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# A Role of PDI in the Reductive Cleavage of Mixed Disulfides

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**We previously reported that protein disulfide isomerase (PDI) can dissociate the glutathione molecule *in vitro* from the mutant human lysozyme (hLZM) C77A-a, which is modified with glutathione at Cys95; however, it seems structurally difficult for PDI to attack either the disulfide bond or the side chain of the cysteine residue of a mixed disulfide. To investigate the function of PDI, we introduced several glutathione and cysteine derivatives at Cys95, instead of the glutathione of C77A-a. Using thiol compounds modified by 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), we could easily modify the free thiol group of C77A-b (C77A with no glutathionylation), without denaturation. For all of the modifications we tested, a negative correlation was found between the initial rate and the acceleration ratio of the reductive cleavage of mixed disulfides with PDI. A mutant PDI (hPDIM), which has no thiol-disulfide exchange activity, suppressed the reductive cleavage of the mixed disulfide of C77A-a with hPDI, suggesting that hPDI non-covalently interacted with the substrates. Taking account of the results of the structural analysis, we conclude that one of the functions of PDI *in vivo* lies in relaxing the structure around the disulfide bond, as well as in exchanging the thiol-disulfide bonds.**

**Key words: glutathione, human lysozyme, protein disulfide isomerase, protein folding, reductive cleavage of disulfide bond.**

Protein disulfide isomerase (PDI), one of the most abundant proteins in the lumen of the endoplasmic reticulum (ER), was first isolated from liver microsomes more than 30 years ago (*1*) and has been found in most vertebrate tissues and in several plants. PDI was purified as a dimeric form, consisting of subunits with a molecular weight of 57,000 (*2*). PDI shows broad substrate specificity and catalyzes disulfide bond formation in many proteins, including multi-domain and multi-subunit proteins, from reduced or scrambled forms. Based on a study of PDI-depleted microsomes (*3*), it was suggested that PDI was involved in the formation of higher-order protein structure, perhaps by catalyzing disulfide-bond formation in the nascent protein in the ER. PDI is also the $\beta$-subunit of prolyl 4-hydroxylase (*4*), and is a component of the microsomal triglyceride transfer complex (*5*). PDI also has thyroid hormone binding affinity (*6, 7*) and catalytic activity in the GSH-dependent reduction of dehydro-L-ascorbate (*8*).

PDI is a member of the thioredoxin superfamily, and has two thioredoxin-like sequences. One cysteine residue of each sequence (WCGHCK) has an unusually high reactivity and a lower p$K_a$ value than normal (*9-11*). It was suggested that these sequences are the PDI active sites that catalyze the thiol-disulfide exchange reaction. These two active sites act independently (*12*). From studies using protein and peptide inhibitors of the glutathione-insulin transhydrogenase activity of PDI, it appears that PDI recognizes either a disulfide bond or the side chain of a cysteine residue (*13*).

Human lysozyme (hLZM) has four disulfide bonds: Cys6-Cys128, Cys30-Cys116, Cys65-Cys81, and Cys77-Cys95. The mutant hLZM C77A, in which Cys77 is replaced by alanine, is secreted by *Saccharomyces cerevisiae* as two forms (*14*). One (C77A-a) contains a mixed disulfide with glutathione attached to Cys95, and the other (C77A-b) has a free Cys95. Glutathionylation is thought to occur during folding (*14*). Structure analysis of C77A-a (*15, 16*) and studies of oxidizing environment in the ER with glutathione disulfide (*17*) suggested that this glutathionylated protein (C77A-a) mimics the folding intermediate just prior to the formation of the disulfide bond Cys77-Cys95. PDI can remove the glutathione molecule of the glutathionylated protein *in vitro* more efficiently than thioredoxin (*15*). The structure of C77A-a was confirmed by X-ray crystal structure analysis, which indicated that the sulfur atoms within the disulfide bond between Cys95 and the attached glutathione have no solvent-accessible surface area (*15, 16*). The structure around Cys95 and the attached

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Abbreviations: $\alpha$-Glu-Cys-Gly, $\alpha$-glutamyl-L-cysteinylglycine; C77A, the mutant of hLZM in which Cys77 is replaced by alanine; C77A-a(GS-C77A), C77A derivative that has a glutathionylated Cys95; C77A-b, C77A derivative that has a free Cys95; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); ER, endoplasmic reticulum; $\gamma$-Glu-Cys, $\gamma$-L-glutamyl-L-cysteine; GSH, glutathione; GSSG, glutathione disulfide; hLZM, human lysozyme; PDI, protein disulfide isomerase; hPDI, human PDI; hPDIM, a mutant of hPDI; TNB, 2-nitro-5-mercaptobenzoic acid.

glutathione is fairly rigid. Thus, PDI was considered to be unable either to access or to recognize the disulfide bond or sulfur atoms directly. To catalyze the reductive cleavage of this disulfide bond, PDI must recognize some structure of glutathione and relax the structure around it. To investigate the function of this reaction, we introduced several glutathione and cysteine derivatives at Cys95 of C77A-b, and studied the reductive cleavage of the disulfide bonds between the additive compounds and Cys95 of C77A.

Here we show that the activity of PDI for the reductive cleavage of a disulfide bond is not strong, and its main role of PDI may lie in its function to relax the structure around the mixed disulfide bond. Furthermore, we report that hPDIM, the mutant of hPDI containing two WSGHCK sequences instead of WCGHCK, and which shows no thiol-disulfide exchange activity (*12, 18*), non-covalently interacts with its substrates, because hPDIM inhibited the reductive cleavage of disulfide bonds, either with or without hPDI.

## MATERIALS AND METHODS

*Materials*—Bovine PDI was purified to homogeneity according to the published method (*19*). hPDI and the mutant hPDI (hPDIM), harboring two WSGHCK sequences instead of the native WCGHCK sequences, were kindly provided by Dr. T. Hayano. Glutathione (GSH), glutathione disulfide (GSSG), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), L-cysteine (Cys), and acetic anhydride were purchased from Wako Pure Chemical Industries (Osaka). L-Cysteinylglycine (Cys-Gly), carboxypeptidase Y, and *Micrococcus lysodeikticus* cells were from Sigma Chemical (St. Louis, USA). The CM-Toyopearl 650C column and the TSK-gel ODS-120T column for reverse-phase HPLC were obtained from Tosoh (Tokyo), and the Asahipak ES-502C column was from Asahi Kasei (Tokyo). All other chemicals were of reagent quality. The yeast *Saccharomyces cerevisiae* AH22R$^-$ (MAT$\alpha$ *leu2 his4 can1 pho80*) (*20*) was used as the host strain. The mutant hLZM (C77A) expression plasmid, in which Cys77 is replaced by Ala, was previously described (*14*). Modified Burkholder minimal medium (*21*), supplemented with 8% sucrose, was used for yeast growth. The secreted C77A was purified as separate forms, C77A-a and C77A-b, with a CM-Toyopearl 650C column and an Asahipak ES-502C cation-exchange column, as described (*14*).

*Synthesis of Peptides*—Glycyl-L-cysteinyl-glycine (Gly-Cys-Gly) was synthesized using an Applied Biosystems 430A Peptide Synthesizer.

*Preparation of TNB-Activated Compounds*—GSH, Gly-Cys-Gly, Cys-Gly, and Cys were modified by Ellman's reagent (DTNB) according to the published method (*22*). Each compound (2.7 mM) was incubated with DTNB (5.3 mM) in 100 mM Tris-HCl (pH 8.0) and 10 mM EDTA at room temperature. The products were purified by reverse-phase HPLC. $\gamma$-Glutamyl-L-cysteine-TNB ($\gamma$-Glu-Cys-TNB) was synthesized from GSH. After activation by TNB, the carboxyl terminal glycine of glutathione-TNB (GS-TNB) was removed by carboxypeptidase Y. $\gamma$-Glu-Cys-TNB was purified by reverse-phase HPLC. *N*-Acetyl-$\gamma$-Glu-Cys-Gly-TNB was synthesized from GS-TNB. The amino group of GS-TNB was acetylated by acetic anhydride in 0.3 M phosphate buffer (pH 8.0) on ice. The resulting *N*-acetyl-$\gamma$-Glu-Cys-Gly-TNB was purified by reverse-phase HPLC. Structures were confirmed by NMR and Fab-mass spectroscopy.

*Addition of TNB-Activated Thiol Compounds to the Mutant hLZM C77A-b*—C77A-b was reacted with TNB-compounds instead of DTNB as described (*22*). The C77A proteins possessing mixed disulfides with various compounds were purified by HPLC (Asahipak ES-502C, Asahi Chemical) as described previously (*14*).

*Reductive Cleavage of Mixed Disulfides*—PDI-mediated conversions of mixed disulfides between various compounds and C77A to C77A-b (reductive cleavage) were analyzed according to the published method (*15*). Purified mixed disulfides (0.35 $\mu$M) were incubated at 37°C for an appropriate time, either with or without an equimolar amount of purified bovine PDI, hPDI, or hPDIM, in GSH/GSSG reaction buffer, containing 100 mM sodium phosphate (pH 7.5), 2 mM GSH, and 0.2 mM GSSG. The products of these reactions were analyzed by reverse-phase HPLC on a TSK-gel ODS-120T column (4.6 mm i.d.×25 cm) equilibrated with 15% acetonitrile, and were eluted at a flow rate of 1 ml/min with a linear gradient of acetonitrile (15-55%) containing 0.1% trifluoroacetic acid for 45 min.

*X-Ray Crystal Structure Analyses of Cys-C77A*—Crystallization of Cys-C77A was carried out using a vapor diffusion technique, as described previously (*23*). To obtain a crystal of Cys-C77A, the protein solution, containing 20.0 mg protein/ml, 1.8 M NaCl, and 30 mM sodium phosphate (pH 4.0), was diffused against a solution of 2.0 M NaCl and 30 mM sodium phosphate at 4°C. The crystal grew to a size large enough for X-ray diffraction experiments within a week. The space groups were determined to be $P2_12_12_1$, and the unit cell parameters were $a=57.8$ Å, $b=61.0$ Å, $c=32.7$ Å. The intensity data collection was carried out by an automated oscillation camera system, DIP-320 (MAC Science), equipped with a cylindrical imaging plate detector (*24, 25*) on a Cu rotating anode generator operated at 50 kV, 90 mA at 4°C. The diffraction intensities were recorded on the area detector, evaluated by the program WELMS (*26*), and processed by the program PROTEIN (*27*). The structure refinement was carried out using the program package X-PLOR, version 3.0 (*28*). The resolution for Cys-C77A was 2.2 Å.

*Assay of Lysozyme Activity*—The lytic activities of hLZM and the modified C77A were measured as described previously (*29*). The lysis of *M. lysodeikticus* cells (0.2 mg/ml) in 0.1 M potassium phosphate buffer (pH 6.2) at 25°C was monitored at 450 nm. Activities were normalized by assuming the activity of wild-type hLZM to be 100%.

## RESULTS

*Modification of the Free Thiol Group of C77A-b by TNB-Activated Thiol Compounds*—Before we attempted to modify the free thiol group of C77A-b, which has only one free thiol group (Cys95), with several compounds, we first tested its modification by DTNB. As shown in Fig. 1a, this thiol group was modified by DTNB without any denaturant, although with lower efficiency than in the presence of a denaturant. The free thiol group of C77A-b was also modified with the mixed disulfide between glutathione and TNB (GS-TNB) without any denaturation, and the glutathione was transferred to the Cys95 of C77A-b

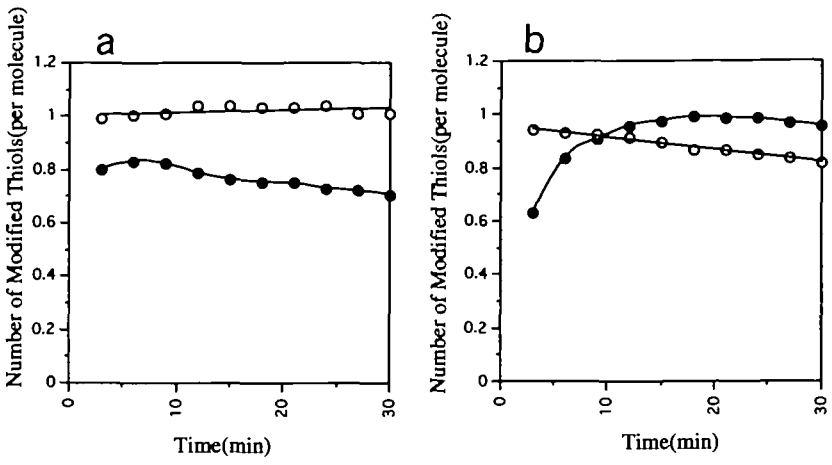


Fig. 1. **Modification of the free thiol group in C77A-b.** The free thiol group of C77A-b (0.01 mM) was modified by 0.5 mM Ellman's reagent (DTNB) (a) and by 0.5 mM disulfide between GSH and TNB (GS-TNB) (b) in 0.01 M EDTA and 0.1 M Tris-HCl (pH 8.0), with (○) or without (●) 8 M urea as a denaturant. The concentration of TNB anion was determined from the molar absorption coefficient value of 14,000 $M^{-1} \cdot cm^{-1}$ at 412 nm.

TABLE I. **Rate of reductive cleavage to C77A-b.** Mixed disulfides between various compounds and C77A-b were converted to C77A-b, by bovine PDI, as described in the "MATERIALS AND METHODS." The initial rate of the increase of C77A-b, with or without PDI, was used for calculation of the relative rate of conversion and the ratio of acceleration. The rates of conversion without PDI were subtracted from the rates of conversion with PDI, and were normalized by assuming the case of the mixed disulfide between glutathione and C77A-b as 100%.

| Mixed disulfide | Rate of reductive cleavage with PDI (nmol/min/μmol PDI) | Rate of reductive cleavage without PDI (nmol/min) | Relative rate of reductive cleavage (%) | Ratio of acceleration (with PDI/without PDI) |
|---|---|---|---|---|
| GS-C77A (C77A-a) | 21.9 | 1.23 | 100 | 17.9 |
| Gly-Cys-Gly-C77A | 35.0 | 2.02 | 159 | 17.3 |
| Cys-Gly-C77A | 51.5 | 3.62 | 231 | 14.2 |
| γ-Glu-Cys-C77A | 21.0 | 1.10 | 96 | 19.1 |
| Cys-C77A | 117.8 | 10.44 | 519 | 11.3 |
| α-Glu-Cys-Gly-C77A | 73.5 | 5.21 | 330 | 14.1 |

(Fig. 1b). The resultant protein (GS-C77A) which was purified using an Asahipak ES-502C cation-exchange HPLC column, had the same retention time on reverse-phase HPLC and the same lytic activity as that of the C77A-a secreted by yeast (data not shown). Other TNB-activated compounds such as Gly-Cys-Gly-TNB, Cys-Gly-TNB, γ-Glu-Cys-TNB, Cys-TNB, and α-Glu-Cys-Gly-TNB, also modified the free thiol group of C77A-b (data not shown), and the products were purified in the same manner. Products with mixed disulfides between various compounds and the Cys95 of C77A-b had 40–70% of the lytic activities of the wild type hLZM towards *M. lysodeikticus*, suggesting that subtle structural changes in the proteins were caused by the modifications. Other compounds larger than glutathione, such as *N*-acetyl-γ-Glu-Cys-Gly, could also be transferred to the Cys95 of C77A-b, but the resultant products had little lytic activity. This observation suggests that there were some deviations from the structure of native hLZM. Therefore, we did not study these compounds further.

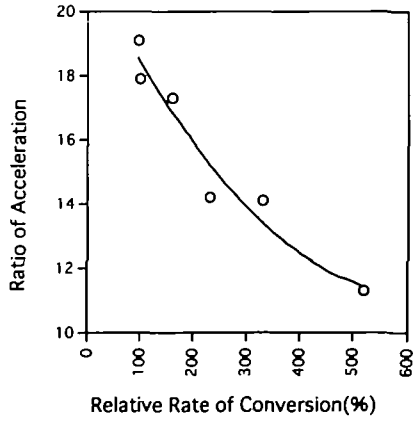


Fig. 2. **Relationship between the relative rate and the acceleration ratio of conversion to C77A-b.** The ratio of acceleration was plotted against the relative rate of conversion.

*Reductive Cleavage of Modified C77A Proteins*—The PDI-mediated reductive conversions of modified C77A proteins to C77A-b were analyzed, and the results are shown in Table I. Shortening of the glutamic acid moiety of the glutathione generally increased the conversion rate catalyzed by PDI. This result may be explained by the stability of glutathione in GS-C77A (C77A-a). The structure around glutathione in GS-C77A (C77A-a) is stabilized by several hydrogen bonds between the glutamic acid moiety of the glutathione and Arg98, Tyr93, and Trp64 of hLZM (*15*). Shortening of the glutamic acid moiety of glutathione or the replacement of glutathione with other additives causes loss of hydrogen bonds and a decrease in the stability of the additives. It appeared that the loss of the hydrogen bonds causes thermal drift of additives and that a low-molecular reductant, such as glutathione, can easily access the disulfide bond, because the conversion rate without PDI also increased when the glutamic acid moiety of the glutathione was shortened. The conversion rate of PDI with α-Glu-Cys-Gly-C77A was larger than that with GS-C77A (C77A-a). This may be the result of disorder of the hydrogen bonds in the glutamic acid moiety. The

acceleration ratio of the conversion decreased when the conversion rate catalyzed by PDI increased (Fig. 2). Mixed disulfides, such as γ-Glu-Cys-C77A, which showed relatively low rates of PDI-catalyzed conversion, exhibited higher acceleration value ratios. This may be because these disulfide bonds were structurally blocked from attack by a reductive agent such as glutathione, as in GS-C77A (*15, 16*), whereas PDI could relax the structure around them, thereby allowing the attack and the subsequent reduction. These results suggest that PDI has some additional role besides the reductive cleavage of mixed disulfides.

*The Crystal Structure of Cys-C77A*—We analyzed the crystal structure of Cys-C77A, which has the highest conversion rate. The final crystallographic *R*-factor was 0.18, the root mean-square deviations of the bonds were 0.013 Å, and those of the angles were 1.8°. The structure is shown in Fig. 3, for comparison with that of GS-C77A (C77A-a). The cysteine residue that binds with Cys95 of C77A in Cys-C77A is shifted to the outside of the molecule, as compared with the cysteine moiety of the glutathione in GS-C77A (C77A-a). This result suggests that the disulfide bond between cysteine and Cys95 of C77A in Cys-C77A is more exposed to the solvent than that of GS-C77A (C77A-a). We calculated the solvent-accessible surface area (ASA), using a solvent probe with a radius of 1.4 Å (the radius of a water molecule). As shown in Table II, the ASA of the Cα in Cys-C77A is much larger than that of GS-C77A (C77A-a), and its Cβ has a small ASA value, whereas that of GS-C77A (C77A-a) lacks a solvent-accessible surface. These results show that a reductive reagent such as glutathione could have partial access to the disulfide bond between the cysteine molecule and Cys95 of C77A in the case of Cys-C77A. From this observation, it is concluded that reductive cleavage of mixed disulfides occurs with glutathione when the disulfide bond is sufficiently exposed to the solvent region. The role of PDI may be to bind the protein and relax its structure to allow reductive reagents, such as glutathione, access to the target disulfides. There might not be a need to relax the structure around the mixed disulfide bond of Cys-C77A for reductive cleavage.

*The Role of hPDIM in the Reductive Cleavage of Cys-C77A and GS-C77A (C77A-a) by PDI*—We next investigated the function of hPDIM. In analyses of the conversion from Cys-C77A and GS-C77A (C77A-a) to C77A-b, using hPDI and hPDIM, hPDI and bovine PDI showed the same conversion rate. However, hPDIM, which has no thiol-disulfide exchange activity, suppressed the reductive cleavage of Cys-C77A with glutathione as the reducer (Fig. 4a), and suppressed the reductive cleavage of GS-C77A (C77A-a) with hPDI (Fig. 4b). These results suggest that

TABLE II. **Solvent-accessible surface area (ASA) of the atoms in the cysteine residues attached to Cys95.** The ASA values of the cysteine residue atoms attached to Cys95 of Cys-C77A were calculated by the program ASA (Mizuno, H. and Go, N., personal communication) using a solvent probe with a 1.4 Å radius. The ASA data of the wild-type hLZM and GS-C77A (C77A-a) were previously reported (*1*).

| Atom name | Solvent-accessible surface area (Å) | | |
|---|---|---|---|
| | Wild-type (Cys77) | GS-C77A (C77A-a) | Cys-C77A |
| Cys N | 0.00 | 0.00 | 5.74 |
| Cα | 4.73 | 1.58 | 23.68 |
| C | 0.00 | 0.00 | 0.00 |
| O | 12.67 | 0.00 | 30.61 |
| Cβ | 0.00 | 0.00 | 1.15 |
| Sγ | 0.00 | 0.00 | 0.00 |

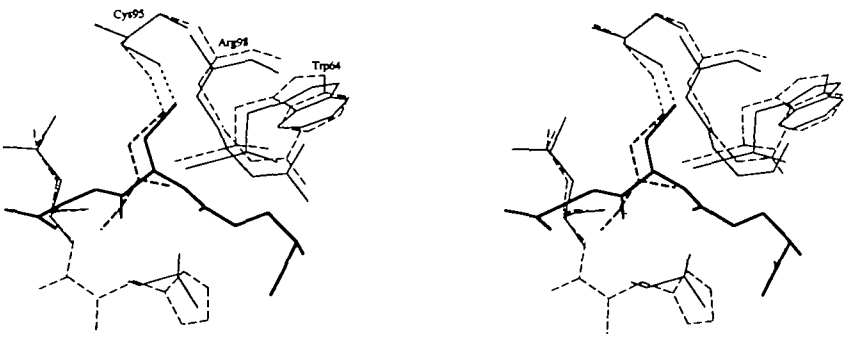


Fig. 3. **Crystal structure of Cys-C77A.** The structure around the Cys95 of Cys-C77A was compared with that of GS-C77A (C77A-a) (*1*). The solid line shows the structure of GS-C77A (C77A-a) and the dashed line shows the structure of Cys-C77A. Bold lines show glutathione (solid line) and cysteine (dashed line). Dotted lines show disulfide bonds between Cys95 of C77A and glutathione or cysteine.

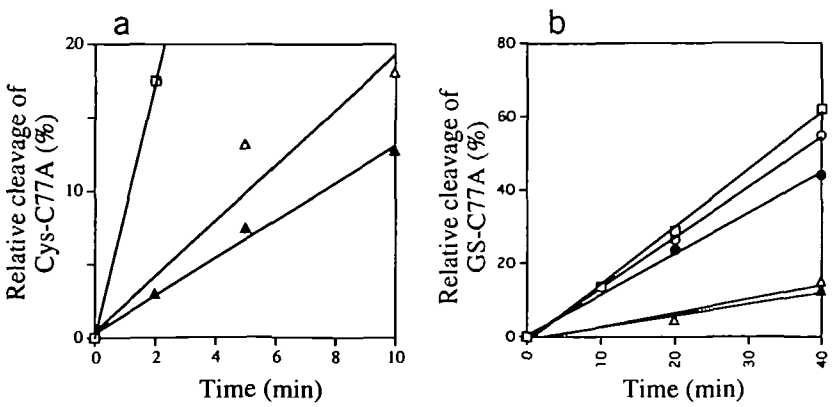


Fig. 4. **Reductive cleavage of Cys-C77A (a) and GS-C77A (C77A-a) (b) by hPDI and hPDIM.** (a) 0.36 μM Cys-C77A was incubated at 37°C in the GSH/GSSG reaction buffer containing 100 mM sodium phosphate (pH 7.5), 2 mM GSH, and 0.2 mM GSSG with either 0.36 μM hPDI (□), 0.36 μM hPDIM (▲), or without any PDI (△) for 0, 2, 5, or 10 min. (b) 0.36 μM GS-C77A (C77A-a) was incubated at 37°C in the same reaction buffer with either 0.36 μM hPDI (□), 0.36 μM hPDIM (▲), 0.36 μM hPDI and 0.36 μM hPDIM (○), 0.36 μM hPDI and 0.72 μM hPDIM (●), or without any PDI (△) for 0, 10, 20, or 40 min. The rate of conversion was estimated by reverse-phase HPLC.

hPDIM inhibits the PDI-catalyzed reduction by competitive binding at the same site. These observations also suggest that hPDIM non-covalently interacted with the target proteins, thereby preventing the access of both glutathione and hPDI to the target disulfide bond.

## DISCUSSION

To investigate the function of PDI, we introduced several TNB-activated thiol compounds to Cys95 of C77A-b. In such TNB-activated thiol compounds, a more stable thiolate anion is the preferred leaving group, so that attack of lysozyme thiol occurs predominantly on the alkyl sulfur of the mixed disulfide. The free thiol group (Cys95) of C77A-b could not be modified by iodoacetate without denaturation (*14*). As shown in Fig. 1a, Cys95 of C77A-b could easily be modified by DTNB or TNB-activated thiol compounds. The different results obtained using iodoacetate and DTNB could be explained by their different reactivities.

PDI is believed to recognize the disulfide bond and/or the side chain of a cysteine residue (*13*). However, our results seem inconsistent with this recognition mechanism for PDI. Although PDI could neither access the disulfide bond between glutathione and Cys95, nor contact the side chain of each cysteine residue of GS-C77A (C77A-a), PDI dissociated glutathione from C77A-a (*15*). How did PDI recognize and approach the disulfide bond? Our results suggest that PDI catalyzes the reductive cleavage of the mixed disulfide of GS-C77A (C77A-a) by relaxing the structure around the disulfide bond. This chaperone-like function was observed only for PDI, and not for thioredoxin (*15*).

PDI assists in the refolding of D-glyceraldehyde-3-phosphate dehydrogenase, a protein lacking disulfide bonds, to its active state (*30*), and the chaperone activity reported for PDI is independent of its disulfide-isomerase activity (*31*). From our results, the oxidative formation and the reductive cleavage of disulfide bonds would also occur in non-enzymatic and chemical reactions when reagents such as glutathione are present. The role of PDI *in vivo* may lie in the function to change the protein structure or to keep it relaxed when the disulfide bond is formed through the mixed disulfide between glutathione and the nascent polypeptide. We found that hPDIM, the mutant of hPDI, suppressed the reductive cleavage by glutathione of the disulfide bond between the cysteine and Cys95 in Cys-C77A. This effect is not due either to a decrease of the product C77A-b by disulfide bonding between C77A-b and the remaining cysteine residues of hPDIM, or to the consumption of glutathione by the remaining cysteine residues of hPDIM, because the total recovery of Cys-C77A and C77A-b was much the same, and the molar ratio of GSH to hPDIM was 2 mM/0.36 mM. Furthermore, no heterodimer between C77A-b and hPDIM was detected (data not shown) although a disulfide-bonded complex between PDI and a substrate protein was reported (*32*). This effect of hPDIM was also observed in the reductive cleavage of GS-C77A (C77A-a). These observations suggest that hPDIM inhibits the PDI-catalyzed reduction of GS-C77A (C77A-a) by non-covalently binding at the same site. The role of PDI is also expanded, in that PDI binding will render the disulfide bond susceptible to reaction with PDI itself. This interaction between hPDIM and Cys-C77A or GS-C77A (C77A-a) might be ionic, rather than hydrophobic, because Cys-C77A and GS-C77A (C77A-a) have an almost native-like structure (*16*).

GS-C77A (C77A-a) seems to mimic a folding intermediate (*15, 16*). Glutathione exists in the ER at millimolar concentrations and maintains the relatively oxidative condition of the luminal environment (*17*). In view of these findings, it seems likely that the free thiol groups of the nascent polypeptides are modified by glutathione after translocation to the ER, and then PDI catalyzes native disulfide bond formation using these mixed disulfides as substrates. The mixed disulfides between the glutathione and the nascent polypeptides would have some distinct structural conformation. PDI could recognize such mixed disulfides (*15*), possibly through the glutathione, and then the conformational change would be introduced. hPDIM, which lacks the thiol-disulfide exchange activity (*12, 18*), non-covalently interacted with the mixed disulfide of GS-C77A (C77A-a), and did not accelerate, but instead suppressed, the dissociation of glutathione from GS-C77A (C77A-a). Non-covalent interaction between PDI and a tripeptide affinity probe which has no cysteine residue was described, and the peptide appeared to bind at a site other than putative PDI active sites (*33*). From these observations, the following may be deduced. PDI non-covalently interacts with the protein to change its conformation. This conformational change is necessary to dissociate the glutathione molecule bound to the cysteine residue of the folding intermediate and to form a disulfide bond through the thiol-disulfide exchange activity of PDI. hPDIM has no thiol-disulfide exchange activity, but would change the conformation of the protein as PDI does. The disulfide bond of Cys-C77A protrudes towards the solvent. Nevertheless, the approach of both PDI and glutathione to the disulfide bond was interfered with by hPDIM, and the dissociation of cysteine from Cys-C77A was inhibited. hPDIM did not assist in the dissociation of other glutathione derivatives, even though its recognition ability and chaperone-like function are normal.

There are several possible reasons why hPDIM suppresses the dissociation of glutathione from GS-C77A. First, hPDIM would covalently interact with mixed disulfide of GS-C77A and interfere with the approach of GSH or PDI to the disulfide bond. The complex of the substrate and hPDIM was not detected by our HPLC system, but this possibility cannot be ruled out. Second, the conformation of GS-C77A might be changed through interaction with hPDIM, but it might easily return to the normal conformation after hPDIM leaves GS-C77A because of the absence of thiol disulfide exchange activity of hPDIM. Thus, GSH or PDI could not approach the disulfide bond. The functions of PDI and PDIM require further investigation.

We modified Cys95 of C77A by using TNB-activated thiol compounds. The methods for modifying cysteine residues in peptides by using DTNB, and their use for structural analyses were previously reported (*34*). Recently, Ruoppolo and Freedman generated mixed disulfides between glutathione and the reduced forms of disulfide-bonded proteins with GSSG, to model the unfolded state of newly synthesized secretory proteins (*35*). They carried out the reaction at room temperature for 3-10 h under a nitrogen atmosphere (moles GSSG/moles thiol groups of protein = 100/1-500/1). Their reaction generated only the

GSH-modified protein, and its reaction could not be monitored. In our method, the modification was carried out within 30 min at room temperature, under a normal atmosphere (TNB-activated compounds/thiol groups of protein = 2/1-50/1), and the reaction was traced by monitoring the absorbance of the TNB anion. This method is useful not only for the analysis of various reactions involving cysteine residues and disulfide bonds, but also for studies of protein complexes, because it can easily be used to link proteins and/or domains.

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