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Reductive Depolymerization of Bovine Thyroglobulin Multimers *via* Enzymatic Reduction of Protein Disulfide and Glutathionylated Mixed Disulfide Linkages

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The nascent thyroglobulin (Tg) multimer molecule, which is generated during the initial fate of Tg in ER, undergoes the rapid reductive depolymerization. In an attempt to determine the depolymerization process, various types of Tg multimers, which were generated from deoxycholate-treated/reduced Tg, partially unfolded Tg or partially unfolded/reduced Tg, were subjected to various GSH (reduced glutathione) reducing systems using protein disulfide isomerase (PDI), glutathione reductase (GR), glutaredoxin or thioredoxin reductase. The Tg multimers generated from deoxycholate-treated/reduced Tg were depolymerized readily by the PDI/GSH system, which is consistent with the reductase activity of PDI. The PDI/GSH-induced depolymerization of the Tg multimers, which were generated from either partially unfolded Tg or partially unfolded/reduced Tg, required the simultaneous inclusion of glutathione reductase, which is capable of reducing glutathionylated mixed disulfide (PSSG). This suggests that PSSG was generated during the Tg multimerization stage or its depolymerization stage. In particular, the thioredoxin/thioredoxin reductase system or glutaredoxin system was also effective in depolymerizing the Tg multimers generated from the unfolded Tg. Overall, under the net GSH condition, the depolymerization of Tg multimers might be mediated by PDI, which is assisted by other reductive enzymes, and the mechanism for depolymerizing the Tg multimers differs according to the type of Tg multimer containing different degrees and types of disulfide linkages.

Key words: Depolymerization, Thyroglobulin (Tg), Protein disulfide isomerase (PDI), Reductase, Glutathione, Thiol-disulfide exchange

INTRODUCTION

The overall structure of thyroglobulin is characterized by the complexity of the protein (Tg dimer M.W., 660 kDa) with some heterogeneity (Gentile *et al.*, 1995). Another characteristic is represented by the presence of 122 cysteinyl residues for each Tg monomer, most of which are involved in the intra-chain disulfide bonds (Malthiery and Lissitzky, 1987; Mercken *et al.*, 1985).

Previously, it was reported that in thyrocytes, a multimeric form of Tg appeared rapidly as transient aggregates (Kim *et al.*, 1993; Kim and Arvan, 1995), which was generated from the newly formed Tg through the enzymatic or non-enzymatic disulfide bond formation of unfolded/reduced Tg in the ER (Nigam *et al.*, 1994). The transition aggregates were then observed to dissociate into monomers under reducing conditions, and then assembled to dimers prior to intracellular transport and secretion (Kim *et al.*, 1993).

Although the reducing power of the cellular redox state is believed to be important for reductive reversal, it was suggested that some enzymes might also be involved in the reductive process. Hayano *et al.* (1993) reported that the microenvironment around the disulfide bond of proteins is a limitation to facilitated reduction, which may differ according to the nature of the proteins involved. Therefore, it is important to know if there is any specific enzyme system for the reductive depolymerization of multimeric Tg form in the redox environment of the ER. For example, the PDI molecule might be one such protein because it was found to be associated with the Tg

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multimers (Freedman *et al.*, 1989; Gething and Sambrook, 1990). Moreover, the reductase activity of PDI was reported to be involved in the reduction of intraprotein disulfide (Freedman *et al.*, 1994) or GSH mixed disulfide (Lundstr-Öm-Ljung *et al.*, 1999; Hayano *et al.*, 1993; Gravina and Mieyal, 1993). In addition, BiP was observed to be one of chaperones that bound both disulfide-linked aggregates and unfolded monomers of the nascent Tg. However, because the degree of BiP binding declined progressively during the folding of individual Tg molecules (Kim *et al.*, 1992; Kim and Aryan, 1993), it appears that the access and binding of other helper proteins to Tg might alter with time. According to a recent review (Pagani *et al.*, 2000), there is a reductive pathway associated with the formation of protein disulfide bonds in *E. coli*; periplasmic DsbC, which is responsible for reducing incorrect disulfide bonds. In addition, this reductive pathway was reported to involve the accepting of electrons from cytoplasmic thioredoxin (Rietsch *et al.*, 1997). In yeast, GSH is utilized as a reductant for reducing mis-oxidized disulfide by PDI (Wang and Chang, 1999). In particular, the isomerization or reducing activity of PDI in the GSH redox condition has been suggested to be responsible for the rearrangement or reduction of mispaired disulfide bonds (Freedman, 1984; Freedman *et al.*, 1994) in the normal folding process of proteins. Alternatively, the PDI homologues (Tachibana and Stevens, 1992) might serve as a catalyst of disulfide reshuffling, resulting in the reductive reversal of the multimeric form, probably following the kinetics of the protein protein interactions. In addition, there are cellular reduction systems (Hayano *et al.*, 1993; Stehr *et al.*, 2001; Gravina and Mieyal, 1993; Holmgren, 1989), such as the glutaredoxin, or thioredoxin/thioredoxin reductase system, whose actions are governed by not only by the reduction potential but also by the redox state. Nevertheless, there are no reports on the reductive systems responsible for the depolymerization of the multimeric Tg forms.

Therefore, this study examined the effects of the different reductive systems on the depolymerization of the various Tg multimeric forms, which were prepared by treating Tg with urea and/or thiol. The results suggest that the reductive depolymerization of the Tg multimers in the net GSH condition might be mediated by PDI, which is assisted by other reductive enzymes.

MATERIALS AND METHODS

Materials

Sephadex G 25, Sephacryl S-400 HR, EDTA, urea, mercaptoethanol, DTT, GSH, GSSG, cystine, deoxycholate, gluthathione reductase (GR, 300 unit/mg), glutaredoxin (GX, 127.2 unit/mg), thioredoxin (Trx, 4.8 unit/mg), thioredoxin reductase (TR, 5.4 unit/mg) and Protein Assay Reagent were purchased from Sigma Chemicals Co (St. Louis, MO, U.S.A.). The various products used for electrophoresis were purchased from Bio-Rad Laboratories (St. Louis, MO, U.S.A.).

Preparation of thyroglobulin (Tg)

The bovine thyroid was obtained from a local slaughterhouse at Ohjungdong, Taejon, Korea. The bovine Tg was purified using a method reported elsewhere (Gentile *et al.*, 1997; Herzog *et al.*, 1992). Briefly, the tissue extract was subjected to fractional precipitation with 1.4-1.8 M ammonium sulfate, followed by gel chromatography on Sephacryl S-400 HR. Separately, the Tg fractions, which appeared at void volumes, were pooled, freeze-dried, and used as the native Tg multimers.

Purification of protein disulfide isomerase (PDI)

The PDI from a bovine liver was purified using a procedure published elsewhere (Freedman *et al.*, 1995). The purified PDI was identified as being relatively pure by SDS- PAGE analysis, and the redox state was analyzed using the AMS (Molecular Probes) derivatization method (Tu *et al.*, 2000).

Preparation of partially unfolded/reduced Tg or deoxycholate-treated/reduced Tg

Tg (16 mg/mL) was incubated with urea (3 M) and high concentrations of DTT (3-50 mM) in 1 mL of 50 mM Tris-HCl (pH 7.6) at 38°C. After 30 min, the mixture was passed through a sephadex G-25 column in 50 mM Tris-HCl, pH 7.2, and the aliquot was subjected to 5% acrylamide SDS-PAGE to determine the molecular forms of Tg. Partially unfolded/reduced Tg was prepared using 3 M urea and 10 mM DTT. Separately, Tg (16 mg/mL) was incubated with 0.3 % deoxycholate and 10 mM DTT in 1 mL of 50 mM Tris-HCl (pH 7.6) for 30 min at 38°C. After gel filtration, the mixture was used as the deoxycholate-treated/reduced Tg.

Preparation of partially unfolded Tg, extensively unfolded Tg, or deoxycholate-treated Tg

The partially unfolded Tg and extensively unfolded Tg were prepared by incubating Tg (16 mg) in 50 μL of a 50 mM Tris buffer (pH 7.6) containing 3 M urea and 8 M urea, respectively, for 30 min at 38°C. The deoxycholate-treated Tg was prepared by incubating Tg (16 mg) in 50 μL of 50 mM Tris-HCl (pH 7.6) containing 0.3% deoxycholate. Each type of Tg (16 mg/mL) was first incubated with urea (3 M) and GSH (1 mM) in 50 μL of 50 mM Tris-HCl (pH 7.6) containing 1 mM EDTA at 38°C for 30 min to produce the multimeric Tg forms, and a mixture after a 24-fold dilution with the same buffer.

Preparation of purified Tg multimers

The Tg multimers were formed by simultaneously exposing Tg (16 mg/mL) to both urea (3 mM) and GSH (1 mM) in 4 mL of 50 mM Tris-HCl (pH 7.6) at 38°C for 30 min. The sample containing the Tg multimer after concentration was applied to a Sephacryl S 500 column (2 × 60 cm), and eluted with 50 mM Tris-HCl (pH 7.2).

Depolymerization of multimeric Tg forms generated from PDI-induced multimerization of deoxycholate-treated/reduced Tg or partially unfolded/reduced Tg

The deoxycholate-treated/reduced Tg (0.66 mg/mL) was first incubated with PDI (6 μM) in 50 μL of 50 mM Tris-HCl (pH 7.2) containing 1 mM EDTA for 30 min at 25°C to generate the multimeric forms of Tg, and the depolymerization was initiated by adding GSH (final concentration, 2 mM) to the mixture in the presence or absence of GSSG (0.03-0.3 mM), followed by incubation for 30 min. Separately, the partially unfolded/reduced Tg (0.66 mg/mL) was first incubated with PDI (6 μM), and depolymerization was performed as described above.

Depolymerization of multimeric Tg forms generated from urea/GSH-induced multimerization of Tg

Tg (16 mg/mL) was first incubated with 3 M urea in the presence of GSH (1 mM) in 50 μL of 50 mM Tris-HCl (pH 7.6) containing 1 mM EDTA at 38°C for 30 min to produce the multimeric Tg forms, and after a 24-fold dilution with the same buffer, the mixture was further incubated with each reduction system in 50 mL of 50 mM Tris-HCl (pH 7.6) containing 1 mM EDTA at 38°C for 30 min.

Depolymerization of multimeric Tg forms, generated from GSH-induced multimerization of partially unfolded Tg, extensively unfolded Tg or deoxycholate-treated Tg

Separately, the partially unfolded Tg, extensively unfolded Tg or deoxycholate-treated Tg (0.66 mg/mL) was incubated with 1 mM GSH and PDI (2 μM) in 50 μL of 50 mM Tris-HCl (pH 7.6) containing 1 mM EDTA at 38°C to form the multimeric Tg forms. After 30 min, each reduction system was added to the mixture.

Depolymerization of purified Tg multimer

The purified Tg multimer (0.6 mg/mL) was incubated with each reduction system in 50 μL of 50 mM Tris-HCl (pH 7.6) containing 1 mM EDTA for 30 min at 38°C.

Other procedures

SDS-PAGE was performed under non-reducing conditions (Laemmli, 1970) using 5% acrylamide. The amount of protein was determined using the Bradford method (Bradford, 1976) or by measuring the absorbance at a wavelength of 280 nm. Scanning quantitative analyses were carried out using the Quantity One program (Gel Doc 2000, Bio-Rad, Hercules, CA). The amount of reduced cysteine residues in the Tg molecule was determined using the method reported by Ellman (Ellman, 1959).

RESULTS

Depolymerization of the multimeric Tg forms generated from the PDI-induced multimerization of the deoxycholate-treated/ reduced Tg

The depolymerization of various types of Tg multimer molecules was examined in a reconstitution system in an attempt to determine the process for the *in vitro* depolymerization of Tg multimers. This study examined the role of PDI in the depolymerization of multimeric Tg forms under various reducing conditions because the reductase activity of PDI was reported to be responsible for reducing the intraprotein disulfide (Freedman, 1984; Freedman *et al.*, 1994) or GSH mixed disulfide (LundstrÖm-Ljung *et al.*, 1999; Hayano *et al.*, 1993; Gravina and Mieyal, 1993) in the presence of GSH. First, the depolymerization of the multimeric Tg forms, generated from deoxycholate-treated/ reduced Tg was investigated using a consecutive process. The deoxycholate-treated/reduced Tg was initially exposed to PDI (6 $_{m}$M) at 38°C to form the multimeric forms. Thirty minutes later, 2 mM GSH was added to the incubation mixture to cause the depolymerization of the Tg multimer. As shown in Fig. 1, approximately 79.8 ± 1.6% (n=3) of the multimeric Tg form, which is produced from the PDI-mediated multimerization, was reduced in the presence of 2 mM GSH within 30 min. Although the appearance of the monomer form was not apparent, the bands of Tg at the

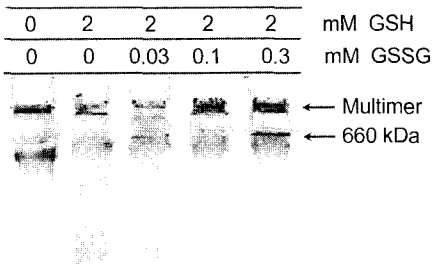

Fig. 1. Depolymerization of the multimeric Tg forms generated from the PDI-induced multimerization of deoxycholate-treated/reduced Tg or partially unfolded/reduced Tg. The deoxycholate-treated/reduced Tg (0.66 mg/mL) was first incubated with 6 μM PDI for 30 min at 25°C to generate the multimeric forms of Tg. Depolymerization was then initiated by adding GSH (final concentration, 2 mM) to the mixture in the presence or absence of GSSG (0.03-0.3 mM) as indicated, followed by incubation for a further 30 min.

lower molecular weights were distributed along the running gel. Under the above conditions, 0.03 mM GSSG had no significant effect on depolymerization, but it appeared to interfere with it at 0.1 or 0.3 mM. This confirms that the depolymerization of the Tg multimer depends on the reducing power of the PDI/GSH system.

Depolymerization of the multimeric Tg forms generated from the PDI-induced multimerization of partially unfolded/reduced Tg

The partially unfolded/reduced Tg, which was prepared by exposing Tg to urea/DTT (Liu and Sok, 2004), was first incubated with PDI for 30 min to form the Tg multimers. Subsequently, the reducing agents were consecutively included in the same reaction mixture to cause the depolymerization of the Tg multimer. Fig. 2 shows that 2 mM GSH alone has no marked effect on the depolymerization of multimeric Tg form (<10%), whereas the inclusion of the GR system in combination with GSH achieved remarkable depolymerization. Therefore, the PSSG was generated non-specifically during the PDI/GSH-induced depolymerization of the Tg multimer. In a related study, the PDI-induced multimerization of the partially unfolded/reduced Tg was performed for different times (2-30 min), and GSH (2 mM) was then added to the mixture, which was then treated in the same manner described above. After 20 min reduction, when each sample was analyzed by SDS-PAGE, the degree of depolymerization differed according to the multimerization time; 2, 5, 10, or 30 min multimerization products showed 47%, 36%, 21%, and 7% depolymerization, respectively (data not shown). Therefore, some part of the depolymerization of the Tg multimer did not require a GR system during a short period (2 or 5 min). This suggests that the reductive depolymerization of the Tg multimers by the PDI/GSH system is governed by the degree of disulfide bonding in the Tg multimers; specific disulfide formation at limited sites *vs.* non-specific disulfide formation at multiple sites.

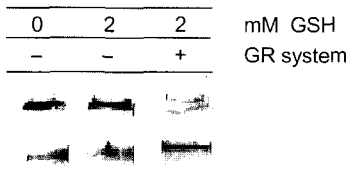

Fig. 2. Depolymerization of the multimeric Tg forms generated from the PDI-induced multimerization of partially unfolded/reduced Tg. Partially unfolded/reduced Tg (0.66 mg/mL) was first incubated with 6 μM PDI in 50 μL of 50 mM Tris-HCl (pH 7.2) containing 1 mM EDTA for 30 min at 25°C to generate the multimeric forms of Tg. The depolymerization was initiated by adding GSH (final concentration, 2 mM) to the mixture in the presence or absence of GR system (GR, 1 unit/mL + NADPH, 1 mM), followed by incubation for a further 30 min as indicated.

Depolymerization of the multimeric Tg forms generated from the urea/GSH-induced multimerization of Tg

The depolymerization of the multimeric Tg forms, which were produced from urea/GSH-induced multimerization of Tg, were examined because the urea/GSH-induced multimerization of Tg could generate the glutathionylated mixed disulfide bond (PSSG) in the Tg multimers. For this purpose, Tg was initially incubated simultaneously with 3 M urea and 1 mM GSH for 30 min, which produced the Tg multimer. Subsequently, after a 24-fold dilution, the above reaction mixture was exposed to additional GSH (final concentration, 2 mM) in the presence or absence of PDI. As shown in Fig. 3, GSH (2 mM), either alone or in combination with PDI, did not produce any significant depolymerization. In particular, the inclusion of GR system in the above incubation mixture caused remarkable depolymerization in a time-dependent fashion; the extent of depolymerization was greater after 30 min than after 10 min. Therefore, PSSG, which was generated during the urea/GSH-induced multimerization of Tg, interfered with the reductive depolymerization of the Tg multimers by PDI/GSH. Although it was initially believed that GSSG

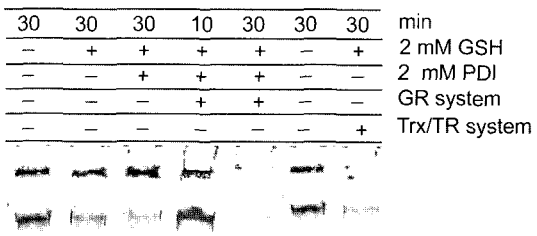

Fig. 3. Depolymerization of the multimeric Tg forms generated from the urea/GSH-induced multimerization of Tg. Tg (16 mg/mL) was first incubated with 3M urea in the presence of GSH (1 mM) in 50 μL of 50 mM Tris-HCl (pH 7.6) containing 1 mM EDTA at 38°C for 30 min to produce multimeric Tg forms, and the mixture, after 24-fold dilution with the same buffer, was further incubated with each reduction system as indicated. GR system (GR, 1 unit/mL + NADPH, 1 mM); Trx/TR system (0.25 unit/mL thioredoxin + 3 unit/mL thioredoxin reductase + 1 mM NADPH).

might affect the rate of depolymerization, the concentration (~10 μM) of GSSG produced under the conditions used was below the minimal concentration (30 μM) needed to inhibit the depolymerization (data not shown). It should be noted that thioredoxin reductase in combination with thioredoxin, which was reported to be involved in the reversible formation of PSSG (Casagrande, 2002), was so effective in depolymerizing the Tg multimer.

Depolymerization of the multimeric Tg forms generated from the PDI/GSH-induced multimerization of the unfolded Tg

Because the isomerase activity of PDI could produce PSSG during Tg multimerization *via* thiol-disulfide exchange (Liu and Sok, 2004), the next focus of this study was to examine the depolymerization of the multimeric Tg forms, which were generated by exposing the partially unfolded Tg, the extensively unfolded Tg or the deoxycholate-treated Tg to GSH in the presence of PDI. Each type of unfolded Tg was initially incubated with 1 mM GSH/PDI (2 μM) to produce the multimeric Tg forms. Each reduction system was added to the mixture 30 min later (Fig. 4). While the addition of GSH (final concentration, 2 mM) showed only slight depolymerization (Fig. 4, left), the additional inclusion of the GR system in the above mixture accelerated the depolymerization process remarkably (83.1 ±4 .7%, n=3), which is in good agreement with the existence of PSSG in the Tg multimer. In contrast, the multimeric Tg forms, which were generated by exposing the extensively unfolded Tg to GSH (1 mM), were resistant to the reductive system (PDI/GSH) including GR (Fig. 4, center). Independently, the accelerating action of the GR system was also observed with the multimeric Tg forms, which were prepared by exposing the deoxycholate-treated Tg to GSH in a sequential process (Fig. 4, right). Therefore, the depolymerization of the Tg multimers differs greatly according to the type of Tg multimers used. Moreover, the mild unfolding of Tg appears to be essential for the reversible multimerization of Tg.

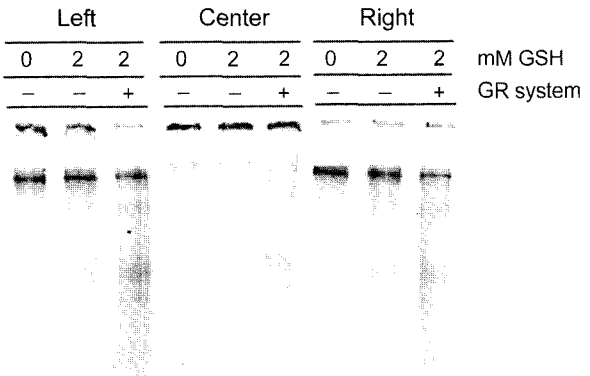

Fig. 4. Depolymerization of the multimeric Tg forms, generated from GSH-induced multimerization of partially unfolded Tg, extensively unfolded Tg or deoxycholate-treated Tg. Partially unfolded Tg, extensively unfolded Tg or deoxycholate-treated Tg (0.66 mg/mL) was incubated with 1 mM GSH and PDI (2 μM) in 50 μL of 50 mM Tris-HCl (pH 7.6) containing 1 mM EDTA at 38°C to form the multimeric Tg forms. Thirty minutes later, each reduction system was added to the mixture as indicated. GR system (GR, 1 unit/mL + NADPH, 1 mM). Left, partially unfolded Tg; center, extensively unfolded Tg; right, deoxycholate-treated Tg.

Depolymerization of the purified Tg multimer

In an attempt to determine the role of the reduction systems in the depolymerization of the multimeric forms, a large amount of Tg multimers was prepared *via* the urea/GSH-induced multimerization of Tg, which generated a mixed disulfide linkage *via* thiol disulfide exchange. After purification by Sephacryl S 500 gel chromatography, the Tg multimer was subjected to various depolymerization conditions. Fig. 5 shows that 2 mM GSH (lane 2) and a combination of 2 mM GSH with PDI produced only slight depolymerization (lane 3), while the GSH/GR system had no effect. Again, the inclusion of the GR system in combination with PDI accelerated the depolymerization of the Tg multimers significantly (lane 4). In addition, the thioredoxin/TR system was also effective in depolymerizing the purified Tg multimers (lane 5). Independently, glutaredoxin (5 units/mL), which is another enzyme capable of reducing the GSH-mixed disulfide (Beer *et al.*, 2004) in the presence of GSH, also caused effective depolymerization of the Tg multimers (lanes 7 & 8) irrespective of the presence of PDI. Therefore, the PDI/GSH-induced reductive depolymerization of the Tg multimers containing PSSG was remarkable in the presence of another reducing enzyme, GR, TR or glutaredoxin.

Fig. 5. Depolymerization of the purified Tg multimer. Purified Tg multimer was incubated with the respective reduction system. Lane 1, purified Tg multimer; lane 2, incubation with GSH (2 mM); lane 3, with 2 mM GSH/2 μM PDI; lane 4, with 2 mM GSH/2 mM PDI/GR system (GR, 1 unit/mL + NADPH, 1 mM); lane 5, with 2 mM GSH/Trx/TR system (thioredoxin, 0.25 unit/mL + TR, 3 units/mL); lane 6, the same as lane 1; lane 7, with 2 mM GSH/glutaredoxin (5 units/mL); lane 8, 2 mM GSH/glutaredoxin (5 units/mL)/2 mM PDI.

DISCUSSION

Similar to the reductase activity of PDI (Freedman, 1984; Debarbieux and Beckwith, 1999), the Tg multimer produced from the PDI-mediated multimerization of detergent-treated/reduced T, was successfully reduced to either the monomeic or dimeric form by PDI under GSH reducing conditions. This suggests that GSH plays a role in the ER as a net source of reducing equivalents to maintain PDI in its reduced form. However, the Tg multimers that were generated from either the partially unfolded/reduced Tg or partially unfolded Tg were resistant to reduction by PDI, indicating that in addition to the intermolecular disulfide linkage, those multimers may contain other linkages such as a glutathionylated mixed disulfide (PSSG) bond, which might be generated during the multimerization or depolymerization of Tg. Either the intramolecular disulfide bonds produced from the excess PDI-induced oxidation of the partially unfolded/reduced Tg or the intramolecular disulfide bonds of the partially unfolded Tg appear to be the targets for glutathionylation in this experiment. This is well supported by the requirement of GR, which reduces GSSG or PSSG (Kumari *et al.*, 1994), for the effective reduction of these types of Tg multimers. Nevertheless, the Tg multimers that were prepared from the 8 M urea-unfolded Tg were not depolymerized even in the reducing system containing both PDI and GR. As indicated by the requirement of deoxycholate in the depolymerization of extensively disulfide-linked Tg multimers by GSH, the extensive disulfide linkage at multiple sites, including the non-polar region, might result in the non-specific intermolecular disulfide linkage embedded in a tightly- associated state (data not shown). Therefore, the difference in the reductive reversal of various Tg multimers might be due to the different microenvironments around the intermolecular disulfide linkage. On the other hand, the facile intermolecular disulfide linkage at a specific region of the Tg molecule may be important for the reversible multimerization of Tg. For this purpose, an association between some chaperones and the newly-synthesized unfolded Tg in the ER might contribute to the reductive reversal of Tg multimerization by preventing non-specific binding between the unfolded Tg molecules or hindering the formation of GSH-mixed type disulfide in the Tg molecule. In this respect, GroEL, BiP and other ER chaperones, which were reported to be upregulated in response to Tg unfolding in the ER (Kuznetsov *et al.*, 1994; Kim *et al.*, 1996), might be necessary for the facilitated reductive depolymerization of newly synthesized Tg multimers. The requirement of GR, which is commonly observed for the effective reversal of most multimeric Tg forms, suggests that PSSG and/or GSSG can be produced during the incubation of the Tg multimer with GSH. Since the GSSG concentration was too low to affect depolymerization, the action of GR is believed to be related to the reductive removal of an intermediate, probably PSSG. This is further supported by another report showing that glutaredoxin (Beer *et al.*, 2004), in combination with GSH, was also effective in depolymerizing the Tg multimer. Nevertheless, there is some doubt as to the role of GR or glutaredoxin in the ER system because they are mainly limited to the cytosolic compartment. Although the depolymerization of the Tg multimer by glutaredoxin alone was not further examined, its action might be extended to the transfer of the reducing equivalent to the disulfide linkage hidden in the intermolecular contact as was reported in the glutaredoxin-catalyzed protein disulfide reduction process (Berardi and Bushewller, 1999). For this purpose, the thioredoxin/TR system utilizing NADPH with a high reduction potential appeared to be more effective. This is analogous to the earlier observation that thioredoxin, a small protein, can reduce intraprotein disulfides (Jung and Thomas, 1996; LundstrÖm-Ljung *et al.*, 1995). However, there is no evidence for such a role of the thioredoxin/TR system in the ER. According to a recent review (Pagani *et al.*, 2000), there is a reductive pathway associated with the formation of protein disulfide bonds in *E. coli*;.Periplasmic DsbC, which is responsible for reducing incorrect disulfide bonds, receives electrons from cytoplasmic thioredoxin (Rietsch *et al.*, 1997). However, in yeast, GSH is used as a reductant for reducing mis-oxidized disulfide bonds by PDI (Wang and Chang, 1999). Alternatively, PDI homologues (Tachibana and Stevens, 1992) might act as a catalyst for disulfide reshuffling. This would result in the reductive reversal of the multimeric form, possibly following the kinetics of the protein-protein interactions. A recent review suggests a possible role of the transmembrane thiredoxin-related molecule as a reducing equivalent (Masutani, 2005). Taken together, the overall rate of reductive dissolution of the Tg multimers is governed by the combined effect of the redox potential, the level of thiol-disulfide oxidoreductases as well as the structural properties of the Tg multimers. Since a combination of thiol disulfide oxidoreductases with some chaperones might be essential for the effective reduction of the Tg aggregates in the normal Tg maturation (Kim and Arvan, 1995), a shortage of such reduction systems or a disturbance in the sequential chaperone function in the ER might lead to prolonged Tg aggregation as was observed with delayed Tg aggregation under some pathological conditions (Kim *et al.*, 1993; Muresan and Arvan, 1998).

In conclusion, PDI participates in the depolymerization of multimeric Tg molecules, which mimics the nascent multimeric Tg in the ER. PDI participates in the primary multimerization as an oxidase or isomerase, and acts as a

reductase in the reductive reversal of Tg multimerization. A specific site, possibly a thioredoxin box-rich region, might be involved in the reversible Tg multimerization. The formation of PSSG in Tg molecules might be due to either an interaction between the unfolded/reduced Tg and GSSG, or an attack of GSH on the intramolecular disulfide link in an incompletely folded Tg molecule. In this respect, the multimerization of unfolded/reduced Tg *via* excess PDI followed by rapid reshuffling and reduction, which is presumably assisted by chaperones or reducing proteins, will be important for reductive reversal of the multimeric Tg molecule. More studies will be needed to define multimeric intermediates of the Tg molecule that are associated with specific chaperones in the ER system.

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