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Multimerization of Bovine Thyroglobulin, Partially Unfolded or Partially Unfolded/Reduced; Involvement of Protein Disulfide Isomerase and Glutathionylated Disulfide Linkage

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Fate of the nascent thyrolglobulin (Tg) molecule is characterized by multimerization. To establish the formation of Tg multimers, the partially unfolded/reduced Tg or deoxycholate-treated/reduced Tg was subjected to protein disulfide isomerase (PDI)-mediated multimerization. Oxidized glutathione/PDI-mediated formation of multimeric Tg forms, requiring at least an equivalent molar ratio of PDI/Tg monomer, decreased with increasing concentration of reduced glutathione (GSH), suggesting the oxidizing role of PDI. Additional support was obtained when PDI alone, at a PDI/Tg molar ratio of 0.3, expressed a rapid multimerization. Independently, the exposure of partially unfolded Tg to GSH resulted in Tg multimerization, enhanced by PDI, according to thiol-disulfide exchange. Though to a lower extent, a similar result was observed with the dimerization of deoxycholate-pretreated Tg monomer. Consequently, it is implied that intermolecular disulfide linkage may be facilitated at a limited region of unfolded Tg. In an attempt to examine the multimerization site, the cysteine residue-rich fragments of the Tg were subjected to GSH-induced multimerization; a 50 kDa fragment, containing three vicinal dithiols, was multimerized, while an N-terminal domain was not. Present results suggest that the oxidase as well as isomerase function of PDI may be involved in the multimerization of partially unfolded Tg or deoxycholate-treated Tg.

Key words: Multimerization, Thyroglobulin (Tg), Partially unfolded reduced, Protein disulfide isomerase (PDI), Disulfide

INTRODUCTION

Thyroglobulin (Tg), a precursor protein of thyroid hormones, is the most abundant protein in the thyroid gland (Chernoff and Rawich, 1981; Gentile *et al.*, 1995). The overall thyroglobulin structure is characterized by both its own complexity (Tg dimer M.W., 660 kDa) and heterogeneity (Gentile *et al.*, 1995). The presence of 122 cysteinyl residues per Tg monomer, most of which are involved in intra-chain disulfide bonds (Malthiery and Lissitzky, 1987; Mercken *et al.*, 1985) is another characteristic of the protein.

Regarding the multimerization of Tg through disulfide formation in endoplasmic reticulum (ER), it had been previously reported that in thyrocytes, the multimeric form of Tg usually appeared as transient aggregates (Kim *et al.*, 1993; Kim and Arvan, 1995), linked by disulfide bonds. Moreover, PDI was found to be associated with unfolded/reduced Tg (Nigam *et al.*, 1994), resembling newly formed Tg in ER. Thus, the multimeric Tg form in ER was supposed to be a product of PDI-mediated oxidative multimerization. The active site cysteines of PDI participate in dithiol-disulfide exchange reactions catalyzing dithiol oxidation, disulfide reduction, or disulfide isomerization depending on the nature of substrate protein and the redox condition (Freedman *et al.*, 1994; Gilbert, 1997). Recent data (Frand *et al.*, 2000; Pollard *et al.*, 1998; Frand and Kaiser, 1999; Tu *et al.*, 2000) provided positive findings for the participation of PDI, coupled with Ero1p, in the oxidative folding of proteins in conditions requiring no glutathione redox system, despite the relative abundance of GSSG in ER. Interestingly, some cysteine residues in the Tg molecule were present in the region compatible with thioredoxin box (CXXC) sequence. Its peculiarity is that thyroglobulin found in various mammalian species commonly contain three highly conserved thioredoxin boxes

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(Malthiery and Lissitzky, 1987; Mercken *et al.*, 1985; Klein *et al.*, 2000). Recently (Klein *et al.*, 2000), it was observed that long time exposure of human goiter Tg to a surplus of GSH resulted in the self-assisted multimerization of Tg, which was supposed to be mediated by thiol disulfide exchange probably in the thioredoxin box region. However, the thioredoxin box region may exist in a buried state within the normal bovine Tg. Prior unfolding of normal Tg appears necessary for effective multimerization thus enabling exposure of specific sites, such as thioredoxin box region, to the surface. However, there has been no attempt to validate this hypothesis.

In this study, we employed various Tg forms, prepared from the treatment of Tg with urea and/or thiol, to examine the multimerization of the protein. Here, we propose that the oxidative multimerization of partially unfolded/reduced Tg is induced by PDI, an oxidant, while the multimerization of partially unfolded Tg is governed by isomerase activity according to thiol disulfide exchange.

MATERIALS AND METHODS

Materials

Sephadex G 25, Sephacryl S-400 HR, EDTA, urea, mercaptoethanol, DTT, GSH, GSSG, cystine, deoxycholate, and Protein Assay Reagent were purchased from Sigma Chemicals Co (St. Louis, MO, USA). Various products for electrophoresis were purchased from Bio-Rad Laboratories (St. Louis, MO, USA).

Preparation of thyroglobulin (Tg)

Bovine thyroid was obtained from a local slaughterhouse in Ohjungdong, Taejon, Korea. The purification of bovine Tg was performed as previously reported (Gentile *et al.*, 1997; Herzog *et al.*, 1992); the tissue extract was subjected to fractional precipitation with 1.4-1.8 M ammonium sulfate, and subsequently to gel chromatography on Sephacryl S-400 HR. Separately, the Tg fractions, appearing at void volumes, were pooled, freeze-dried, and used as native Tg multimers.

Purification of protein disulfide isomerase (PDI)

Purification of PDI from the bovine liver was preformed according to the procedures published before (Freedman *et al.*, 1995). The purified PDI was identified as a relatively pure using SDS- PAGE analysis, while the redox state was analyzed by AMS (Molecular Probes) derivatization method (Tu *et al.*, 2000).

Preparation of polypeptide 50 kDa, unfolded/reduced and *N*-terminal domain of Tg

Limited proteolysis of Tg with thermolysin was performed as previously described (Gentile *et al.*, 1997), and the polypeptide fragments separated by a preparative continuous-elution SDS-PAGE apparatus (Bio-Rad model 491, Bio-Rad). Fractions containing 50 kDa polypeptide were pooled, lyophilized, and then passed through a Sephadex G 25 column to remove SDS. Separately, *N*-terminal domain (NTD) was separated from bovine Tg as described before (Delom *et al.*, 2001), and NTD fragment was purified by a Sephadex G-200 chromatography, and FPLC using a Superose 12 column (1.3×28 cm).

Preparation of purified Tg multimers

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

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 in 50 mM Tris-HCl, pH 7.2, and the aliquot was subjected to 5% SDS-PAGE to analyze 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, and the mixture, after gel filtration, was used as deoxycholate-treated/reduced Tg.

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

Partially unfolded Tg and extensively unfolded Tg were prepared by incubating the Tg (16 mg) in 50 μL of 50 mM Tris buffer (pH 7.6) containing 3 M urea and 8 M urea, respectively, for 30 min at 38°C. Deoxycholate-treated Tg was prepared by incubating the Tg (16 mg) in 50 mL of 50 mM Tris-HCl (pH 7.6) containing 0.3% deoxycholate. Subsequently, after 24-fold dilution, each type of Tg was used for Tg multimerization.

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

Disulfide-induced multimerization of partially unfolded/reduced Tg or deoxycholate-treated/reduced Tg was performed by incubating partially unfolded/reduced Tg or deoxycholate-treated/reduced Tg (0.66 mg/mL) with each disulfide in 50 μL of 50 mM Tris-HCl (pH 7.2) for 30 min at 25°C. GSSG/PDI-induced multimerization of partially unfolded/reduced Tg was achieved by incubating the Tg (0.66 mg/mL) with PDI (2 mM) and GSSG (30 mM) in the

presence or absence of GSH (0.1-2 mM). PDI-induced multimerization of partially unfolded/reduced Tg or deoxycholate-treated/reduced Tg was carried out by exposing partially unfolded/reduced Tg (0.66 mg/mL) to PDI (6 mM).

Multimerization of Tg after simultaneous exposure of Tg to urea and DTT at low concentrations

Tg (16 mg/mL) was incubated with 3 M urea and various concentrations of DTT (0.1-1 mM) in 1 mL of 50 mM Tris-HCl (pH 7.6) for 30 min at 38°C. The mixture, after gel filtration on Sephadex G-25, was analyzed by SDS-PAGE.

Urea/GSH-induced multimerization of Tg

Tg (16 mg/mL) was simultaneously exposed to urea (3 M) and GSH (0.1-1 mM) in 50 µL of 50 mM Tris-HCL (pH 7.6) for 30 min at 38°C as described above. Separately, the concentration of Tg was varied from 0.66 mg/mL to 16 mg/mL.

GSH-induced multimerization of partially unfolded Tg or deoxycholate-treated Tg

Partially unfolded Tg or deoxycholate-treated Tg (0.66 mg/mL) was incubated with GSH (0.1-1.0 mM) in the presence or absence of PDI (2 µM) in 50 mM Tris-HCl (pH 7.6) for 30 min at 38°C. Subsequently, the mixture was used after 24 fold dilution. Separately, GSH (1 mM)-induced multimerization was performed in the presence of GSSG (0.1-1 mM).

Multimerization of 50 kDa and NTD fragment

50 kDa and NTD polypeptide, at 0.6 or 1 mg/mL, was incubated with GSH (0.3 or 1 mM) and 3 M urea in 50 µL of 50 mM Tris (pH 7.6) for 30 min at 38°C. The aliquot was analyzed by SDS-PAGE (10 % and 15% acrylamide).

Other procedures

SDS-PAGE was performed under non-reducing conditions (Laemmli, 1970) using 5% acrylamide. Protein amount was determined according to Bradford method (Bradford, 1976) or by the measurement of A_{280}. Scanning quantitative analyses were carried out using a Quantity One program (Gel Doc 2000, Bio-Rad, Hercules, CA). The amount of reduced cysteine residue in Tg molecule was determined according to Ellmans method (Ellman, 1959).

RESULTS

Preparation of partially unfolded/reduced Tg

In an attempt to elucidate the process for *in vitro* multimerization of Tg, we examined the multimerization of various types of Tg molecules in the reconstitution system. We first examined the molecular conversion of the partially unfolded/reduced Tg monomer form. To prepare partially unfolded/reduced Tg (Herzog *et al.*, 1992), it was treated with various concentrations of DTT (3-50 mM) in the presence of 3 M urea for 30 min at 38°C (Fig. 1A). After gel filtration on a G-25 sephadex, the resulting Tg was subjected to SDS-PAGE analysis under non-reducing conditions. Fig. 1A demonstrates that native Tg, purified by gel chromatography, showed two major forms (lane 1), monomeric (330 kDa) and dimeric (660 kDa), as had been reported previously (Herzog *et al.*, 1992); the separation of each Tg form without extensive unfolding was impossible. A portion of the dimeric form was unaltered in SDS-PAGE under reducing conditions This proved to be consistent with the existence of linkages other than disulfide bonds in the dimeric Tg form (Delom *et al.*, 1999). Therefore, this experiment focused on the molecular conversion of the monomeric Tg form. The distribution of Tg forms, generated from the pretreatment of Tg with DTT in combination with urea, differed according to DTT

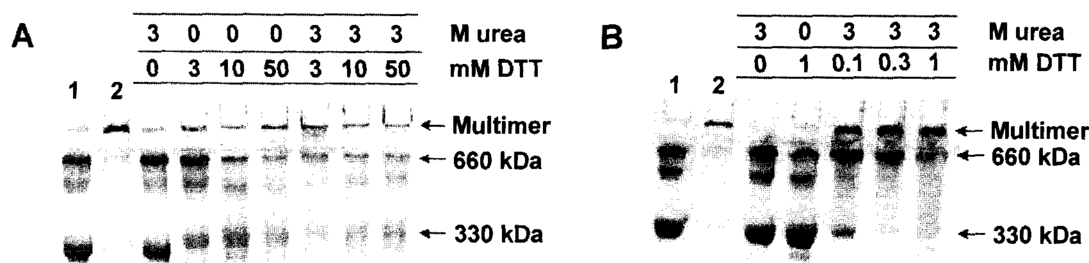

Fig. 1. Exposure of bovine Tg to urea and/or DTT. (A), Native bovine Tg (16 mg/ml) was incubated with urea and /or DTT at higher concentrations (3-50 mM) in 1 mL of 50 mM Tris-HCl (pH 7.6) at 38°C for 30 min as indicated, and then the mixture, after gel filtration, was subjected to SDS-PAGE analysis (5 % acrylamide). Lane 1, native Tg; lane 2, native Tg multimer. (B), Native bovine Tg was incubated with urea and /or DTT at lower concentrations (0.1-1.0 mM).

concentration (Fig. 1A). Though the band, corresponding to the monomeric form (330 kDa) was dispersed, it remained a predominant component in the treatment of Tg with DTT (10 or 50 mM) in combination with 3 M urea. While the multimeric Tg forms, distributed in the stacking gel, appeared after the treatment of Tg with 3 mM DTT in combination with 3 M urea (Fig. 1A). Based on this, 3 M urea/10 mM DTT was chosen for the preparation of partially unfolded/reduced Tg.

Oxidative multimerization of partially unfolded/ reduced Tg

When partially unfolded/reduced Tg was exposed to various disulfides (0.1 mM) as oxidants to non-enzymatic multimerization (Fig. 2), the oxidized DTT or oxidized lipoamide failed to promote a remarkable multimerization (lanes 3 & 4). Conversely, disulfides such as cystine methylester, cystamine or cystine, directly converted the monomeric form of partially unfolded/reduced Tg into multimeric forms (lanes 5, 6, & 7). In this comparison, cystine was much more effective than the oxidized glutathione in multimerization of the partially unfolded/ reduced Tg monomer, indicating a structural requirement of disulfide oxidants to interact with cysteine residue in the Tg molecule. Although GSSG, at 0.3 mM, exhibited a slight multimerization, its effect was negligible in the presence of 1 mM GSH. Thus, it is less likely that the GSH redox buffer at physiological concentrations may cause a remarkable multimerization of partially unfolded/ reduced Tg.

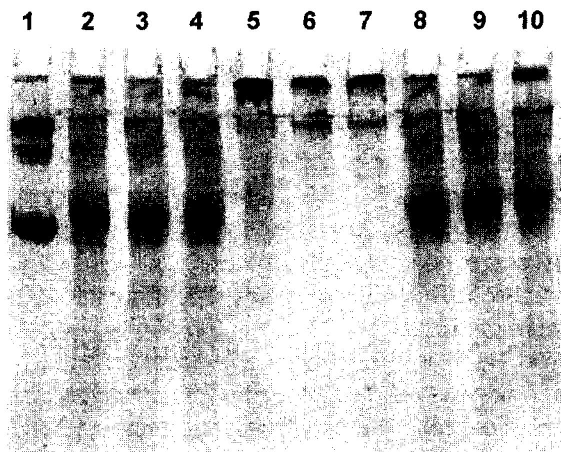

Fig. 2. Disulfide-induced multimerization of partially unfolded/reduced Tg. Partially unfolded/reduced Tg was incubated with each disulfide at 25°C for 30 min. Lane 1, native Tg; lane 2, partially unfolded/reduced Tg only; lanes 3 & 4, with 0.1 mM oxidized DTT and oxidized lipoamide, respectively; lanes 5-7, with 0.1 mM cystine, cystamine, and cystine methylester, respectively; lanes 8-10, with 0.03, 0.1, and 0.3 mM GSSG, respectively.

In the next study, the enzymatic multimerization of partially unfolded/reduced Tg was examined. Since PDI had been reported to be associated with unfolded/ reduced Tg (Nigam *et al.*, 1994), the oxidative function of PDI was examined. Fig. 3A demonstrates that in contrast to no remarkable formation of multimeric form by GSSG (30 μM) alone, a remarkable conversion of a monomer form to a multimeric one was accomplished by the inclusion of PDI (2 μM). The quantification of Tg forms after 30 min incubation indicated that the disappearance of monomer (330 kDa) form amounted to 90.4 ± 2.9 % (n=3), and the percentage of dimeric or multimeric forms was variable. Further, a dose-dependent effect of PDI on GSSG-mediated multimerization of partially unfolded/ reduced Tg (2 μM) was examined. As shown in Figure 3A, PDI at 0.2 μM had a slight effect on multimerization of partially unfolded/reduced Tg monomer, while more than one third of the Tg monomer was converted to its multimeric form in the presence of PDI at 0.6 μM. A maximal multimerization was achieved with 2 μM PDI, suggesting a stoichiometric interaction (molar ratio of PDI/Tg monomer =1.0) between PDI and partially unfolded/reduced Tg. In a

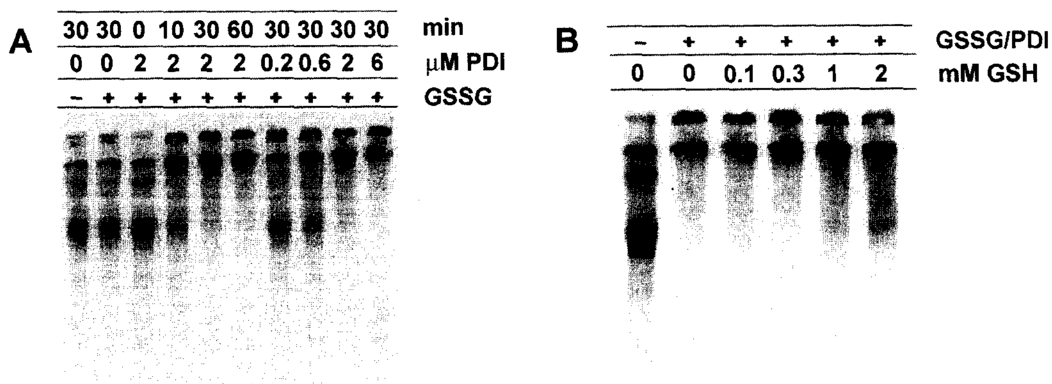

Fig. 3. GSSG/PDI-induced multimerization of partially unfolded/reduced Tg. (A). Partially unfolded/reduced Tg (0.66 mg/ml) was incubated with GSSG (30 mM) and PDI at 25°C for 30 min as indicated. (B). Partially unfolded/reduced Tg (0.66 mg/mL) was incubated with GSSG (30 mM) and PDI (2 mM) in the presence of GSH as indicated.

related study, where the effect of the redox (GSH/GSSG) state on the GSSG/PDI-induced multimerization of Tg was examined (Fig. 3B), GSH at 1 and 2 μM decreased GSSG/PDI-induced Tg multimerization by 11.5±1.7% (n= 3) and 23.7±2.5% (n=3), respectively, compatible with the oxidase role of PDI. However, GSH at physiological concentrations (1 or 2 mM) failed to decrease GSSG (300 mM)/PDI-induced Tg multimerization. To see whether oxidized PDI is directly involved in the oxidative folding of unfolded/reduced Tg, we examined the multimerization of partially unfolded/reduced Tg by PDI; although PDI at 2 μM showed no effect on multimerization of the monomeric Tg form even after 30 min, PDI at 6 μM exhibited a remarkable multimerization of the Tg monomer (>90%) after 5 min (Fig. 4A, lane 4). A quite similar result was observed when PDI, preoxidized with 100 μM GSSG, was employed, confirming that PDI, used in this experiment, was mainly in oxidized form. Independently, Tg was pretreated with 0.3% deoxycholate and 10 mM DTT, and the mixture then, after gel filtration on the G-25 sephadex, was subjected to PDI-induced multimerization; the pattern for the conversion of deoxycholate-treated/reduced Tg to its multimeric form was similar to that of the partially unfolded/reduced Tg, although to a lower extent (Fig. 4B).

Spontaneous multimerization of partially unfolded /reduced Tg *via* thiol-disulfide exchange

Since spontaneous conversion of partially unfolded/ reduced Tg to multimeric form was suspected during the incubation of native Tg with 3 mM DTT/3 M urea (Fig. 1A), it was assumed that the thiol-disulfide exchange, directly led to the intermolecular disulfide formation of the Tg molecule. In this respect, Tg was pretreated with lower concentrations (0.1-1.0 mM) of DTT in combination with 3 M urea, and then resulting Tg forms, after gel filtration on the G-25 sephadex, were analyzed by SDS-PAGE. Fig. 1B indicated that DTT (0.1-1.0 mM), in combination with 3 M urea, caused a spontaneous Tg multimerization in a concentration-dependent manner; the inclusion of 0.1 mM DTT converted monomer form to dimeric or multimeric form, whereas the multimeric form was predominant with 0.3 or 1 mM DTT. This suggested a sequential conversion of a monomeric form to a multimeric form *via* a dimeric one. A similar multimerization was also observed when DTT was substituted with GSH, an endogenous thiol, although GSH was less efficient. In further study, we turned to urea/GSH-induced multimerization of Tg. To this end, where Tg was simultaneously incubated with 3M urea and GSH for 30 min, and then the mixture, after 24-fold dilution, was directly subjected to SDS-PAGE analysis. In the presence of 3 M urea and GSH, the monomeric Tg form was converted to dimeric or multimeric forms (Fig. 5). Despite the multimeric form was one of major products in incubation with 3 M urea/1 mM GSH, the dimeric form seemed to be more predominant with 3 M urea/0.3 mM

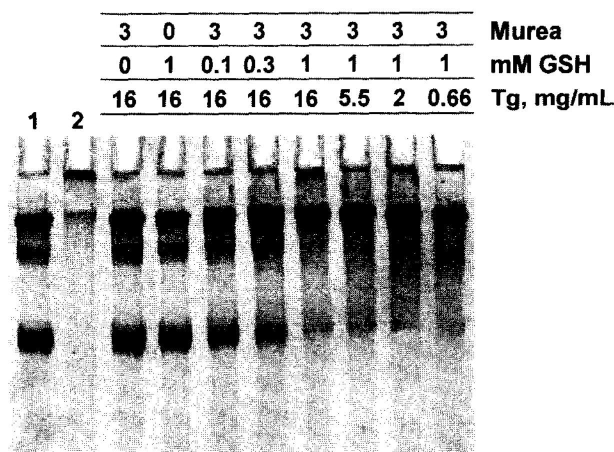

Fig. 5. Urea/GSH-induced multimerization of Tg. Native bovine Tg (16 mg/mL) was incubated with urea and/or GSH at 38°C for 30 min, and then the aliquot, after 24-fold dilution, was subjected to SDS-PAGE analysis. Lane 1, native Tg; lane 2, native Tg multimer.

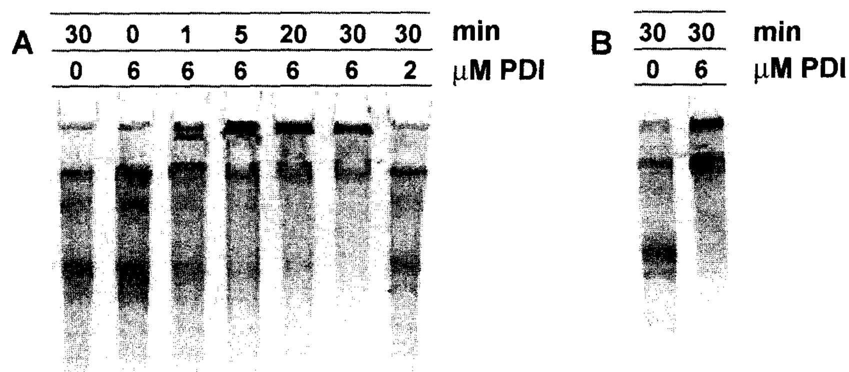

Fig. 4. PDI-induced multimerization of partially unfolded/reduced Tg or deoxycholate-treated/reduced Tg. (A), Partially unfolded/reduced Tg (0.66 mg/mL) was incubated with PDI for various times at 25°C as indicated. (B), Deoxycholate-treated/reduced Tg (0.66 mg/mL) was incubated with PDI.

Table I. Determination of reduced cysteine residues in Tg pretreated with urea/GSH or deoxycholate/GSH. Native bovine Tg (16 mg/mL) was incubated with 3 M urea/1 mM GSH, 8 M urea/1 mM GSH, or 0.3% deoxycholate/1 mM GSH in 1 mL of 50 mM Tris-HCl (pH 7.6) at 38°C for 30 min, and then the mixture, after gel filtration on Sephades G 25, was subjected to the thiol determination according to DTNB method (31)

Treatment	Amount of thiol residue (moles / Tg monomer mole)
3 M urea / GSH	3.9 ± 0.1
8 M urea / GSH	6.4 ± 0.2
Deoxycholate / GSH	1.9 ± 0.2

GSH (lane 6). Separately, when the concentration of urea was varied from 1 to 8 M in the presence of 1 mM GSH, it was found (Table I) that an effective multimerization was achievable with 3 M or 8 M urea, but neither 1 nor 2 M urea (unpublished data), suggesting that at least a partial unfolding of Tg, achievable with at least 3 M of urea, is a prior requirement for the effective multimerization. When the amount of free thiol, generated in the Tg molecule treated with urea and GSH, was determined by DTNB method (Ellman, 1959), it was found that the treatment with 3 M and 8 M urea, in combination with 1 mM GSH, generated approximately 4 and 7 thiol cysteine residues, respectively, per Tg monomer. In contrast to the 2 thiol cysteine residues that were generated in Tg treated with 0.3% deoxycholate, in combination with 1 mM GSH, which failed to cause the unfolding of protein. These results led us to surmise that the treatment with 3 M urea may result in the exposure of some cystine residues, hidden in a specific area of the Tg molecule, to the thiol disulfide exchange with GSH.

Thiol-induced multimerization of partially unfolded Tg or Tg fragment *via* thiol-disulfide exchange

To test this assumption, we employed a sequential process of Tg multimerization; Tg (16 mg/mL) was first exposed to 3 M urea to prepare partially unfolded Tg, and then, the partially unfolded Tg, after 24-fold dilution, was further incubated with various concentrations of GSH for 30 min at 38°C. As shown in Fig. 6A, a concentration-dependent effect of GSH on the conversion of partially unfolded Tg monomer to the multimeric forms *via* the dimeric form was observed. To determine whether the partially unfolded Tg is recognized by PDI, we examined the effect of PDI on GSH-induced conversion of partially unfolded Tg. Noteworthy, at a molar ratio of PDI/Tg monomer of 1.0, PDI enhanced the GSH-induced Tg multimerization at each GSH concentration. However, GSH/PDI-induced multimerization of partially unfolded Tg was irrespective of GSSG up to 0.3 mM. In contrast, the enhancing effect of PDI seemed to be less pronounced in the presence of 2 mM GSH, implying that the PDI-mediated multimerization may be overwhelmed by GSH-mediated self-assisted multimerization. These results led to the assumption that the Tg molecule itself may contain a region, which can mimic the thiol-disulfide exchange activity of PDI. In the subsequent experiment, it was tested whether detergents can replace urea in the GSH-induced Tg multimerization according to a sequential process. First, Tg (16 mg/mL) was incubated with 0.3% deoxycholate for 30 min, and the mixture, after 24-fold dilution, was exposed to GSH. As described in Fig. 6B, the deoxycholate-pretreated Tg monomer, after exposure to GSH (0.3 or 1 mM), was partially converted to dimeric form, and PDI increased GSH-induced dimerization at each concentration of GSH (Fig. 6B). Taken together, it is assumed that a Tg region, sensitive to thiol-disulfide exchange, may also be at least partially surface-exposed in the presence of deoxycholate. In an attempt to support the above possibility, a cysteine-rich fragment of Tg, a 50 kDa fragment (residues 1281-1591) containing 16 cysteine residues in addition to three thioredoxin boxes (Fig. 7A,

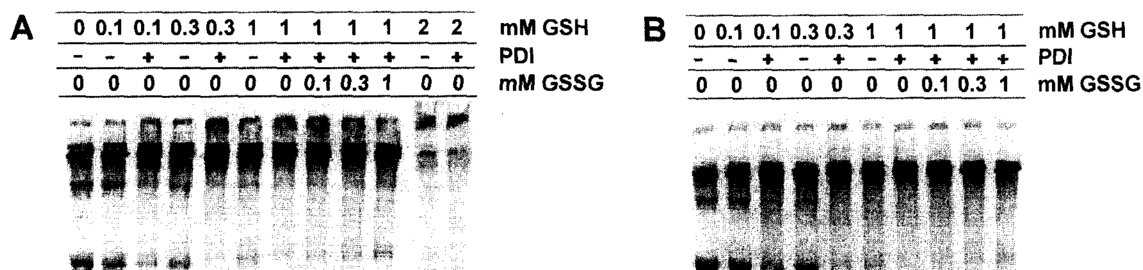

Fig. 6. GSH-induced multimerization of partially unfolded Tg or deoxycholate-treated Tg. (A), Partially unfolded Tg (0.66 mg/mL) was incubated with GSH of various concentrations in the presence or absence of PDI (2 mM) at 38°C for 30 min as indicated. Separately, GSH (1 mM)/PDI (2 mM)-induced multimerization was examined in the presence of GSSG as indicated. (B), Deoxycholate-treated Tg (0.66 mg/mL) was incubated with GSH in the presence or absence of PDI (2 mM). Separately, GSH (1 mM)/PDI (2 mM)-induced multimerization was examined in the presence of GSSG.

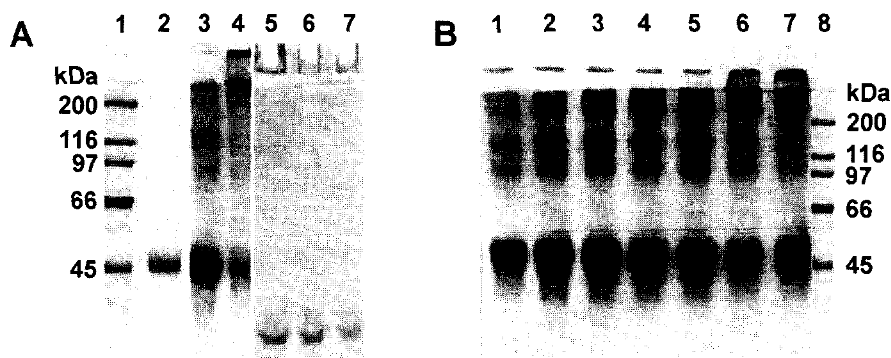

Fig. 7. Multimerization of Tg fragments after exposure to urea and GSH. (A), Each Tg fragment (0.6 mg/mL) was incubated with urea and/or GSH at 38°C for 30 min. Lane 1, marker proteins; lane 2, purified 50 kDa; lane 3, incubation of 50 kDa with 3 M urea; lane 4, 50 kDa with 3 M urea/0.3 mM GSH; lane 5, NTD with 3 M urea; lanes 6 & 7, NTD with 0.3 and 1.0 mM GSH, respectively, in combination with 3 M urea. (B), Time-dependent multimerization of 50 kDa (1.0 mg/mL) during incubation with 3 M urea/0.3 mM GSH. Lane 1, 50 kDa in 3 M urea; lanes 2-7, incubation with 3 M urea/0.3 mM GSH for 0, 1, 5, 10, 20, and 30 min, respectively; lane 8, marker proteins. SDS-PAGE analysis was done using 10% acrylamide gel, except analysis of NTD fragment using 15% acrylamide gel.

lanes 2 & 3), and *N*-terminal domain (24 kDa), a highly cysteine residue-conserved region (lane 5) were prepared, and subjected to GSH-induced multimerization in the same way as was done for the GSH-mediated multimerization of partially unfolded Tg. Fig. 7A (lane 4) shows that the 50 kDa fragment, dissolved in 3 M urea, was multimerized after 30 min incubation with 0.3 mM GSH, and moreover, a time-dependent multimerization was found during the exposure of 50 kDa (1 mg/mL) to 0.3 mM GSH (Fig. 7B), while the NTD fragment was not (Fig. 7A, lanes 6, & 7).

DISCUSSION

Concerning the formation of Tg multimers through disulfide linkage in ER, nascent Tg was detected as transient aggregates, linked by disulfide bonds (kim and arvan, 1995; Ellman, 1959; Kuznetsov *et al.*, 1997). Despite many reports (Freedman *et al.*, 1994; Gilbert, 1997) concerning the role of PDI in protein folding, there has been no reconstitution study to explore PDI-promoted multimerization of Tg molecules.

Oxidative multimerization of partially unfolded/reduced Tg

Present study reveals a high susceptibility of partially unfolded/reduced Tg to disulfide oxidants. The difference in the efficiency among disulfides might be ascribed to the different affinity of disulfides with unfolded/reduced Tg molecule, rather than the oxidizing potential of disulfides, which was important for the multimerization of a small model peptide (Darby *et al.*, 1994). Thus, the initial step, thionylation of cysteine residue in Tg molecule, seems to govern the efficiency of disulfides in the non-enzymatic multimerization of Tg. However, non-enzymatic multimerization of partially unfolded/reduced Tg through the transfer of oxidzing equivalents from GSSG is not likely in the redox state of ER, since no remarkable multimerization was observed in the glutathione redox condition of ER. Instead, the role of PDI in GSSG-mediated multimerization of partially unfolded/reduced Tg is suggested from the catalytic role of PDI in the initial glutathionylation (Ruoppolo *et al.*, 1996). Conversely, since PDI, used in this experiment, is in a predominantly oxidized form, it is more likely that the oxidative activity of PDI is directly involved in the intermolecular disulfide formation. Moreover, the stoichiometry (1:1) of PDI/Tg monomer molar ratio, required for the effective multimerization by a GSSG/PDI system, may support a possible role of PDI as a donor of disulfide bond, assisted by the oxidant GSSG. The encouraging evidence for the immediate relay of oxidizing equivalents from PDI to partially unfolded/reduced Tg may come from the result that PDI alone showed a rapid Tg multimerization at a PDI/Tg monomer molar ratio of 3.0. The same phenomenon was also observed with PDI-induced multimerization of deoxycholate-pretreated/reduced Tg, where a limited region of Tg is exposed to the reduction. Therefore, the transfer of oxidizing capacity from PDI might occur at a specific site in Tg, recognized by PDI, which could commonly be disclosed by the treatment with urea or deoxycholate. Moreover, the physiological role of PDI in Tg multimerization may be well supported by the observation that PDI-mediated multimerization was not diminished in the glutathione redox condition of ER, despite the counteracting effect of GSH on PDI oxidation. Although the necessity of GSSG in the oxidative folding of proteins was negated in UPR stress (Cuozzo and Kaiser, 1999), where a large amount of Ero1p b was induced (Cuozzo and Kaiser, 1999; Pagani *et al.*, 2000), it is not ruled out that GSSG in high concentrations may contri-

bute to an increase in the oxidative capacity of PDI in the very least by causing the formation of protein-GSH mixed type disulfide (PSSG), a predominant oxidant (Bass *et al.*, 2004). In the ER condition containing a low level of Ero1p PDI/GSSG systems, compared to PDI/Ero1p, it may be more responsible for the formation of mismatched aggregates present in nascent Tg (Kim *et al.*, 1993) or GSH-mixed disulfide in incompletely folded Tg. Considering a high concentration of PDI, predominantly oxidized (Pagani *et al.*, 2000), in an ER system (Gilbert, 1990), the primary oxidation by excess PDI may lead to a rapid formation of Tg mutimers, followed by a slow reshuffling process by PDI in net reducing redox state of ER as had been observed in the exposure of thyrocytes to DTT (Kim and Arvan, 1995).

Spontaneous multimerization of partially unfolded /reduced Tg *via* thiol-disulfide exchange

The spontaneous conversion of Tg monomer to multimeric form during the simultaneous exposure to 3 M urea and thiols at low concentrations (0.3-1.0 mM) is noteworthy. It may be in line with the participation of an activity other than the oxidase function of PDI. The urea/thiols-induced multimerization of the Tg molecule is characterized by at least a partial unfolding by urea (3 M), which can lead to an exposure of the hidden region to the surface. Another requirement is a mild reduction condition as suggested from the presence of reduced cysteine residue, corresponding to approximately 4 reduced cysteine residues, present in the Tg molecule treated with 3 M urea and 1 mM GSH. Since most of cysteinyl residues in native Tg molecules are involved in intra-chain disulfide bonds (De Crombrugghe *et al.*, 1966), the formation of an intermolecular disulfide bond may reflect an attack of extraneous thiol at surface-exposed disulfide bonds in a Tg molecule. This observation might point to a function of the Tg molecule itself in the self-assisted formation of intermolecular disulfide linkage *via* thiol-disulfide exchange.

Thiol-induced multimerization of partially unfolded Tg or Tg fragment *via* thiol-disulfide exchange

Since GSH-induced multimerization of partially unfolded Tg was not remarkably interfered in the presence of GSSG at physiological concentrations, the multimerization of partially unfolded Tg monomers through thiol-disulfide exchange could proceed in the ER system. Furthermore, the employment of a sequential process to uncouple unfolding of the Tg molecule and intermolecular disulfide linkage of unfolded Tg divulged that once unfolded, it might expose the disulfide bond in a specific region, susceptible to thiol-disulfide exchange. Probably consistent with this, PDI expressing an isomerase activity in relatively reducing redox buffer (Freedman *et al.*, 1994; Gilbert, 1989), further enhanced the GSH-induced multimerization of partially unfolded Tg. The molar ratio of PDI/Tg of 1.0, required for the effective multimerization, suggests a selective recognition of partially unfolded Tg by PDI in reducing condition. Different susceptibility to GSH-induced multimerization between extensively unfolded Tg and partially unfolded Tg may be explained by the notion that the amount of surface-exposed disulfide bonds, exchangeable with GSH, may differ according to the unfolding condition as manifested by the different thiol levels in urea-treated Tg (Table I). This, likewise, might explain the different magnitude of multimerization between 3 M urea-pretreated Tg and deoxycholate-pretreated Tg. Although the specific region responsible for the Tg multimerization was not identified here, the cysteine-rich region, susceptible to thiol-disulfide exchange, might be involved in the facilitated multimerizatin as demonstrated from the observation that 50 kDa fragment containing three thioredoxin boxes. This view might be supported by an earlier report (Klein *et al.*, 2000) stating that a human recombinant Tg fragment containing three thioredoxin boxes had been reported to exhibit a redox activity corresponding to PDI activity, although to a small extent.

In conclusion, PDI participates in the multimerization of unfolded Tg molecules, as oxidase or isomerase. Either the interaction between unfolded/reduced Tg and GSSG, or the attack of GSH on partially unfolded Tg, might result in the formation of PSSG, an intermediate, in Tg molecules. In this respect, the multimerization of unfolded/reduced Tg by excess PDI will be determined by the availability of the PSSG in the ER system. The fate of the Tg molecules multimeric intermediates remains to be defined in future research.

ABBREVIATIONS

Tg: thyroglobulin; PDI: protein disulfide isomerase; GSSG: oxidized glutathione; GSH: reduced glutathione; PSSG: protein-glutathione mixed disulfide; NTD: *N*-terminal domain; DTT: dithiothreitol; ER, endoplasmic reticulum: DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).

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