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Conformational Dynamics of β_2-Microglobulin Analyzed by Reduction and Reoxidation of the Disulfide Bond

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Although native β_2-microglobulin (β2-m), the light chain of the major histocompatibility complex class I antigen, assumes an immunoglobulin domain fold, it is also found as a major component of dialysis-related amyloid fibrils. In the amyloid fibrils, the conformation of β2-m is considered to be largely different from that of the native state, and a monomeric denatured form is likely to be a precursor to the amyloid fibril. To obtain insight into the conformational dynamics of β2-m leading to the formation of amyloid fibrils, we studied the reduction and reoxidation of the disulfide bond by reduced and oxidized dithiothreitol, respectively, and the effects on the reduction of the chaperonin GroEL, a model protein that might destabilize the native state of β2-m. We show that β2-m occasionally unfolds into a denatured form even under physiological conditions and that this transition is promoted upon interaction with GroEL. The results imply that *in vivo* interactions of β2-m with other proteins or membrane components could destabilize its native structure, thus stabilizing the amyloid precursor.

Key words: amyloid fibril, β_2-microglobulin, chaperonin GroEL, dialysis-related amyloidosis, disulfide bond.

Abbreviations: apoE, apolipoprotein E; β2-m, β_2-microglobulin; CD, circular dichroism; DTT, dithiothreitol; GdnHCl, guanidine hydrochloride; HPLC, high pressure liquid chromatography; NMR, nuclear magnetic resonance; ThT, thioflavin T.

β_2-Microglobulin (β2-m) is a major component of the amyloid fibrils deposited in dialysis-related amyloidosis with clinical manifestations such as carpal tunnel syndrome and destructive arthropathy associated with cystic bone lesions (*1–3*). It consists of a polypeptide chain of 99 residues (molecular weight 11,800) and has a β-sandwich structure stabilized by a single cross-sheet disulfide bond formed between Cys25 and Cys80 (Fig. 1) (*4*, *5*). However, β2-m, existing on the surface of nearly all cells as part of the light chain of the major histocompatibility complex class I antigen (*6*), is a normal constituent of plasma, where its concentration in adults is 1–3 mg/liter (*3*). The production of β2-m is in the range of 150–200 mg/day, of which 97% is excreted in the kidneys. The concentration of β2-m, therefore, is significantly increased in patients with renal failure. Blood dialysis therapy results in the retention of a high concentration of β2-m (~50 mg/liter) in the plasma. Although this retention appears to be a prerequisite, other factors, such as the age of the patient, the duration of dialysis, and the type of dialysis membrane used, may also be involved in the deposition of amyloid fibrils, and the exact mechanism of amyloid fibril formation is unknown.

Recently, β2-m has become one of the most important targets of biochemical and physicochemical research into amyloid fibrils (*7–11*). We have been studying the structure of amyloid fibrils and the mechanism of their formation using recombinant human β2-m produced by the methylotrophic yeast *Pichia pastoris* (*12–16*). For many proteins involved in forming amyloid fibrils, the process is facilitated by destabilization of the native state (*17–19*). In the case of β2-m, the formation of amyloid fibrils by an *in vitro* extension reaction peaks at pH 2.5 (*12*, *20*, *21*), far from physiological conditions, suggesting that destabilization of the native structure caused by unknown factors produces the amyloid precursor under physiological conditions. One of the factors that could play a significant role in destabilizing the native structure under physiological conditions is interaction with transition metals, particularly Cu^{2+} (*4*, *10*, *22*). On the other hand, it has been suggested that a notable fraction, *i.e.* as much as 25%, of the molecules intrinsically assume the destabilized amyloidogenic conformation even under physiological conditions (*7*, *9*). To understand the β2-m fibril formation at neutral pH, it is important to clarify the conformational dynamics of the native structure.

A single disulfide bond buried in the molecule is highly conserved in the immunoglobulin superfamily, stabilizing the immunoglobulin domain fold of these proteins (*12*, *23–25*). The reactivity of the disulfide bond to reducing reagents is useful for analyzing the conformational stability of the protein molecule. The reoxidation of the reduced disulfide bond is also influenced by the conformational dynamics of proteins (*24*). In a previous paper (*15*), we analyzed the reactivity of the disulfide bonds in amyloid fibrils and indicated that they are mostly buried in the interior. In the present paper, to obtain further insight into the conformational stability of β2-m, we

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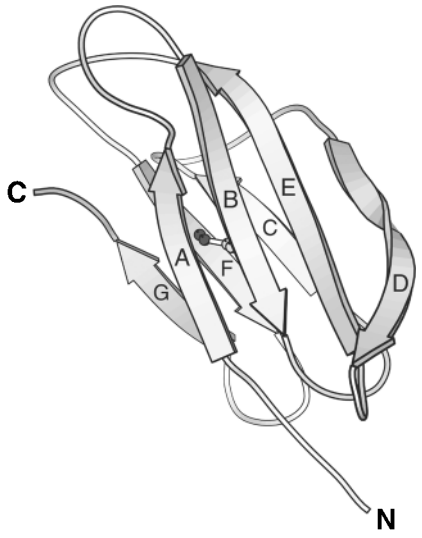

Fig. 1. **Structure of β2-m.** The diagram was created by Molscript (*38*) with the structure (PDB entry 3HLA) reported by Bjorkman *et al.* (*6*).

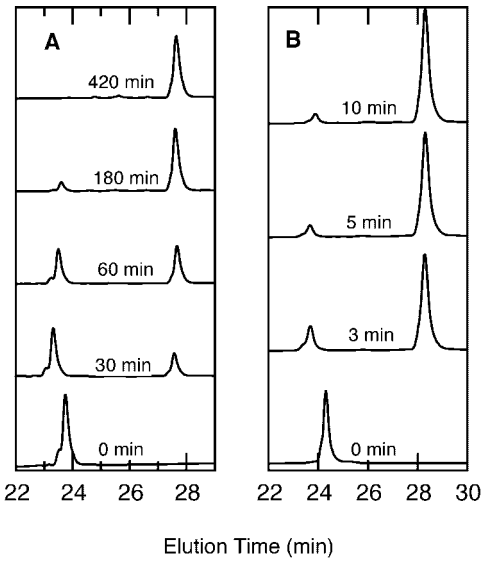

Fig. 2. **HPLC elution profiles for the reduction of the disulfide bond of β2-m.** Conditions were 20 μM β2-m in 180 mM DTT (A) or 0.5 mM DTT and 4 M GdnHCl (B) at pH 8.5 and 37°C. The peak intensity of the oxidized β2-m decreased with time while that of the reduced β2-m increased.

characterized in detail the reduction and reoxidation of the disulfide bond in the monomeric state at pH 8.5. The results indicate that, although highly protected from reduction, the native β2-m occasionally experiences global fluctuations in which the disulfide bond is exposed to the solvent. Moreover, such fluctuation was facilitated upon interaction with chaperonin GroEL. The results suggest that the *in vivo* interaction of β2-m with other hydrophobic components in blood may be an important factor effectively trapping the amyloidogenic precursors.

MAERIALS AND METHODS

Materials—Recombinant β2-m with three signal peptides, Glu-Ala-Glu-Ala-Tyr-Val-, Glu-Ala-Tyr-Val-, and Val-, added at the amino-terminal Leu of intact β2-m were expressed in *Pichia pastoris*, and then purified as described previously (*12*, *13*). In this study, we used β2-m with one additional amino acid residue. The conformational and amyloidogenic properties of this species were indistinguishable from those of the intact β2-m purified from patients.

GroEL was prepared with a GroE-overproducing strain of *Escherichia coli*, DH1/pKY206 (*26*). Purification of GroEL was performed according to the method of Buchner *et al*. (*27*) with some modifications. The entire process was carried out in 50 mM Tris-HCl buffer (pH 7.8), containing 1 mM EDTA, 1 mM 2-mercaptoethanol and 50 mM NaCl. After lysis of the cells by sonication and removal of the insoluble material by centrifugation for 45 min at 12,000 ×*g*, the supernatant was collected. Ammonium sulfate was added to 55% (w/w) saturation and the solution was stirred for 1 h on ice. The precipitated protein was collected by centrifugation for 30 min at 12,000 ×*g* and dissolved in the buffer. This protein solution was applied to a Sephacryl S-300 gel-filtration column and fractions of GroEL were obtained.

Methods—The reduction of the disulfide bond was carried out in the presence of various concentrations of reduced DTT in 50 mM Tris-HCl buffer (pH 8.5) at 25°C. The reduction was also done in 4 M Gdn-HCl. After various reaction periods, an aliquot was taken and the pH was lowered to around 2 by adding 1 N HCl, so that the reaction was quenched. To prevent the oxidation of DTT, the buffers were degassed. The effective concentration of DTT was determined by titrating an aliquot of stock DTT solutions with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) assuming the molar extinction coefficient of reduced DTNB to be 13,600 M^{-1} cm^{-1} at 412 nm (*28*). The extent of the reduction of the disulfide bond of β2-m was monitored by reversed-phase HPLC performed on a GILSON liquid chromatograph equipped with a C4-300Å column (3.3 × 150 mm, Waters) at a flow rate of 0.5 ml/min. Intact and reduced β2-m were eluted separately with a gradient beginning with solvent A (0.05% trifluoroacetic acid) and an increasing percentage of solvent B (0.05% trifluoroacetic acid/acetonitrile).

To observe the reoxidation of reduced β2-m by oxidized DTT, the reduced β2-m was prepared first by incubating β2-m for 30 min in the presence of 4 M Gdn-HCl and 10 mM reduced DTT. The excess reagents were removed by gel filtration on a Sephadex G25 column, which was equilibrated with 10 mM Tris-HCl (pH 8.5). Reoxidation of the disulfide bond was initiated by adding oxidized DTT at 200 mM and the extent of disulfide bond formation was monitored by HPLC as described above.

The kinetics of reduction or reoxidation of the disulfide bond were fitted to first-order kinetics with an apparent rate constant, k_{app}: $f_{ox} = \exp(-k_{app} \times t)$ for the reducing reaction and $f_{ox} = f_{\infty}[1 - \exp(-k_{app} \times t)]$ for the oxidizing reaction, where f_{ox} is the fraction of oxidized species, t is the reaction time, and f_{∞} is the fraction of oxidized species at infinite time. It is noted that f_{∞} is adjustable only for the oxidation reaction (see below). We used a least-squares curve-fitting procedure for the fitting.

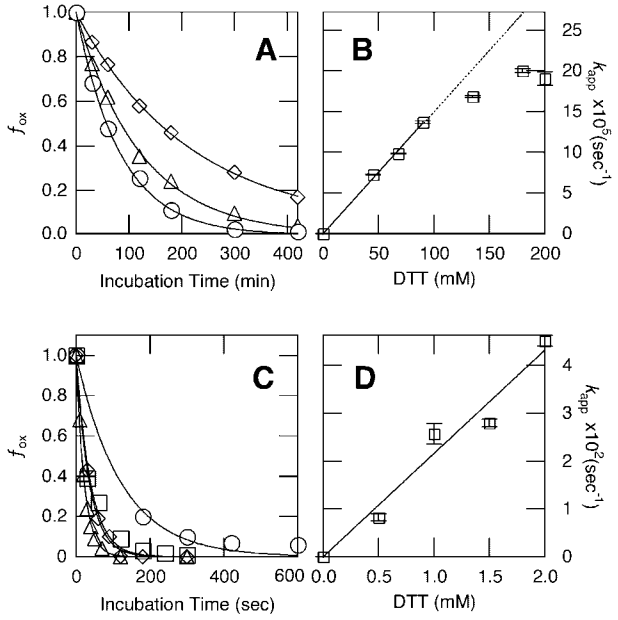

Fig. 3. **Kinetics of the reduction of the disulfide bond of 20 μM β2-m by DTT in the absence (A, B) and presence (C, D) of 4 M Gdn-HCl (pH 8.5) at 37°C.** (A) DTT concentrations were 180 (open circles), 90 (open triangles), and 45 (open diamonds) mM. (C) DTT concentrations were 2.0 (open triangles), 1.5 (open diamonds), 1.0 (open squares), and 0.5 (open cirlces) mM. (A, C) Decrease in the relative fraction of oxidized β2-m (f_{ox}) with time. The lines show the fitted first-order kinetics. (B, D) Dependence of the apparent first-order rate constant on the concentration of DTT.

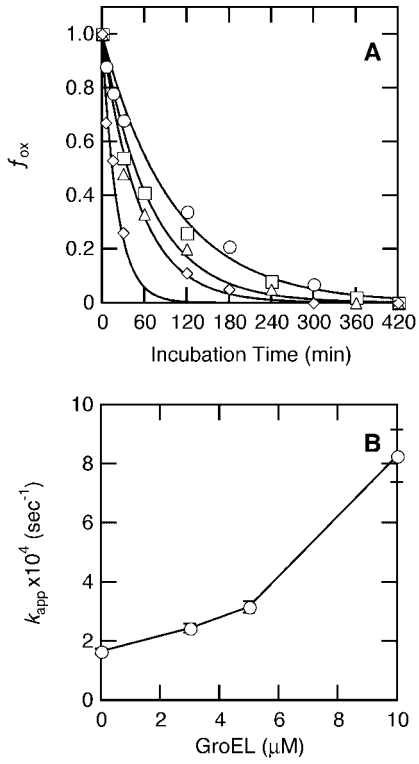

Fig. 4. **Reduction of the disulfide bond of β2-m (20 μM) in the presence of GroEL by 100 mM DTT in 50 mM Tris-HCl (pH 8.5) at 37°C.** (A) Decrease with time in the fraction of oxidized β2-m in the absence (open circles) and presence of 3 μM (open squares), 5 μM (open triangles), and 10 μM (open diamonds) GroEL. The lines show the fitted first-order kinetics. (B) Dependence of the apparent first-order rate constant on the concentration of GroEL.

RESULTS

Reduction of a Disulfide Bond in the Native State—The disulfide bond of β2-m could not be reduced with a low concentration of DTT (*e.g.* 10 mM) at pH 8.5 and 37°C in the absence of denaturant. The inaccessibility of the disulfide bond to the reducing reagents would be common to the immunoglobulin domains, in which the disulfide bond is buried in the interior of the protein molecule (*23–25*). However, as reported by Hong *et al.* (*15*), in the presence of sufficiently high concentrations of DTT, the disulfide bond was progressively reduced with time as can be seen from the HPLC profiles (Fig. 2A). The fraction of oxidized β2-m calculated from its peak area decreased exponentially with incubation time and most of the molecules were reduced after several hours (Figs. 2A and 3A). We did not observe any peaks other than the intact and reduced states, indicating that the reduction by DTT is a cooperative process. The rate of reduction decreased with the decrease in the concentration of DTT (Fig. 3A).

When carried out in the presence of 4 M Gdn-HCl at pH 8.5 and 37°C, where β2-m is unfolded (*12*), the reduction by DTT was very rapid: in 0.5 mM DTT, the reaction took less than one hour (Figs. 2B and 3C). Again, the reaction was cooperative without an accumulation of intermediate. Raising the DTT concentration accelerated the reaction: at 2 mM DTT, the reduction ended in a few minutes. These results confirm that the intrinsic rate of reduction of the exposed disulfide bond by DTT is very high.

Both in the presence and absence of Gdn-HCl, the reduction processes were consistent with first-order kinetics with the apparent rate constant, k_{app} (see Methods). The k_{app} value at 0.5 mM DTT in 4 M Gdn-HCl, pH 8.5, and 37°C was 0.01 s^{-1}, and the value increased linearly with the increase in the concentration of DTT. Since β2-m is largely unfolded in the presence of 4 M Gdn-HCl (*12*), we assumed that the rate in 4 M Gdn-HCl represents that of the solvent-exposed disulfide bond and the second order rate constant (~20 s^{-1} M^{-1}) of the reduction of disulfide bond was obtained. The rate constant thus obtained is comparable to the rate of reduction of the gluthathione dimer by DTT at pH 8.7 (*29*). Our value with β2-m at pH 8.5 and 37°C was slightly higher than the rate (~3 s^{-1} M^{-1}) for the reduction of the disulfide bond of the immunoglobulin domain at pH 8.0 and 25°C (*25*). In contrast, the reaction in the absence of denaturant was very slow with $k_{app} = 1.8 \times 10^{-4}$ s^{-1} even in the presence of 200 mM DTT (Fig. 3B). At DTT concentrations less than 100 mM, k_{app} was linearly dependent on the concentration of DTT, although it showed a saturating tendency above 100 mM DTT. The slope of DTT dependence below 100 mM DTT was distinct from that in the presence Gdn-HCl (Fig. 3D). In other words, at the same DTT concentration, the reduction of the disulfide

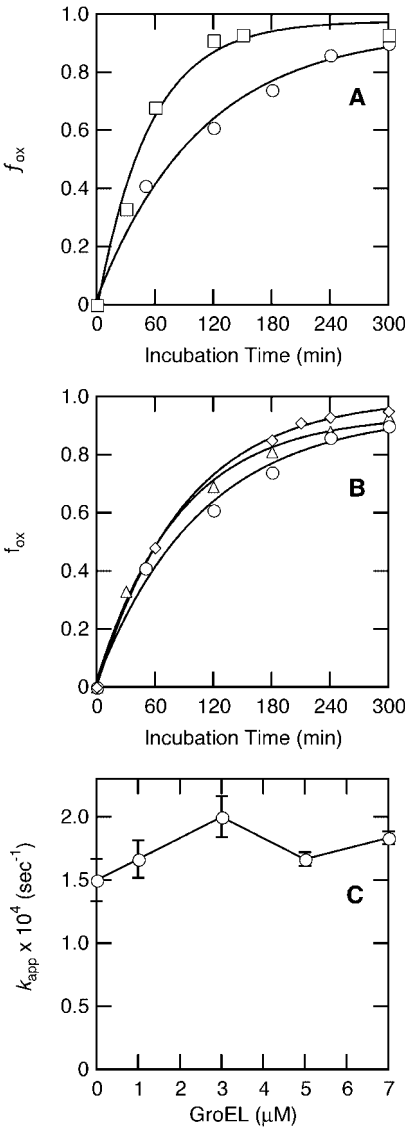

Fig. 5. **Kinetics of the reoxidation of the disulfide bond of reduced β2-m (20 μM) by 200 mM oxidized DTT in 50 mM Tris-HCl (pH 8.5) at 37°C.** (A) Increase with time of the fraction of oxidized β2-m in the absence (open circles) and presence (open squares) of 4 M Gdn-HCl. (B) Effects of GroEL on the reoxidation kinetics. Concentrations of GroEL were 0 (open circles), 3 (open triangles), and 7 (open diamonds) μM. (C) Dependence of the apparent rate constant of reoxidation on the GroEL concentration.

bond of the native state is slower by 1.44×10^4 fold than that of the unfolded state.

Reduction of a Disulfide Bond in the Presence of GroEL—Chaperonin GroEL interacts with various conformational states with exposed hydrophobic surfaces (*30*). Since β2-m shows a greater tendency to form aggregates than other globular proteins (*12*), we expected it to interact with GroEL. However, we did not detect a stable complex by gel filtration HPLC, suggesting that there was no persistent interaction between the two (data not shown). Then, the reduction of the disulfide bond was used as a probe to monitor the dynamic interaction with GroEL. Upon the addition of 3–10 μM GroEL at 100 mM DTT, the rate of reduction was notably increased, while the reaction still followed first-order kinetics (Fig. 4A). The apparent first-order rate constant increased by four-fold at 10 μM GroEL (Fig. 4B). Thus, the binding to GroEL destabilizes the β2-m structure, resulting in facilitation of the reduction of the disulfide bond.

Reoxidation of a Disulfide Bond—The disulfide bond can be reformed by the reaction of reduced β2-m with oxidized DTT at 200 mM (Fig. 5A). The reoxidized species monitored by HPLC increased in a saturating manner with time. However, in contrast to the reduction of the disulfide bond, the oxidation did not always produce full recovery of the oxidized species, although more than a 90% recovery was usually obtained. It is likely that aggregation or misfolding of the reduced species decreased the yield of the reoxidized species. Since such a side reaction is likely to occur, the final yield was set as an adjustable parameter in the curve fitting of the reoxidation kinetics, so that an apparent first-order rate constant of 1.5×10^{-4} s^{-1} was obtained at 200 mM oxidized DTT.

When the thiol groups are buried inside of the protein molecule, the reoxidation slows because of their inaccessibility to oxidizing reagents (*24*, *31*). In such a situation, the denaturation of the reduced protein often results in an acceleration of the reaction. We carried out the reoxidation of the disulfide bond in the presence of 4 M Gdn-HCl. The reaction with an apparent rate constant of 3.0×10^{-4} s^{-1} was two-fold faster than that in the absence of Gdn-HCl, suggesting that the thiol groups were partially buried inside of the molecule. The results are consistent with our previous observation that reduced β2-m in the absence of a denaturant assumes a native-like immunoglobulin fold with buried thiol groups (*12*), although the extent of burial is less than that of the constant domain of the immunoglobulin light chain (*23*, *24*).

We then measured the effects of GroEL on the reoxidation kinetics. However, in the GroEL concentration range examined, no significant effect was observed (Fig. 5, B and C). In contrast to the interaction of GroEL with intact β2-m, the formation of a stable complex of GroEL and reduced β2-m was indicated by gel filtration HPLC (data not shown). Thus, we conclude that the persistent interaction with GroEL does not induce a conformational change of reduced β2-m useful for disulfide bond formation. Probably, the bound β2-m assumes an intermediate conformational state with buried SH groups.

Both GroEL (pI = 5.0) and β2-m (pI = 6.0) are negatively charged at pH 8.5. Therefore, it is likely that electrostatic charge repulsion suppresses the interaction. In such a situation, the addition of salt may promote the interaction, consequently affecting the reduction or reoxidation reactions. However, the addition of salt notably increased neither the reduction nor reoxidation rates, although a slight increase in both rates was detected (data not shown). This suggests that hydrophobic interactions are predominantly responsible for the interaction of GroEL and β2-m in both the oxidized and reduced forms.

DISCUSSION

Mechanism of Disulfide Bond Reduction—The reduction of the disulfide bond by DTT can be represented by

the following scheme including the native state (N), the denatured state (U), and the reduced state (R) (*25*):

$$\mathrm{N} \underset{k_R}{\overset{k_U}{\rightleftarrows}} \mathrm{D} \overset{k_{int}[DTT]}{\rightleftarrows} \mathrm{R} \quad (1)$$

where k_U and k_R represent the unfolding and refolding rate constants, respectively, and k_{int} and [DTT] are the intrinsic rate constant of reduction and DTT concentration, respectively. The equilibrium constant (K_U) between N and U is defined by $K_U = k_U/k_R$. It is noted that the reaction scheme resembles that of the $^1H/^2H$ exchange of amide protons (*32*).

In Scheme 1, under the conditions where $k_U \ll (k_R + k_{int}[DTT])$, the apparent reduction rate, k_{app}, is represented by:

$$k_{app} = k_U k_{int}[DTT]/(k_R + k_{int}[DTT]) \quad (2)$$

When $k_R \gg k_{int}[DTT]$ in Eq. 2, k_{app} is approximated by $K_U k_{int}[DTT]$ (EX2 limit). Then, $(1/K_U)$ corresponds to the protection factor of the $^1H/^2H$ exchange of amide protons and the free energy change of denaturation (ΔG_U) can be estimated by $\Delta G_U = -RT\ln K_U$, where R and T are a gas constant and temperature, respectively. At the other extreme when $k_{int}[DTT] \gg k_R$, k_{app} represents k_U (EX1 limit). Under intermediate conditions where k_R is comparable to $k_{int}[DTT]$, ΔG_U estimated on the basis of the EX2 limit would be larger than the true value.

Below 100 mM DTT in the absence of Gdn-HCl, the dependence on the [DTT] concentration of the rate constant is linear (Fig. 3B), suggesting the EX2 mechanism: while k_{app} in Gdn-HCl is $k_{int}[DTT]$, that in the absence of Gdn-HCl is $K_U k_{int}[DTT]$. Assuming the EX2 limit, we estimated K_U to be $1/(1.44 \times 10^4)$ and then ΔG_U to be 25 kJ/mol at pH 8.5 and 37°C. We previously obtained a value of 31 kJ/mol at pH 8.5 and 10°C from analysis of the Gdn-HCl–induced unfolding curve (*12*). Considering the difference in temperatures, the two values are consistent with each other. This supports the validity of the EX2 approximation and, moreover, implies that the native form is not highly stable, occasionally experiencing substantial denaturation even under native conditions. However, the population (0.07%) of the denatured form estimated from the disulfide reactivity is much less than that (25%) of amyloidogenic species proposed by Heegaard *et al*. (*7*) from experiments with capillary electrophoresis.

The saturation tendency of the reduction rate above 100 mM DTT suggests the conversion from an EX2 mechanism to an EX1 mechanism. On the basis of Eq. 1, with $k_{app} = 1.8 \times 10^{-4}$ s^{-1} at 200 mM DTT, $k_{int}[DTT] = 4$ s^{-1} at 200 mM DTT, and $K_U = [1/(1.44 \times 10^4)]$, k_U and k_R are estimated to be 3.6×10^{-4} s^{-1} and 5.2 s^{-1}, respectively. The estimated k_U value is consistent with the conversion to EX1 limit where k_{app} represents k_U. Although these are very rough estimates of kinetic parameters, the results indicate the usefulness of the present approach using DTT for analyzing the dynamic behavior of β2-m. For comparison, from the unfolding and refolding kinetics dependent on Gdn-HCl at pH 7.4 and 30°C, Chiti *et al.* (*9*) proposed a sequential folding mechanism of β2-m with two kinetic intermediates I_1 and I_2: $U \rightleftarrows I_1 \rightleftarrows I_2 \rightleftarrows N$. The values of the observed rate constants for the fast and slow phases of folding and for unfolding, extrapolated to zero denaturant concentration, are 5.1 s^{-1}, 3.0×10^{-4} s^{-1}, and 4.9×10^{-4} s^{-1}, respectively. Intriguingly, their rate constants for fast refolding (5.1 s^{-1}) and unfolding (4.9×10^{-4} s^{-1}) are very similar to those of k_R (5.2 s^{-1}) and k_U (3.6×10^{-4} s^{-1}), respectively, obtained here from the reactivity of the disulfide bond.

Implications for Amyloid Fibril Formation—The global fluctuation in β2-m as demonstrated by the reactivity of the disulfide bond was accelerated upon interaction with GroEL. Since we could not detect the formation of a stable complex by gel filtration HPLC, the interaction does not result in such a complex. Nevertheless, it is clear that the transient and dynamic interactions stabilize the denatured form. Such a GroEL-dependent denaturation of substrate proteins has been reported before, implying the underlying mechanism for the unfoldase activity of GroEL (*33*–*35*). The GroEL-induced denaturation of substrate proteins has been explained by the passive mass action taking advantage of hydrophobic interaction with GroEL (*33*, *34*) or by the ATP-dependent forced unfolding (*35*).

In the present study, GroEL was used as a model protein that induces the denaturation of β2-m. It is possible that a factor such as GroEL can directly promote amyloid fibril formation *in vivo*. In this context, it should be noted that, in the presence of sub-micellar concentrations of sodium dodecyl sulphate, a detergent that mimics some characteristics of biological membranes, fibril formation by a peptide from human complement receptor 1 is promoted (*36*). *In vivo*, there are various proteins and hydrophobic lipids that might interact with β2-m. These include proteins associated with amyloid fibrils such as apoE and the amyloid P component (*21*, *37*). Although these compounds are generally considered to stabilize amyloid fibrils, it is likely that they also stabilize amyloidogenic precursors through hydrophobic interactions, thus promoting the formation of amyloid fibrils.

Conclusion—Because β2-m has only one disulfide bond, the characterization of the oxidized and reduced states is straightforward by HPLC. In the present paper as well as in a previous paper (*15*), we show that the oxidation and reduction of the disulfide bond is a useful probe with which to monitor the conformation of β2-m. The results indicate that, although highly protected from reduction, the native β2-m occasionally experiences global fluctuations in which the disulfide bond is exposed to the solvent. Such fluctuation is facilitated upon interaction with chaperonin GroEL. Analysis of the change in reactivity of the disulfide bond upon the addition of various physiologically relevant substances will be useful in elucidating the mechanism of amyloid fibril formation by β2-m.

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