished the ability of β2M to form amyloid fibrils, suggesting a critical role of the disulfide bond in amyloid fibril formation by β2M.

MATERIALS AND METHODS

Recombinant β2M—cDNA encoding β2M was amplified by PCR using the primers 5′-aggtcgcgaatacgtaatccagcgtactcc and 5′-cataccgagcggccgccactgtgctg. The amplified DNA fragment was digested with *Eco*105I and *Not*I, and cloned

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Abbreviations: β2M, β_2-microglobulin; ThT, thioflavin T; C_L fragment, isolated constant domain of the immunoglobulin light chain; ΔG_U, Gibbs free energy change of unfolding; Gdn-HCl, guanidine-hydrochloride.

into the *P. pastoris* expression vector pPIC9 (Invitrogen), resulting in pPICβ2M. pPICβ2M was digested with *Aat*I and transformed into *P. pastoris* GS115. The most efficient transformant was selected based on the expression efficiency in small-scale test tube culture. High cell density fermentation of the selected strain was carried out in a 2 L Mitsuwa Biosystem fermentor as described (*16*). The culture was continued for 2 days after the induction of protein expression by the addition of methanol. The supernatant containing secreted β2M was first desalted by passing it through a Sephadex G-25 (Amersham/Pharmacia Biotech) column equilibrated with 10 mM Na phosphate, pH 7.5. The sample was added to a Ca tartrate column (*17*) and eluted with a linear gradient of Na phosphate buffer. The β2M fraction was further purified on DEAE Sepharose CL-6B (Amersham/Pharmacia Biotech) equilibrated with 20 mM Tris-HCl (pH 8.5) eluted with a linear gradient of NaCl. Three peaks were obtained. On the basis of the N-terminal sequence determined with a Hewlett Packard protein sequencer, G1005A, and a molecular weight determined by MALDI-TOF mass spectrometry (PerSeptive Biosystems), all three peaks were identified as β2M species with different N-termini. The three species had 6 (Glu-Ala-Glu-Ala-Tyr-Val-), 4 (Glu-Ala-Tyr-Val-), or 1 (Val-) additional amino acid residues added to the N-terminal (Leu) of intact β2M. These additional residues were derived from the signal sequence in the vector. The second peak with 4 additional amino acid residues was the major peak, and this fraction was used in this study.

Reduction of the Disulfide Bond—Since the intrachain disulfide bond of β2M could not be reduced in the absence of a denaturant, reduction was carried out with 10 mM DTT in 4 M guanidine hydrochloride (Gdn-HCl) and 10 mM Tris-HCl (pH 8.5) as described (*13, 14*). The reduced protein was separated from Gdn-HCl and DTT on a Sephadex G-25 column equilibrated with 10 mM Tris-HCl (pH 8.5) containing 0.3 M KCl. The thiol content of the reduced β2M was determined by titration with DTNB (*18*) in 4 M Gdn-HCl, and was always close to 2.

Alkylation of the thiol groups of reduced β2M was carried out in 4 M Gdn-HCl at pH 8.5 by adding iodoacetate or iodoacetamide to a final concentration of 0.1 M. Modification with cystamine was carried out in the same buffer by the addition of cystamine to a final concentration of 10 mM. Both reactions were continued for 2 h, and the modified proteins were separated from the residual reagents on a Sephadex G-25 column. When analyzed by reverse-phase HPLC, each of these modified species gave a single sharp peak at a position different from that of the intact β2M, confirming the complete reaction.

CD—The standard buffers for spectroscopic measurements at pH 8.5 and 2.5 were 10 mM Tris-HCl containing 0.3 M KCl and 10 mM glycine-HCl, respectively. CD measurements were carried out with a Jasco spectropolarimeter, J-720, at 20°C. The results are expressed as the mean residue ellipticity [θ]. Far- and near-UV CD spectra were measured using cells with light paths of 1 and 10 mm, respectively. The protein concentrations were 25 and 75 μM for the far- and near-UV measurements, respectively.

Fluorescence—Fluorescence spectra were measured with a Hitachi fluorescence spectrophotometer, F4500, at 20°C, and a cell with a 5-mm light path was used. Tryptophan fluorescence was measured with excitation at 295 nm at a protein concentration of 0.05 mg ml^{-1}. The fluorescence of ThT was monitored at 485 nm with excitation at 445 nm.

NMR—1D ^1H NMR spectra were recorded on a 500-MHz spectrometer (Bruker DMX 500) equipped with a triple-axis-gradient triple-resonance probe. The spectral width was 8,012.8 Hz, and 128 scans of 4,096 real time points were collected over 3 min. The temperature was 20°C.

Denaturation by Gdn-HCl—The unfolding curves were obtained from the Gdn-HCl–induced fluorescence change in 10 mM Tris-HCl buffer (pH 8.5, 0.3 M KCl) at 10°C, and analyzed assuming a two-state transition between the folded and unfolded states. We carried out the measurements at 10°C since the reduced β2M showed minimal propensity to aggregate at this temperature. The free energy change of unfolding (ΔG_U) decreases with increases in the concentration of Gdn-HCl ([Gdn-HCl]), as described by the standard relation: $\Delta G_U = \Delta G_U(H_2O) - m[\text{Gdn-HCl}]$, where $\Delta G_U(H_2O)$ is the free energy change in the absence of denaturant and m represents a measure of the cooperativity of unfolding. The unfolding curves monitored by tryptophyl fluorescence were normalized by assuming the appropriate baselines for the folded and unfolded states, and then fitted to the above equation by the least-squares curve fitting program, in which $\Delta G_U(H_2O)$ and m were adjustable parameters.

Polymerization Assay—β2M amyloid fibrils were formed by the fibril extension method established by Naiki *et al.* (*7–10*), in which the fragmented fibrils were extended by the monomeric proteins, and the reaction was monitored by fluorometric analysis with ThT. Seed fibrils of β2M were originally prepared from Baker's cryst wall excised from the popliteal fossa of a patient suffering from dialysis-related amyloidosis (*1*). The seed fibrils used were 3rd to 5th generation from the original fibrils, prepared by the extension reaction with monomeric recombinant β2M. First, a solution of monomeric β2M at 25 μM in 50 mM citrate buffer (pH 2.5) and 100 mM KCl at 4°C was prepared. Then, β2M seed fibrils were added to the monomeric solution to a final concentration of 5 μg ml^{-1}. The reaction was started by increasing the temperature to 37°C by placing the samples in a water bath. From each reaction tube, 7.5 μl was taken and mixed with 1.5 ml of 5 μM ThT in 50 mM glycine-NaOH buffer (pH 8.5), and the fluorescence of ThT was measured. The extension reactions of reduced β2M under various pH and salt conditions were carried out in a similar manner.

Electron Microscopy—Reaction mixtures (2.5 μl) were diluted with 25 μl of distilled water. These diluted samples were spread on carbon-coated grids, allowing the solution to stand for 1 to 2 min before removing excess solution with filter paper. After drying up the residual solution, 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 up. These samples were examined under a Hitachi H-7000 electron microscope with an acceleration voltage of 75 kV.

RESULTS

Conformation—As we first noticed that reduced β2M has a high propensity to aggregate in the absence of Gdn-HCl, various conditions were examined for preparing reduced, soluble β2M. Reduced β2M formed aggregates between pH

6 and 8, even at low protein concentrations of 0.1 mg ml^{-1}. We found that pH 8.5 (10 mM Tris-HCl) in the presence of 0.3 M KCl were the best conditions for minimizing aggregation. Intact β2M with the disulfide bond was soluble at all pH values examined.

The CD spectrum of intact β2M at pH 8.5, with a small negative peak at 220 nm and several fine peaks in the aromatic region (Fig. 1, A and B), was similar to those reported by other investigators (*19–22*). After reduction of the disulfide bond and removal of Gdn-HCl, β2M showed a far-UV CD spectrum typical of a β-structure with a minimum at 218 nm and an intensity greater than that of the intact protein. The near-UV CD spectrum of reduced β2M exhibited intense positive peaks, showing that the rigid tertiary structure was retained. In contrast, the far-UV CD spectrum of β2M alkylated with iodoacetamide, iodoacetate (data not shown) or cystamine indicated that the protein was substantially denatured. The near-UV spectra of these species were weak and monotonous in comparison with that of the intact protein, indicating disordering of the side chains. The far-UV spectra indicated that all of the intact β2M, reduced β2M, and modified β2M were largely unfolded at pH 2.5 (Fig. 1C). However, a comparison with the spectrum obtained in 4 M Gdn-HCl suggested some remaining secondary structure. The near-UV spectra of these species confirmed the disordering of tertiary structures (Fig. 1D).

Intact β2M at pH 8.5 showed a 1D NMR spectrum with a high dispersion of sharp peaks, reflecting the tightly packed native structure (Fig. 2). The results are consistent with those reported previously (*12, 20, 21, 23*). The 1D spectrum of reduced β2M in the absence of Gdn-HCl was similar to that of intact β2M, suggesting the existence of a native-like structure. In contrast, the 1D spectrum of β2M modified with cystamine at pH 8.5 was typical of an unfolded protein, without peaks above 8.5 ppm or below 0 ppm. The spectra at pH 2.5 were similar for all three species, showing substantial disordering.

β2M has two tryptophan residues at positions 60 and 95, and these residues are partially exposed to the solvent (*11*). The fluorescence spectrum of the native state at pH 8.5 shows a maximum at 340 nm (Fig. 3A), consistent with the partial burial of the tryptophan residues. Reduced β2M after removal of Gdn-HCl showed a maximum at 340 nm, the same wavelength as intact B2M, and the intensity was slightly increased. In the case of the C_L fragment, reduction of the disulfide bond increased the fluorescence intensity markedly (*13, 15*). This can be explained by the dequenching of the tryptophan fluorescence of buried Trp150, which is almost completely quenched by the disulfide bond located in its vicinity in the native C_L fragment. Although β2M has two tryptophan residues at positions 60 and 95, neither occupies the position (Leu39 in β2M) corresponding to the quenched tryptophan residue in the C_L fragment.

In the case of the reduced C_L fragment, the native-like structure was also confirmed by the slow reactivity of thiol groups toward DTNB (*13, 15*) or glutathione (*14*). We examined the reactivity of the thiol groups of reduced β2M with DTNB at pH 8.5. The titration reaction was slow in comparison with the reaction in 4 M Gdn-HCl (data not shown), further confirming the native-like structure of reduced β2M with buried thiol groups.

Stability—The normalized unfolding transition curves induced by Gdn-HCl were constructed on the basis of the

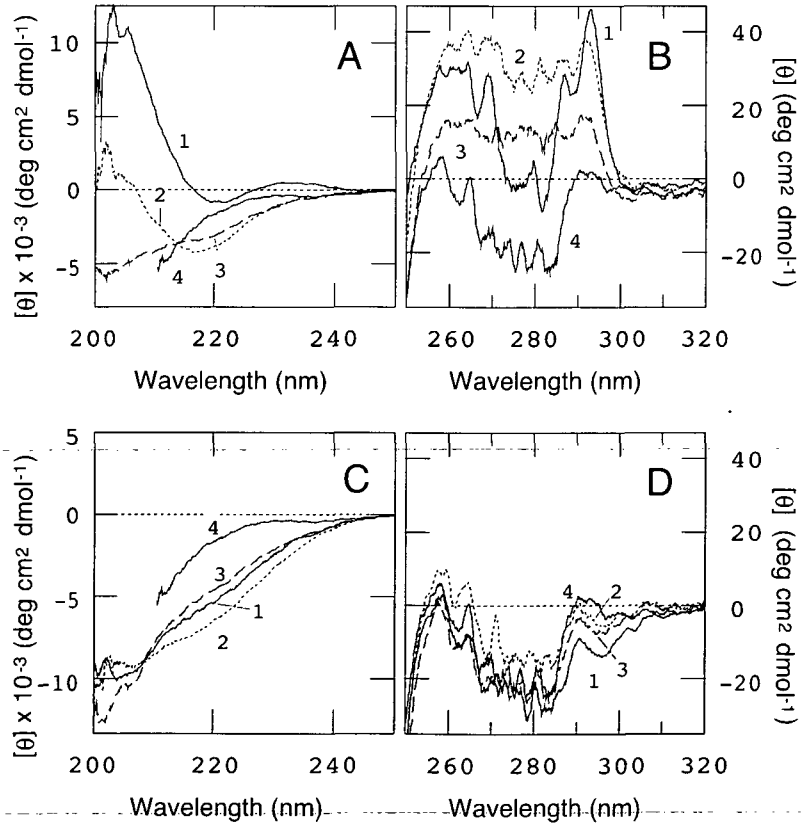

Fig. 1. **CD spectra of β2M at pH 8.5 (A, B) and 2.5 (C, D) at 20°C.** (A, C) Far-UV and (B, D) near-UV CD spectra of intact β2M (1), reduced β2M (2), β2M modified with cystamine (3), and intact β2M in 4 M Gdn-HCl (4).

changes in fluorescence at 350 nm in 10 mM Tris-HCl buffer (pH 8.5) at 10°C (Fig. 3B). The unfolding transition of intact β2M occurred between 2 and 3 M Gdn-HCl, consistent with previous reports (*19, 20, 22*). Although reduced β2M assumed a folded conformation similar to that of intact β2M in the absence of denaturant, its stability was much less than that of intact β2M. The unfolding transition curves were analyzed on the basis of a two-state transition mechanism. $\Delta G_U(H_2O)$, m and C_m values for intact β2M were 33.8 kJ mol^{-1}, 13.3 kJ mol^{-1} (mol of Gdn-HCl)$^{-1}$, and 2.5 M, respectively. Those of reduced β2M were 13.5 kJ mol^{-1}, 25.4 kJ mol^{-1} (mol of Gdn-HCl)$^{-1}$ and 0.5 M, respectively. Thus, reduction of the disulfide bond resulted in a decrease in stability by 20 kJ mol^{-1}. In the case of the C_L fragment, C_m values for intact and reduced proteins were 1.2 and 0.6 M, respectively, and reduction of the disulfide bond resulted in a decrease in $\Delta G_U(H_2O)$ of 16 kJ mol^{-1} (*13*).

Amyloid Fibril Formation—The effects of disulfide bond reduction on amyloid fibril formation were examined by the extension reaction at pH 2.5 (Fig. 4). The extension reaction of the recombinant β2M was completed in 2 h, consistent with the results with β2M obtained from patients (*9*). On electron micrographs, the extended fibrils, with a diameter of about 10 nm and a longitudinal periodicity, were seen to be the same as the extended fibrils prepared with β2M purified from patients (Fig. 5). Polarized micrographs of fibrils after staining with Congo-red showed an orange-green birefringence, typical of amyloid fibrils (data not shown). These results confirm that the recombinant β2M with an additional 4 residues at the N-terminus is indistinguishable from β2M obtained from patients with respect to amyloid fibril formation.

The results obtained with the disulfide modified β2M species were markedly different (Fig. 4). None of these species formed evident amyloid fibrils as determined by ThT fluorescence assay. Electron micrographs showed no clear fibrils as observed for intact β2M (Fig. 5). Instead, electron micrographs of reduced or alkylated β2M showed a small number of thin curled filaments, similar to the filaments generated at pH 4.0 or in the presence of high salt (*21*). Although these might have been protofibrils, typical β2M fibrils were rarely observed with the disulfide bond-cleaved species. The formation of curled filaments was observed even in the absence of seed fibrils, suggesting that the filaments can be formed spontaneously without seed.

As reduction of the disulfide bond increased the propensity to form aggregates at neutral pH, we suspected that the inability of disulfide bond–cleaved β2M to form amyloid

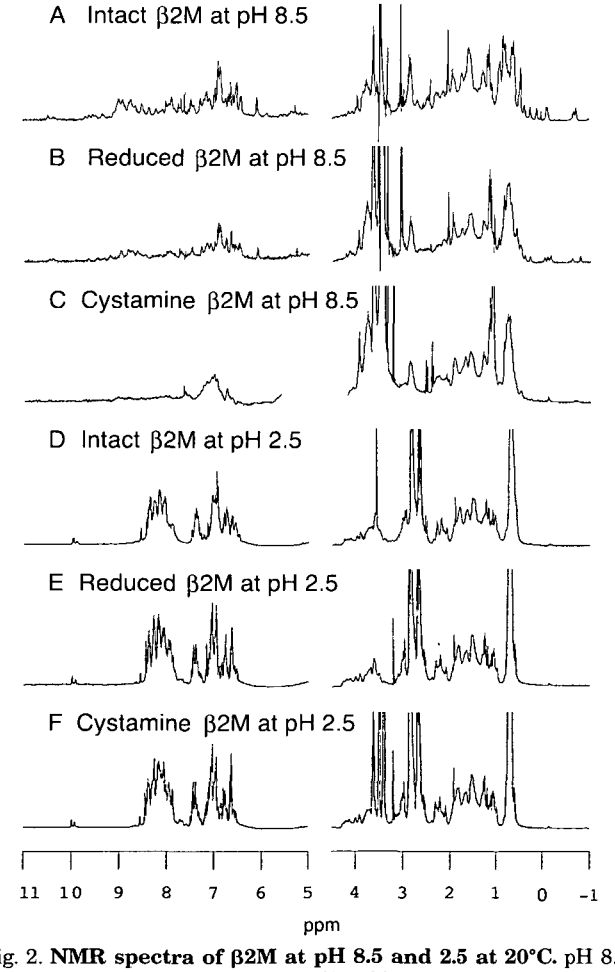

Fig. 2. **NMR spectra of β2M at pH 8.5 and 2.5 at 20°C.** pH 8.5: (A) intact β2M, (B) reduced β2M, (C) β2M modified with cystamine. pH 2.5: (D) intact β2M, (E) reduced β2M, (F) β2M modified with cystamine.

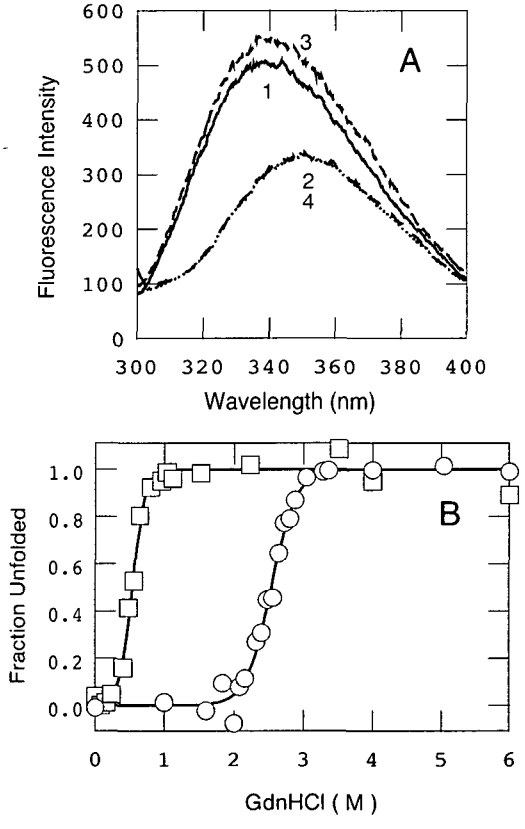

Fig. 3. **Fluorescence spectra and unfolding transitions of β2M at pH 8.5.** (A) Spectra of intact β2M (1, 2) and reduced β2M (3, 4) in the presence (2, 4) and absence (1, 3) of 4 M Gdn-HCl at 20°C. (B) Unfolding transitions of intact β2M (○) and reduced (□) β2M induced by Gdn-HCl at 10°C. Lines are theoretical curves drawn on the basis of a two-state mechanism. $\Delta G_U(H_2O)$, m, and C_m values for intact β2M are 33.8 ± 2.4 kJ mol^{-1}, 13.3 ± 0.9 kJ mol^{-1} (mol of Gdn-HCl)$^{-1}$, and 2.5 M, respectively. Those of reduced β2M are 13.5 ± 1.9 kJ mol^{-1}, 25.4 ± 3.6 kJ mol^{-1} (mol of Gdn-HCl)$^{-1}$, and 0.5 M, respectively.

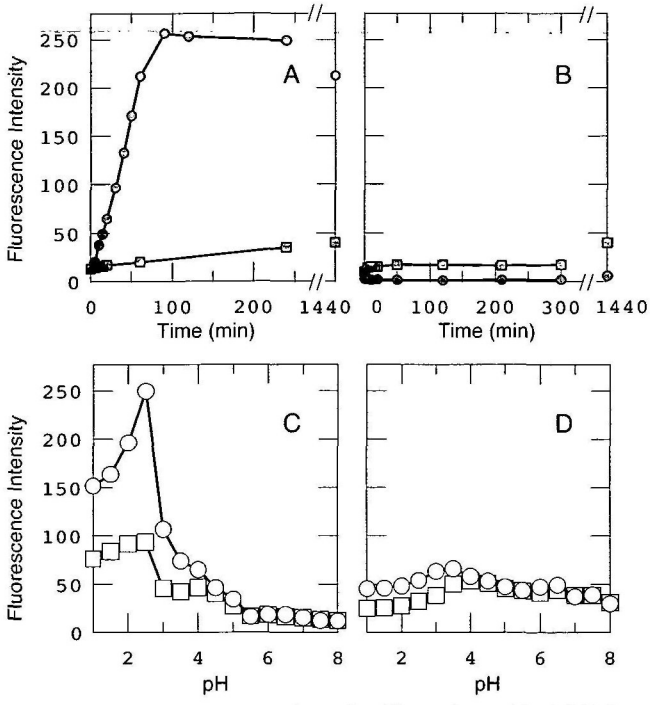

Fig. 4. **Amyloid fibril formation of β2M monitored by ThT fluorescence.** Time course of fibril formation of intact β2M (●) and reduced β2M (■) at pH 2.5 (A) and pH 7.5 (B). pH dependence of fibril formation of intact β2M (C) and reduced β2M (D) at the extension times of 30 min (□) and 4 h (○).

fibrils was due to the formation of amorphous aggregates. To increase the solubility, we prepared modified β2M in which thiol groups formed a mixed disulfide with cystamine, a positively charged thiol reagent. The extent of aggregation of β2M with mixed disulfide bonds with cystamine, measured by sedimentation equilibrium, was less than that of other species (data not shown). Nevertheless, we observed no amyloid fibril formation.

In addition, we examined the extension reaction of the disulfide reduced species under various pH conditions, including neutral pH (Fig. 4). Although the basal level of ThT fluorescence was higher than that of intact β2M, amyloid fibrils were not detected. We also carried out an experiment in which the reduced β2M in 4 M Gdn-HCl was directly diluted in the buffer for fibril formation. While intact β2M formed amyloid fibrils as started by the sample in the absence of denaturant, the reduced β2M did not. None of the conditions examined were successful in promoting fibril formation of the disulfide-cleaved species.

We then examined the effects of reduced β2M on amyloid fibril formation of intact β2M. The addition of a small amount of reduced β2M inhibited the extension reaction of intact β2M, slowing down the rate of fibril formation (Fig. 6). Intriguingly, the final fluorescence level of ThT was independent of the amount of reduced β2M added. This suggests reversible competitive inhibition of extension, in which reduced β2M interacts with the ends of fibrils reversibly so as to inhibit the extension reaction (see below).

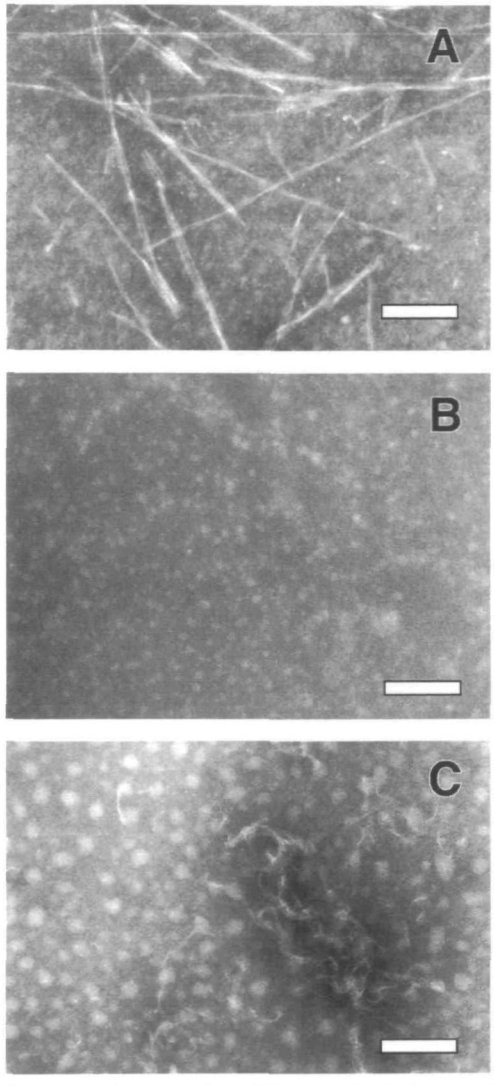

Fig. 5. **Electron micrographs of the products formed by the extension reaction.** Intact β2M (A), reduced β2M (B), and β2M alkylated with iodoacetamide (C). The bars indicate a length of 100 nm.

DISCUSSION

We studied the role of the disulfide bond (Cys25-Cys80) in the conformational stability of β2M and its ability to form amyloid fibrils. We found that the disulfide bond of β2M is not essential for maintaining the immunoglobulin fold. On the other hand, we found that the disulfide bond is essential for forming amyloid fibrils. Although the formation of amyloid fibrils has been reported for many proteins (*24–27*) and peptides (*28–32*) under various conditions, implying that amyloid fibril formation can occur readily, the present results argue that amyloid fibril formation by β2M has stringent sequence or conformational requirements.

Conformation—The role of the disulfide bond in the conformational stability of β2M was first examined by Isenman *et al.* (*19*). The denaturation transition of intact β2M at pH 7.8 and 24°C monitored by optical rotation was similar to that observed in the present study. Although they examined the conformations of various disulfide-modified β2M species, including reduced β2M without modification,

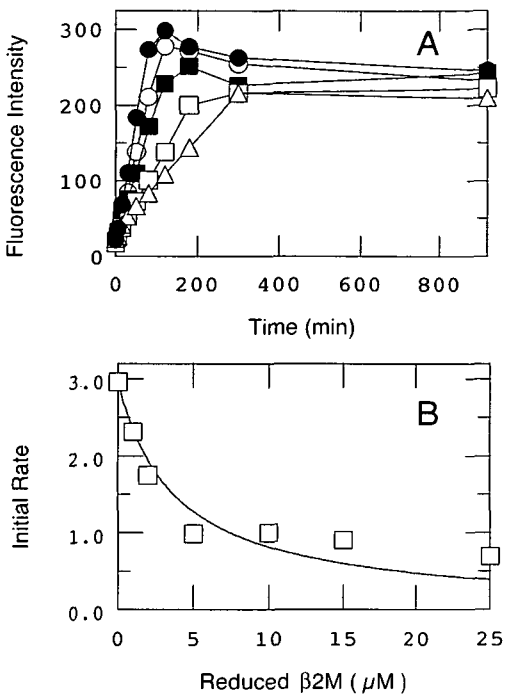

Fig. 6. **Competitive inhibition of the extension of intact β2M by reduced β2M.** (A) Time course of the extension reaction measured by ThT fluorescence, in the presence of 0 (●), 1 (○), 2 (■), 5 (□), and 25 (△) μM of reduced β2M. (B) Dependence of the initial rate on the concentration of reduced β2M. The initial rate is defined as the initial increase of fluorescence intensity per minute. The line indicates the theoretical line drawn on the basis of Eq. 5 and K_i = 3.80 μM.

the lowest concentration of Gdn-HCl was 1.0 M because the reduced protein showed a high tendency to undergo aggregation. They concluded that reduced β2M cannot assume a folded conformation. As shown in Fig. 1, although reduced β2M unfolded in 1.0 M Gdn-HCl, it was folded in the absence of Gdn-HCl. However, upon modification of the thiol groups, β2M could not form the folded structure, probably because the introduced groups prevented the tight packing of the immunoglobulin fold.

At pH 8.5 in the absence of denaturant, the reduced β2M showed a far-UV CD spectrum typical of a β-structure, a well-dispersed 1D-NMR spectrum, a native-like Trp fluorescence spectrum, and cooperative unfolding transition, indicating that the reduced β2M forms a tightly-packed folded structure, although its stability is decreased. The results were essentially the same as those reported for the reduced C_L fragment (*13–15*). In addition, formation of the native-like structure after cleavage of the disulfide bonds has also been reported for the intermediates of disulfide bond formation of bovine pancreatic trypsin inhibitor and bovine pancreatic ribonuclease A (*33, 34*). The decrease in stability upon reduction of the disulfide bond can be explained by an increase in conformational entropy in the unfolded state (*13, 14, 35*). Taken together, under the conditions that do not cause aggregation, the formation of a native-like structure with decreased stability may be common to the disulfide bond–reduced immunoglobulin domains.

Amyloid Fibril Formation—The inability of reduced β2M to form amyloid fibrils by the extension reaction suggests the importance of "intrachain" disulfide bonds for the amyloid fibrils of β2M. The role of "interchain" disulfide bonds has recently attracted attention in studies of prion proteins (*36, 37*). Although reduced β2M does not form amyloid fibrils by itself, it inhibits the extension reaction of intact β2M. Characterizing the inhibition by reduced β2M will be useful in understanding the mechanism of amyloid fibril formation.

Amyloid fibril formation consists of two processes of binding and extension. Amyloid fibril extension of β2M can be explained by a first-order kinetic model (*9*):

$$[P] + [M] \underset{k_{-1}}{\overset{k_2}{\rightleftharpoons}} [P] \quad (1)$$

where [P] and [M] are the concentrations of fibrils and monomers and k_2 and k_{-1} are the rate constants of polymerization and depolymerization, respectively. If t is the reaction time, $f(t)$ is the amount of β2M newly polymerized into fibrils during the reaction, and $[M]_0$ is the initial β2M concentration, the rate of amyloid fibril extension, $f'(t)$, can be described as

$$f'(t) = [P]([M]k_2 - k_{-1}) = [P]([M]_0 k_2 - k_{-1} - k_2 f(t)), \quad (2)$$

where $[M]_0 = [M] + f(t)$.

Several models can be proposed for the inhibition by reduced β2M. If the reduced β2M forms a non-productive complex with monomeric β2M either irreversibly or reversibly, the extension rate will be reduced because the effective concentration of intact β2M monomer is decreased. In this case, the final ThT value should be decreased depending on the affinity of reduced β2M to intact β2M. Reversible inhibition of this type has been observed for the apolipoprotein E–dependent inhibition of Alzheimer's β-amyloid fibril formation (*6*), in which the final ThT fluorescence decreases with the increase in apolipoprotein E.

Naiki *et al.* (*9*) previously estimated the equilibrium constant of fibril extension, $K = k_2/k_{-1}$, from the dependence of the initial rate on the concentration of β2M to be around 10^5 M^{-1}. Inhibition by reduced β2M occurred at less than 10 μM, suggesting a similar strength of affinity between intact β2M and reduced β2M. If this is true, more than half the intact β2M is excluded from amyloid formation in the presence of 25 μM reduced β2M. However, the final ThT fluorescence values were mostly independent of the presence of reduced β2M, indicating that all intact β2M molecules participate in amyloid fibril formation. This can not be explained by the mechanisms described above.

On the other hand, a competitive inhibition model can satisfactorily explain the results observed here, in which both reduced β2M and intact β2M bind to the same ends of fibrils, but only intact β2M extends fibrils. In this mechanism, final ThT values should be independent of reduced β2M, because the amount of intact β2M available is not changed by the addition of reduced β2M. Thus, we used a competitive inhibition mechanism to analyze the inhibitory reaction of reduced β2M. The directionality of extension is not yet clear, although several observations suggest that amyloid fibrils grow without polarity by elongating at both of the free ends (*38, 39*): see also Ref. *40* conflicting results. It is likely that although reduced β2M binds to the extending ends located at both ends of the fibril, the bound β2M cannot provide the binding site for other monomers.

When reduced β2M inhibits the extension reaction com-

petitively by interacting with [P], Model 1 becomes:

$$[P] + [M] \underset{k_{-1}}{\overset{k_2}{\rightleftharpoons}} [P] \quad (3)$$

$$[P] + [R] \underset{k_i}{\rightleftharpoons} [PR]$$

where [R] and [PR] are the concentrations of reduced β2M and its complex with fibrils, respectively, K_i = [P][R]/[PR], $[P]_0$ = [P] + [PR], and $[R]_0$ = [R] + [PR].

The monomeric concentration of β2M used as seeds in Fig. 6 was 0.4 μM. As only the free end(s) of fibrils would be effective as elongation sites, the effective concentration of the seeds should be less than the monomeric concentration. Thus, we can assume that the concentration of seeds is much less than the concentration of inhibitor, *i.e.* $[P]_0 \ll [R]$, and [R] is approximated by $[R]_0$. Therefore, [P] is represented as: $[P] = [P]_0K_i/(K_i + [R]_0)$ and the rate of amyloid fibril formation is:

$$f'(t) = [P]_0K_i/(K_i + [R]_0)([M]_0k_2 - k_{-1} - k_2 f(t)). \quad (4)$$

The initial rate at $t = 0$ is:

$$f'(0) = [P]_0K_i/(K_i + [R]_0)([M]_0k_2 - k_{-1}). \quad (5)$$

At $t = \infty$, $f'(\infty) = 0$, $f(\infty) = [M]_0 - k_{-1}/k_2$ from Eq. 4, indicating that the equilibrium value of amyloid fibrils is independent of the inhibitor concentration.

By fitting with Eq. 5 the initial rate of fibril formation at various concentrations of reduced β2M, K_i was estimated to be 3.80 ± 0.66 μM. The dissociation constant of intact β2M to the fibril ends is reported to be about 10 μM (*9*). Thus, the binding of reduced β2M to the fibril is as tight as the productive binding of β2M for fibril extension. This suggests that, although reduced β2M can interact tightly and reversibly with extension sites or fibrils, it cannot assume the template conformation necessary for subsequent fibril elongation. Consequently, it becomes a competitive inhibitor.

This raises the question of why reduced β2M cannot take part in fibril extension. It is likely that the conformation defined by the disulfide bond is important for amyloid formation of β2M. As intact β2M at pH 2.5 retains some residual structures, such residual structures may play critical roles in amyloid formation. On the other hand, recent studies with other amyloid proteins have suggested the presence of key residues in amyloid formation. For example, in the case of transthyretin, which is composed of 124 residues, a peptide as short as 10 residues can produce amyloid fibrils (*32*). It is possible that even a small peptide of β2M can form amyloid fibrils. In fact, we have preliminary data suggesting that one of the proteolytic fragments of β2M without the disulfide bond can form amyloid fibrils. Considering these observations, several regions of β2M may have the potential to inhibit amyloid formation, and reduction of the disulfide bond may result in the activation of the inhibitory effects because of the increased flexibility of the entire molecule.

After the submission of this manuscript, Smith and Radford (*41*) reported the conformational and amyloidogenic properties of reduced β2M, which contrast with ours. From CD, analytical ultracentrifugation, and 1-anilinonaphthalene-8-sulphonic acid binding analyses, they concluded that the reduction of the disulfide bond results in the denaturation of the native structure, with an accumulation of the non-native conformation at pH 7. However, our results on reduced β2M, in particular the native-like CD and 1D NMR spectra, as well as the native-like conformation of the reduced CL fragment (*13–15*), suggest that, under conditions associated with no aggregation, the formation of a native-like structure with decreased stability may be common to the disulfide bond–reduced immunoglobulin domains.

Smith and Radford (*41*) reported that reduced β2M forms short curled filaments at acidic pH in the presence 0.4 M NaCl. McParland (*21*) also reported the formation of similar filaments with disulfide intact β2M in the presence of high salts. However, the morphology of these filaments was very different from that of typical fibrils formed by the seed-dependent extension reaction, or the filaments taken from patients. Thus, we do not consider that the filaments occasionally observed for the reduced β2M or those formed in the presence of high salt should be called β2M amyloid fibrils. Although the curled filaments might correspond to protofibrils, productive intermediates of fibril formation, they are rather likely to be dead-end products that fail in cooperative fibril formation.

Concluding Remarks—In 1975, Isenman *et al.* (*19*) examined the role of the disulfide bond in the conformational stability of β2M and concluded that the disulfide bond is essential for maintenance of the immunoglobulin fold. Later, Goto *et al.* (*13, 14*) showed that the disulfide bond is not essential for maintaining the immunoglobulin fold of the C_L fragment, a protein with a similar structure to β2M. As Isenman *et al.* (*19*) examined the conformation in the presence of 1 M Gdn-HCl, it is possible that, in the absence of denaturant, reduced β2M would assume the native conformation. With recombinant β2M, we showed that reduced β2M does assume a native-like conformation in the absence of denaturant. β2M has become an important target of amyloid research. Intriguingly, the disulfide bond is essential for the formation of amyloid fibrils of β2M. Further understanding of the role of the disulfide bond will shed light on the mechanism of β2M amyloid fibril formation.

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