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PDI and glutathione-mediated reduction of the glutathionylated variant of human lysozyme

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A mutant human lysozyme, designated as C77A-a, in which glutathione is bound to Cys95, has been shown to mimic an intermediate in the formation of a disulfide bond during folding of human (h)-lysozyme. Protein disulfide isomerase (PDI), which is believed to catalyze disulfide bond formation and associated protein folding in the endoplasmic reticulum, attacked the glutathionylated h-lysozyme C77A-a to dissociate the glutathione molecule. Structural analyses showed that the protein is folded and that the structure around the disulfide bond, buried in a hydrophobic core, between the protein and the bound glutathione is fairly rigid. Thioredoxin, which has higher reducing activity of protein disulfides than PDI, catalyzed the reduction with lower efficiency. These results strongly suggest that PDI can catalyze the disulfide formation in intermediates with compact structure like the native states in the later step of in vivo protein folding.

Protein disulfide isomerase; Protein folding; Glutathione; Lysozyme

1. INTRODUCTION

Protein disulfide isomerase (PDI), one of the most abundant proteins in the endoplasmic reticulum (ER) of various cells, has been shown to catalyze formation, reduction, and isomerization of disulfide bonds in vitro and is believed to catalyze formation of disulfide bonds and associated protein folding in vivo[1,2]. The redox state of the ER is more oxidative than that of the cytosol, and glutathione disulfide may maintain the oxidative environment [3]. However, little is known about the mechanism of PDI-mediated disulfide bond formation during protein folding in the ER and about the involvement of glutathione in this step.

We have focused on the analysis of mutants as a model, which mimic folding intermediates of human lysozyme (h-lysozyme) with four disulfide bonds: Cys6–Cys128, Cys30–Cys116, Cys65–Cys81 and Cys77–Cys95. One of the mutants, designated as C77A, in which Cys77 is replaced by alanine, was secreted from yeast in two distinct forms, C77A-a and C77A-b. The former has a Cys95 modified with glutathione and the latter has a free thiol at Cys95 [4]. The oxidation step in disulfide bond formation in C77A was suggested to involve mixed disulfide with glutathione. Together with the recent finding that glutathione disulfide is the source of the oxidizing equivalent in the ER [3], C77A-a drew our attention because this modification was suggested to occur during in vivo folding and the glutathionylated protein mimics the actual intermediate thought to be a substrate of PDI.

Here we report a new finding that PDI attacks the glutathionylated substrate, which is the first demonstration of the in vivo glutathionylation of a protein. The characteristics of PDI as an in vivo 'foldase' is discussed on the basis of the structural analyses of the substrate.

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2. MATERIALS AND METHODS

Bovine PDI was purified to homogeneity according to the published method [5]. *E coli* thioredoxin was purchased from Takara Shuzo (Kyoto, Japan); glutathione (GSH), glutathione disulfide (GSSG) and dithiothreitol (DTT) were from Wako Pure Chemical Industries (Osaka, Japan). A CM-Toyopearl 650C column and a TSK-gel ODS-120T column for reverse-phase HPLC were obtained from Tosoh (Tokyo, Japan); an Asahipak ES-502C column was from Asahikasei (Tokyo, Japan). All other chemicals were of reagent quality.

The yeast *Saccharomyces cerevisiae* AH22R$^-$ (MAT**a** *leu2 his4 can1 pho80*)[6] was utilized as a host strain. The expression plasmid of mutant h-lysozyme (C77A), in which Cys77 is replaced by Ala, was previously described [4]. Modified Burkholder minimal medium [7], supplemented with 8% sucrose was used for growth of yeast.

Secreted mutant h-lysozyme (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 [4].

PDI- and thioredoxin-mediated conversion of C77A-a to C77A-b

were analyzed as follows. Purified C77A-a was incubated at 37°C for an appropriate time with 2-fold the amount of purified bovine PDI or that of *E. coli* thioredoxin in the GSH/GSSG reaction buffer containing 100 mM sodium phosphate, pH 7.5, 2 mM GSH and 0.2 mM GSSG or in the DTT reaction buffer containing 100 mM sodium phosphate, pH7.5, and 0.1 mM DTT. 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 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.

Amino acid compositions of the products of PDI- or thioredoxin-mediated conversion were determined with a Beckman Model 6300E amino acid analyzer. Analyses were performed on protein samples hydrolyzed for 22 h, in vacuo, at 110°C in 6 N HCl.

Rod shaped crystals of C77A-a were obtained from a buffer solution which contained 20 mg/ml protein, 30 mM sodium phosphate, pH 6.0, and 2.5 M NaCl using a modification of the published method [8] and seeding technique. The space group was $P2_12_12_1$ and its cell dimensions were a = 57.2 Å, b = 61.0 Å, c = 33.2 Å. The variation of cell dimension was so slight that the C77A-a crystal was considered isomorphous to the wild-type h-lysozyme crystal. Diffraction data were collected with an automated oscillation camera system equipped with Imaging Plate (MAC Science, DIP-100) on a rotating anode generator with a 0.3 × 3.0 mm focus. Indexing, evaluation of diffraction intensities, scaling and merging were done using the program systems ELMS [9] and PROTEIN [10]. The diffraction data were 84% complete to 1.8Å resolution and the final merging *R*-value was 6.67%. The structure of C77A-a was determined by a molecular replacement method with atomic coordinates of wild-type h-lysozyme [11] as a starting model using the programs PROTEIN and TRAREF [12]. After several cycles of the rigid group refinements, 2Fo-Fc and Fo-Fc maps were calculated. These maps showed clearly the electron densities of the bound glutathione molecule. The initial model of the glutathione molecule was constructed from the atomic model of the crystal structure of γ-L-glutamyl-L-cysteinyl-L-glycine at 120 K [13]. The glutathione model was fitted to the respective electron densities on an Evans & Sutherland PS-300 graphics system using the program FRODO [14]. Structural refinements of C77A-a were carried out using the stereochemically restrained least squares refinement method, as implemented in the program package PROTIN/PROLSQ [15] at 1.8 Å resolution. The refined model finally consisted of the protein, the glutathione and 113 water molecules, and the crystallographic *R*-factor was 0.146 for 8,982 independent reflections between 5.0 and 1.8 Å. The root mean square (rms) bond deviation from target value is 0.017 Å, and 2.6° from ideal bond angles. The rms difference in atomic B factors for covalently bonded pairs was 1.4 Å^2. The coordinate error was estimated to be about 0.12 Å based on the statistics of Luzzati [16].

3. RESULTS

To elucidate the possible involvement of PDI in the folding pathway of h-lysozyme, we investigated the effect of PDI on C77A-a and C77A-b. As shown in Fig. 1A, PDI was found to convert C77A-a to C77A-b by dissociating the glutathione molecule bound to Cys95 in C77A-a in the presence of glutathione and glutathione disulfide. The conversion of C77A-a to C77A-b was also confirmed by analyzing the amino acid composition of the product obtained from C77A-a after incubation with PDI (Table 1). Nevertheless, the reverse reaction was not observed at all (data not shown). In the time course of the reaction, any other protein except C77A-a and C77A-b was not found, indicating that C77A-a was converted to C77A-b without any shuffling of other three disulfide bonds. Figs. 1B and 1C show the

Table I

Amino acid composition of the product of PDI-mediated reduction of C77A-a

Amino acid	Theoretical values	Wild-type	C77A-a	C77A-b	The product
Asx	18	17.0	17.1	16.8	16.5
Thr	5	4.3	4.3	4.3	4.3
Ser	6	4.5	4.5	4.5	4.5
Glx	9	8.7	9.7	8.7	9.1
Gly	11	10.3	11.2	10.3	10.2
Ala	14	13.5	14.5	14.3	13.8
Val	9	7.7	7.7	7.6	7.5
Cys	8	5.7	5.6	4.7	4.2
Met	2	1.9	1.9	1.9	1.9
Ile	5	4.4	4.4	4.4	4.3
Leu	8	8.0	8.0	8.0	8.0
Tyr	6	5.6	5.4	5.6	5.4
Phe	2	1.8	1.8	1.8	1.9
Lys	5	4.7	4.9	4.9	4.9
His	1	1.1	1.1	1.0	1.0
Arg	14	14.2	14.1	13.6	14.0
Pro	2	1.9	1.9	1.9	2.1

effect of PDI and thioredoxin on the time course of the conversion in the GSH/GSSG- and DTT-containing reaction buffers, respectively. PDI catalyzed the dissociation of the glutathione with higher efficiency in the GSH/GSSG reaction buffer, whereas thioredoxin had almost no effect on the reaction (Fig. 1B). The redox potentials of thioredoxin and PDI are -270 mV and -190 mV, respectively [17,18]. Although thioredoxin has been shown to have a much higher protein disulfide reductase activity in the presence of dithiothreitol than PDI [17–20], PDI catalyzed the reduction of the disulfide bond more efficiently than thioredoxin, as shown in Fig. 1C. These results indicate that there must be some unknown mechanism in the PDI-mediated reduction. To the best of our knowledge, this is the first time that such an in vivo glutathionylated protein has been shown to be a substrate for PDI. Our observations suggest that PDI acts on its substrates as an actual 'foldase' in the cell, using GSH and GSSG as the source of the reducing and oxidizing equivalents, because C77A-a and C77A-b may mimic a folding intermediate and the wild-type protein, respectively.

Until now, there has been no information on the three-dimensional structure of the glutathionylated protein, especially on the in vivo folding intermediate. Thus, the three-dimensional structure of C77A-a was determined and refined by X-ray crystallography at 1.8 Å resolution. The overall structure of C77A-a was essentially identical to those of C77A-b [21] and the wild type, because the rms deviation of main chain atoms was 1.0 Å. These results indicate that the binding of the glutathione molecule to h-lysozyme does not affect the overall main chain folding. The atomic structure of the glutathione molecule and structural environment around the glutathione in C77A-a are shown in Fig. 2.

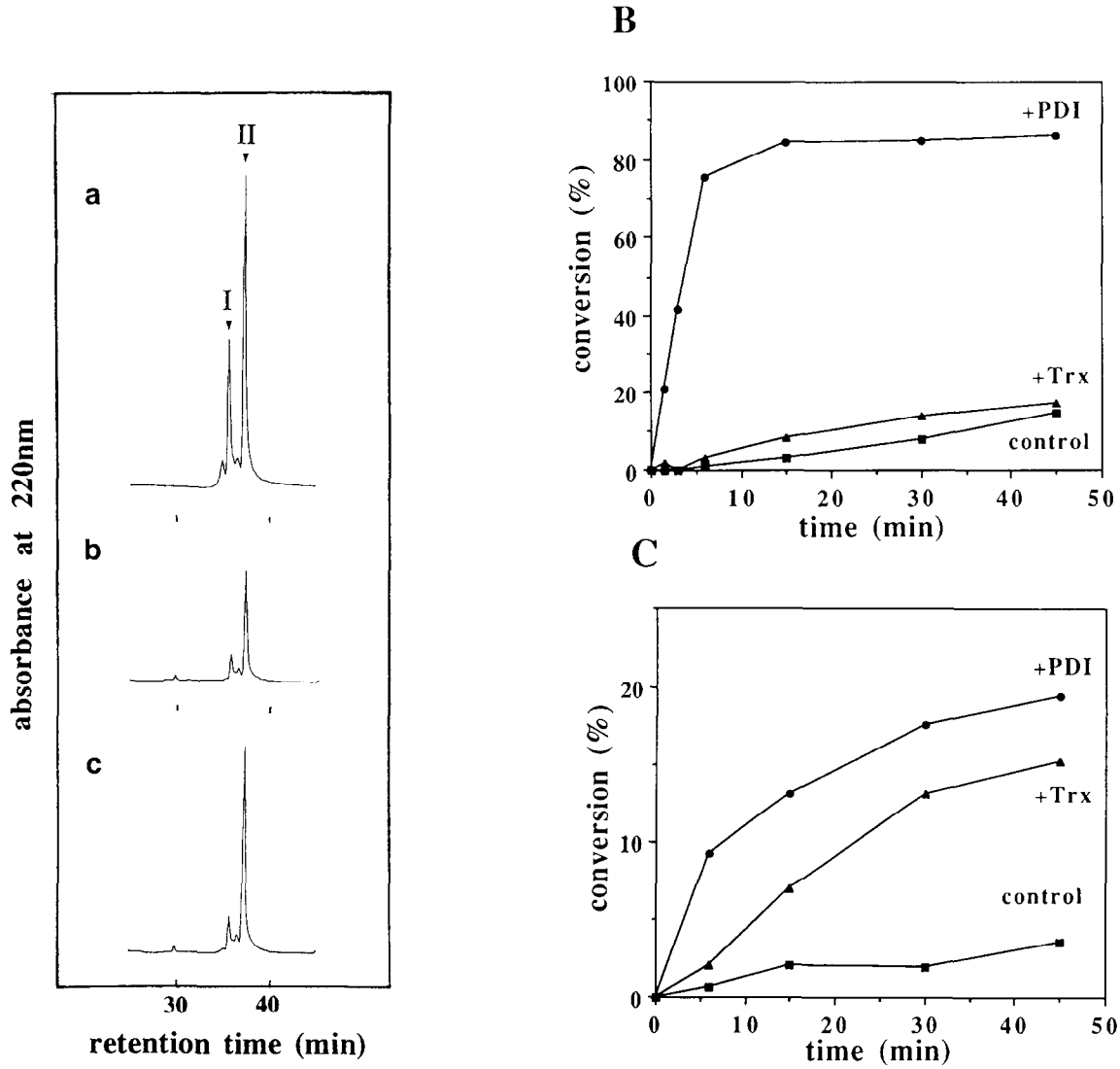

Fig. 1. Dissociation of glutathione bound to Cys^{95} in C77A-a by PDI or thioredoxin. (A) Trace a: 2 1 μM of C77A-a and 4.2 μM of C77A-b were separated by reverse-phase HPLC on a TSK-gel ODS-120T column as described in Section 2. Trace b: 2.1 μM of C77A-a was incubated in the GSH/GSSG reaction buffer as described in Section 2 containing 4.2 μM of bovine PDI at 37°C for 45 min, followed by separation as above Trace c: the reaction product obtained as described in trace b was separated as above soon after mixing with 2.1 μM of C77A-b. Peaks I and II correspond to C77A-a and C77A-b, respectively. Parts of chromatograms are shown here. (B), 1.4 μM of C77A-a was incubated at 37°C in the GSH/GSSG reaction buffer with 2.8 μM of bovine PDI (●), with 2.8 μM of *E coli* thioredoxin (▲), and without the enzymes (■) for 0, 1.5, 3, 6, 15, 30 and 45 min, respectively, followed by reverse phase HPLC to estimate the rate of conversion (C) 1.4 μM of C77A-a was incubated at 37°C in the DTT reaction buffer as described in Section 2 with 2.8 μM of bovine PDI (●), with 2.8 μM of *E.coli* thioredoxin (▲), and without the enzymes (■) for 0, 1.5, 3, 6, 15, 30, and 45 min, respectively, followed by reverse-phase HPLC.

The cysteine residue in the glutathione has almost the same geometry of the disulfide bond compared with that of Cys^{77} in the wild-type protein which has a disulfide bond with Cys^{95}. The glutathione molecule makes a close interaction with the h-lysozyme molecule by many hydrogen bondings and hydrophobic interactions (Fig. 2). The glutathione molecule had no interaction with neighboring protein molecules in the crystal. However, the cysteine residue, except the Cα atom, was not exposed to the solvent-accessible surface of C77A-a (Table II). Especially, the side chain atoms of the cysteine and those of Cys^{95} were completely buried in the molecule. Atomic thermal factors of the glutathione molecule are also shown in Table II. Although Glu and Gly residues exposed to the solvent region have high termal factors, the factor of the sulfur atom (15.8 $Å^2$) was the lowest in the glutathione. Further, the disulfide bond between Cys^{95} and the glutathione was surrounded by many hydrophobic residues: Phe^{57}, Ile^{59}, Trp^{64}, Leu^{79}, Leu^{84}, Val^{99} and Trp^{109} (data not shown). The average thermal factor of the side chain atoms in the hydrophobic core was 13.6 $Å^2$, indicating that the structure around the disulfide bond is fairly rigid.

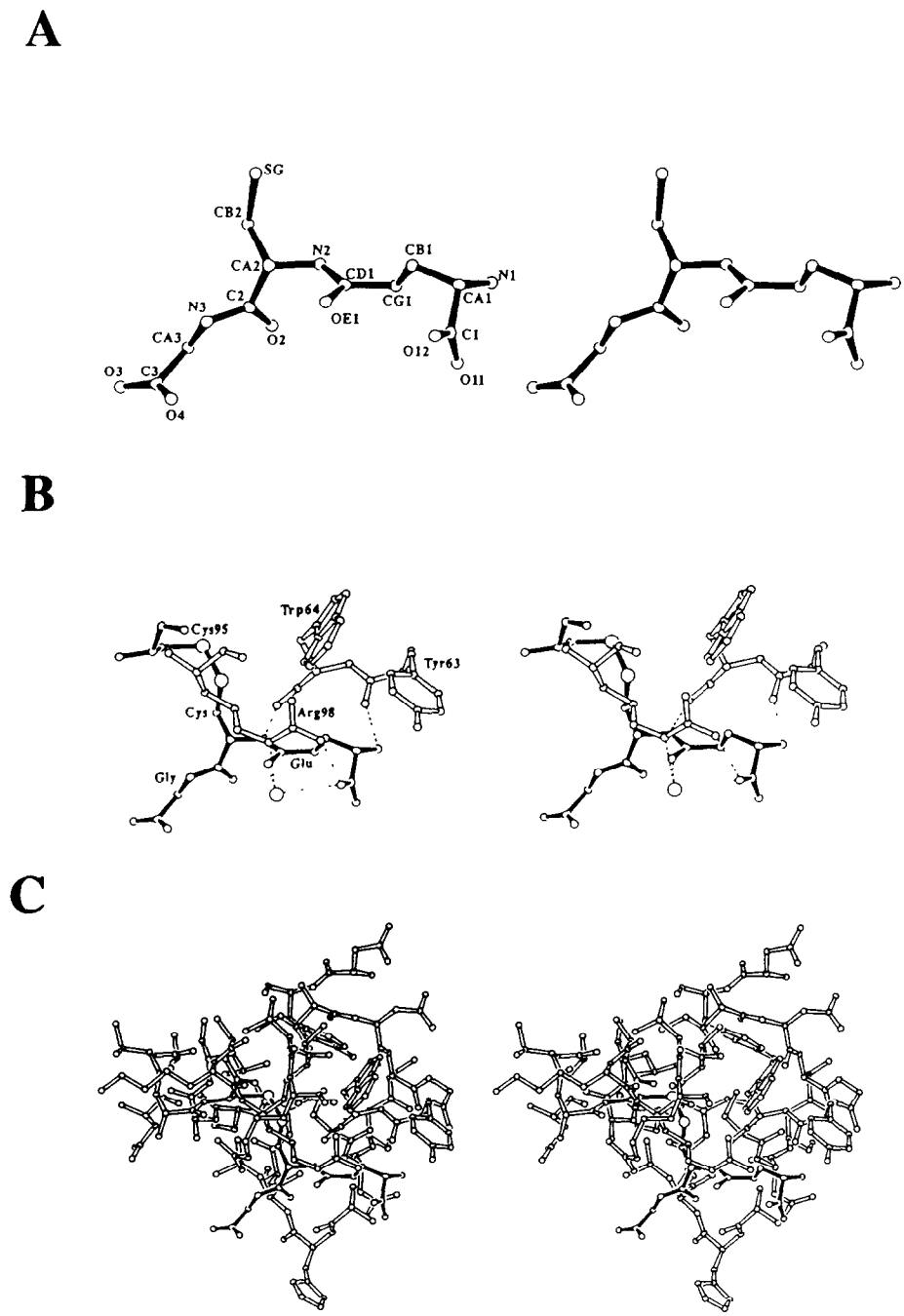

Fig. 2. Stereo drawings of the atomic structure of the glutathione molecule in C77A-a. (A) a ball and stick model of the glutathione molecule bound to C77A-a is shown. Each atoms are named and labeled as shown. (B) interactions between the glutathione molecule and surrounding residues, Tyr^{63}, Trp^{64}, Cys^{95} and Arg^{98} are shown. Models of the glutathione and Cys^{95} are indicated by closed bonds and other residues are indicated by open bonds Six hydrogen bonds, including those that are water mediated, are indicated by broken lines. Two sulfur atoms and the water molecule are indicated by large circles. (C) an atomic model of the glutathione and the surrounding residues within 12 Å from the Sγ atom of the cysteine residue of the glutathione is shown. The glutathione and Cys^{95} are also indicated as shown in B.

4. DISCUSSION

There are several examples of glutathione binding free cysteine residues in proteins in vitro, but the glutathionylation of Cys^{95} in C77A-a is believed to occur in the ER [4]. It has been suggested that the disulfide bond between Cys^{77} and Cys^{95} may be formed in the last step of the folding process of h-lysozyme [22,23]. The C77A-a possibly mimics the intermediate just prior to the formation of the Cys^{77}–Cys^{95} bond, which contributes to stabilization of the folded h-lysozyme [24]. Together with the recent finding that an oxidative environment in the ER is maintained by glutathione disulfide, glutathione is supposed to be involved in the oxidative

formation of disulfide bonds during the protein folding step. The participation of PDI in this step is also supported by the observation that the glutathione bound to Cys^{95} is dissociated by PDI. Thus the PDI-mediated reduction would lead to the formation of the disulfide bond between Cys^{77} and Cys^{95} in the wild-type h-lysozyme. Further, Cys^{95} in C77A-b was not glutathionylated by PDI, allowing us to suspect that the cysteine residues in the folding intermediates are glutathionylated spontaneously under the oxidative conditions in the ER rather than by catalysis of PDI.

Results obtained from the crystallographic analyses showed that the disulfide bond between Cys^{95} and the glutathione in C77A-a was surrounded by hydrophobic residues and that the structure around the disulfide bond was fairly rigid. Thus it is unlikely that the disulfide bond is exposed to the accessible surface even by its thermal vibration, indicating that PDI may induce structural change to its substrate prior to the reduction of the disulfide bond. There are two possible explanations how PDI recognizes its substrate: (1) PDI recognizes some specific amino acid sequences. No sequence specificity has been found for the binding of peptides or proteins to PDI [25]. However, cysteine-containing peptides are found to bind 4- to 8-fold tighter than no-cysteine-containing peptides, suggesting that the cysteine residue contributes to the interaction with PDI [26]. Based on our structural analyses, side chain atoms of the cysteine residues are buried in a hydrophobic core. In consequence PDI cannot attack the sulfur atoms of the disulfide bond directly. This observation clearly indicates that the cysteine is not involved in the first step of recognition of the substrate by PDI. However, PDI may recognize the structural features of bound glutathione exhibited by glutamic acid and/or glycine, because they are exposed to the surface of the protein. (2) PDI can recognize the energetic state of the substrates, as suggested from the study of S protein [27]. The S protein lacks 20 amino acid residues of its amino-terminal peptide (S peptide) of ribonuclease A. Disulfide bonds of S protein are found to be shuffled by PDI, while those of native ribonuclease A are not, suggesting that PDI does not distinguish substrates by their specific sequences but by their energetics [28]. According to this model, the observed conversion might be explained by the energy state of the C77A-a protein, which is probably high enough to be susceptible to structural change induced by PDI.

Table II

Thermal factors and solvent accessible surface area of atoms in the glutathione

Atom name	Thermal factor (Å^2)		Solvent accessible surface area (Å^2)*	
Glu N	30.82		0.	
Cα	30.61		1.57	
C	32.05		0.	
O1	33.64		23.22	
O2	32.04		22.17	
Cβ	27.28		0.	
Cγ	23.79		0.	
Cδ	22.70		0.	
Oε	23.10	Cys77 in wild-type	9.57	Cys77 in wild-type
Cys N	21.49	12.58	0.	0.
Cα	19.58	12.51	1.58	4.73
C	21.37	12.13	0.	0.
O	19.68	12.30	0.	12 67
Cβ	16.81	11.66	0.	0.
Sγ	15.84	9.73	0.	0.
Gly N	24.84		10.33	
Cα	29.39		7.89	
C	32.86		3.45	
O1	34.27		29.56	
O2	35.47		38.00	

*Solvent accessible surface area was calculated by the program ASA (H. Mizuno and N. Go, unpublished) using a solvent probe of 1.4 Å radius.

In the in vitro experiments, PDI acts on pre-folded or completely denatured proteins or short polypeptides [29], which correspond to molecular species in the early step during protein folding. In the present study the dissociation of the glutathione bond to Cys^{95} in C77A-a with a folded structure was found to be catalyzed by PDI more efficiently than by thioredoxin (Fig. 1B and C). Disulfides in various proteins are shown to be good substrates of thioredoxin with no substrate specificity, whereas structurally buried disulfides are not [30]. Our finding described here is the first example that PDI appears to catalyze the disulfide formation in intermediates, which have become compact, like the native states in the later step of in vivo protein folding, while most disulfide bonded intermediates already studied were open in conformation [31]. From the present evidence it is tempting to say that PDI is the real catalyst during in vivo protein folding, while thioredoxin may not. We believe that these observations will be helpful for a detailed understanding of the actual mechanism of PDI-mediated disulfide bond formation in vivo.

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REFERENCES

[1] Bulleid, N.J. and Freedman, R.B. (1988) Nature 335, 649–651.
[2] Freedman, R.B. (1989) Cell 57, 1069–1072.
[3] Hwang, C., Sinskey, A.J. and Lodish, H.F. (1992) Science 257, 1496–1502.
[4] Taniyama, Y., Seko, C. and Kikuchi, M. (1990) J. Biol. Chem. 265, 16767–16771.
[5] Lambert, N. and Freedman, R.B. (1983) Biochem. J. 213, 225–234
[6] Miyanohara, A., Toh-e, A., Nozaki, C., Hamada, F., Ohtomo, N. and Matsubara, K. (1983) Proc. Natl. Acad. Sci. USA 80, 1–5.
[7] Toh-e, A., Ueda, Y., Kakimoto, S. and Oshima, Y. (1973) J. Bacteriol. 113, 727–738.

[8] Inaka, K , Kuroki, R., Kikuchi, M. and Matsushima, M. (1991) J. Biol. Chem. 266, 20666–20671.
[9] Tanaka, I., Yao, M., Suzuki, M., Hikichi, K., Matsumoto, T., Kozasa, M. and Katayama, C (1990) J. Appl. Crystallogr 23, 334–339.
[10] Steigeman, W (1974) Ph D thesis, Technische Universität, München.
[11] Kidera, A., Inaka, K , Matsushima, M and Go, N (1992) J. Mol. Biol. 225, 477–486
[12] Huber, R. and Schneider, M (1985) J. Appl. Crystallogr 18, 165–169.
[13] Görbitz, C.H. (1987) Acta Chem. Scand. B.41, 362–366.
[14] Jones, T.A. (1978) Acta Crystallogr. A34, 931–935
[15] Hendrickson, W.A. (1985) Methods Enzymol. 115, 252–270.
[16] Luzzati, V. (1952) Acta Crystallogr 5, 802–810.
[17] Krause, G., Lundström, J., Lopez-Barea, J , Pueyo, C. and Holmgren, A (1991) J Biol. Chem. 266, 9494 9500.
[18] Lundström, J. and Holmgren, A. (1990) J. Biol. Chem 265, 9114–9120.
[19] Holmgren, A (1979) J. Biol Chem. 254, 9113 9119
[20] Holmgren, A (1979) J. Biol. Chem 254, 9627–9632
[21] Kuroki, R., Inaka, K., Taniyama, Y., Kidokoro, S., Matsushima, M., Kikuchi, M. and Yutani, K. (1992) Biochemistry 31, 8323–8328.
[22] Kikuchi, M , Taniyama, Y., Kanaya, S , Takao, T. and Shimonishi, Y. (1990) Eur. J. Biochem. 187, 315–320
[23] Inaka, K , Taniyama, Y , Kikuchi, M., Morikawa, K. and Matsushima, M. (1991) J Biol Chem. 266, 12599–12603.
[24] Taniyama, Y , Ogasahara, K , Yutani, K and Kikuchi, M (1992) J. Biol. Chem 267, 4619–4624
[25] Noiva, R., Kimura, H., Roos, J. and Lennarz, W.J (1991) J. Biol. Chem 266, 19645–19649.
[26] Morjana, N.A and Gilbert, H.F. (1991) Biochemistry 30, 4985–4990.
[27] Freedman, R B. (1991) in: Conformations and Forces in Protein Folding (Nall, B.T. and Dill, K A. eds.) American Association for the Advancement of Science, pp 204–214
[28] Kato, I. and Anfinsen, C.B. (1969) J Biol Chem. 224, 1004–1007.
[29] Freedman, R.B., Brockway, B E. and Lambert, N (1948) Biochem. Sci. Transactions 12, 929–932.
[30] Homgren, A. (1985) Annu Rev Biochem. 54, 237–271.
[31] Freedman, R.B. (1984) Trends Biochem. Sci. 9, 438 441