THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 47, Issue of November 19, pp. 48830–48835, 2004

Printed in U.S.A.

The Acidic C-terminal Domain Stabilizes the Chaperone Function of Protein Disulfide Isomerase*

Received for publication, June 24, 2004, and in revised form, September 7, 2004
Published, JBC Papers in Press, September 9, 2004, DOI 10.1074/jbc.M407076200

Rui Tian‡§, Sheng-Jian Li¶, Dong-Liang Wang‡, Zhen Zhao¶, Ying Liu‡, and Rong-Qiao He‡‖

From the ‡Laboratory of Visual Information Processing and the ¶National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, 15 Da Tun Road, Chaoyang District, Beijing 100101 and the §Graduate School, Chinese Academy of Sciences, Yuquan Road A19, Shijingshan District, Beijing 100039, China

Protein disulfide isomerase (PDI, EC 5.3.4.1) is a chaperone and catalyzes the formation and rearrangement of disulfide bonds in proteins. Domain c-(463–491), containing 18 acidic residues, is an interesting and important C-terminal extension of PDI. In this study, the PDI mutant abb′a′, in which domain c is truncated, was used to investigate the relationship between the C-terminal structure and chaperone function. Reactivation and light-scattering experiments show that both wild-type PDI and abb′a′ interact with lactate dehydrogenase (LDH, EC 1.1.1.27), which tends to self-aggregate during reactivation. The interaction enhances reactivation of LDH and reduces aggregation. According to these results, it seems as if domain c might be dispensable to the chaperone function of PDI. However, abb′a′ is prone to self-aggregation and causes increased aggregation of LDH during thermal denaturation. In contrast, wild-type PDI remains active as a chaperone under these conditions and prevents self-aggregation of LDH. Furthermore, measurements of intrinsic fluorescence and difference absorbance during denaturation show that abb′a′ is much more labile to heat or guanidine hydrochloride denaturation than wild-type PDI. This suggests that domain c is required for the stabilization and maintenance of the chaperone function of PDI under extreme conditions.

Protein disulfide isomerase (PDI)[1] contains 491 amino acid residues and is a multifunctional protein (1, 2). As an enzyme, it catalyzes the formation and rearrangement of disulfide bonds during oxidative protein folding. As a chaperone, PDI inhibits aggregation of the denatured protein and assists the refolding. The substrates of PDI can be proteins with or without disulfide bonds, such as D-glyceraldehyde-3-phosphate dehydrogenase (3), rhodanese (4), lysozyme (5), and acidic phospholipase A_2 (6). Under certain circumstances, PDI shows a so-called anti-chaperone effect in which substoichiometric concentrations of PDI facilitate aggregation and inhibit reactivation of denatured proteins (7), presumably by interacting and stabilizing the aggregates (8–10).

PDI consists of consecutive domains (a, b, b′, and a′) and the acidic C-terminal extension, domain c (8, 9, 11). Domains a and a′ are similar to thioredoxin, and each contains the sequence CGHC, forming two independently acting catalytic sites (8, 11). Domains b and b′ show low homology with domain a and have little sequence similarity to any member of the thioredoxin family but also adopt a thioredoxin fold (9). Domain c, also termed the C-terminal extension (residues 463–491), represents a putative Ca^{2+}-binding region (11) and is rich in acidic amino acids. A C-terminal KDEL motif is necessary and sufficient for the retention of a polypeptide within the lumen of the endoplasmic reticulum (12). Koivunen *et al.* (10) have investigated the function of the PDI domain c using different mutants of PDI with deletions in the C-terminal sequence. They found that under routine conditions, domain c plays no significant role in the chaperone and disulfide isomerase activities of PDI. In contrast, Noiva *et al.* (2) have hypothesized that domain c is vital for PDI to function as a chaperone. Whether domain c is involved in the chaperone function of PDI is an interesting problem worthy of investigation. The data presented in this study indicate that domain c plays a critical role in stabilization of the functional conformation of PDI and in preventing its self-aggregation under extreme conditions, although the deletion of the entire C-terminal extension (463–491) does not suppress chaperone activity toward guanidine hydrochloride (GdnHCl)-denatured lactate dehydrogenase (LDH, EC 1.1.1.27). Thus, our results provide a unifying picture of the functional role of PDI domain c.

EXPERIMENTAL PROCEDURES

Materials—Full-length human PDI cDNA was provided by Prof. K. Kivirikko, University of Oulu, Finland. The cDNA was inserted into the plasmid pUC19. Ni^{2+} chelating Sepharose™ Fast Flow resin was from Amersham Biosciences. Q-Sepharose™ Fast Flow resin and NADH were from Amersham Biosciences. pQE-30 and *Escherichia coli* M15 (pREP4) were from Qiagen. GdnHCl, dithiothreitol (DTT), EDTA, and bovine serum albumin (BSA, fraction V) came from Sigma. Other reagents were analytical grade and used without further purification. Rabbit muscle LDH was also from Sigma. The enzyme showed a single band on SDS-PAGE with a specific activity of ~1000 units. The protein concentration was determined using an absorption coefficient $\epsilon_{280} = 1.40 \pm 0.02$ cm²/mg (13). To avoid the interaction of disulfide bonds of PDI and LDH, a reducing buffer (0.1 M phosphate buffer, pH 7.4, containing 10 mM DTT) was used for all experiments, unless otherwise stated. Absorbance was measured on a Hitachi VIS/UV 2010 spectrophotometer. 90° light scattering and fluorescence were measured on a Hitachi F-4500 fluorescence spectrophotometer. The theoretical pIs of abb′a′ and PDI were calculated by the ExPASy-Compute pI/Mw tool online.

Expression and Purification of Wild-type abb′a′ and PDI—The coding regions of PDI and abb′a′ were amplified by PCR from the pUC19-PDI construct (10, 14). The PCR products (BamH/HindIII) were ligated

* This project is supported by grants from the Knowledge Innovation Project of the Chinese Academy of Sciences (Grant KSCX2-SW214-1) and the National Natural Science Foundation of China (Grants 90206041 and 30170297). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‖ To whom correspondence should be addressed. Tel.: 86-10-6488-9876; Fax: 86-10-6485-3625; E-mail: herq@sun5.ibp.ac.cn.

[1] The abbreviations used are: PDI, protein disulfide isomerase; abb′a′, PDI mutant with domain c (c, 463–491) deleted; ABB′A′, PDI mutant with domain C (C, 441–491) deleted; LDH, lactate dehydrogenase; BSA, bovine serum albumin; GdnHCl, guanidine-HCl; DTT, dithiothreitol.

into pQE-30 to generate an N-terminal extension including a His_6 tag (MRGSHHHHHHGS). To overexpress the two resultant proteins, *E. coli* M15 (pREP4) was transformed with either the pQE-30-PDI or the pQE-30-abb′a′ construct. Transformed cells were grown in LB medium at 37 °C with 100 μg/ml ampicillin and 25 μg/ml kanamycin. An overnight culture, produced from a single colony, was diluted 100-fold and grown to an absorbance (600 nm) of 0.8–1.0 and then induced with isopropyl-1-thio-β-D-galactopyranoside (final concentration, 1 mM). The culture was incubated for another 4 h, and then the bacteria were harvested by centrifugation (6500 rpm, 4 °C, 30 min) and resuspended in buffer A (50 mM Tris, pH 8.0, containing 500 mM NaCl, and 10 mM imidazole). The bacteria were lysed by sonication before centrifugation (6500 rpm, 4 °C, 30 min). The supernatant was transferred to the Ni^{2+} chelating Sepharose™ Fast Flow column, which had been pre-equilibrated with buffer A. The column was continuously washed with buffer A until the absorbance at 280 nm reached a baseline, and then buffer B (50 mM Tris, pH 8.0, containing 500 mM NaCl and 50 mM imidazole) was used to remove nonspecifically bound proteins. The resulting protein was eluted with buffer C (50 mM Tris, pH 8.0, containing 500 mM NaCl and 250 mM imidazole). Eluted protein was dialyzed extensively against 50 mM Tris, pH 8.0, at 4 °C. Then it was applied to a Q-Sepharose™ Fast Flow column (15 × 1.5 cm), which had been pre-equilibrated with 50 mM Tris buffer (pH 8.0). A linear gradient (0–1 M NaCl) was adopted to allow further purification (total volume, 250 ml). Fractions containing the protein of interest were identified by SDS-PAGE and dialyzed extensively against distilled water at 4 °C. Finally, the protein was lyophilized and stored at −20 °C.

Reactivation of GdnHCl-denatured LDH in the Presence of abb′a′ or PDI—Enzyme concentrations are stated in terms of monomers, and a stoichiometry of 1:1 was used although PDI is most active in its dimeric form (15). LDH (50 μM) was denatured completely by incubation in 3 M GdnHCl at 4 °C overnight (16). Reactivation was initiated by a 50-fold dilution of the denatured enzyme into phosphate buffer containing different concentrations of PDI (or abb′a′) and 10 mM DTT (17–19). The reactivation mixture was kept at 4 °C for 30 min and then at 25 °C for 2 h before an aliquot of 50 μl was taken for activity assay at 25 °C. The enzymatic assay was carried out in phosphate buffer (containing 0.72 mM pyruvate, 0.2 mM NADH, 1 mM DTT, and 1 mM EDTA) by monitoring the absorbance at 334 nm at 25 °C, unless otherwise stated.

For assay of chaperone activity after GdnHCl incubation, PDI or abb′a′ (final concentration, 100 μM) was incubated at 37 °C overnight in phosphate buffer containing different concentrations of GdnHCl. The LDH reactivation process and conditions were as described above, except that the GdnHCl-incubated PDI or abb′a′ was added at a molar ratio [chaperone]:[LDH] of 10:1, and the reactivation mixtures were kept at 25 °C for 2 h before the assays.

Aggregation of GdnHCl-denatured LDH Monitored by Light Scattering in the Presence of abb′a′ or PDI—The conditions used were the same as above, except that 90° light scattering (slit widths: $E_x = E_m = 500$ nm) was monitored after a 50-fold dilution of the denatured LDH (17).

Delayed Addition of abb′a′ or PDI during Reactivation of GdnHCl-denatured LDH—LDH (50 μM) was incubated in 3 M GdnHCl overnight at 4 °C to allow complete denaturation, and then the mixture was diluted 50-fold into phosphate buffer containing 10 mM DTT at 25 °C (3, 20). Either PDI or abb′a′ (1.0 μM) was added into the reactivation mixture at different time intervals after dilution. The reactivation mixture was kept at 4 °C for 30 min and then at 25 °C for 2 h before an aliquot (50 μl) was taken for activity assay at 25 °C.

Effects of abb′a′ or PDI on Aggregation of Thermally Denatured LDH Monitored by Light Scattering—LDH (0.2 μM) in the presence of PDI (or abb′a′) at different molar ratios ([chaperone]/[LDH] = 0, 1, 2, 4, 8, 10, or 16) was incubated at 80 °C in phosphate buffer (18, 19) containing 10 mM DTT, and 90° light scattering (slit widths: $E_x = E_m = 500$ nm) was monitored.

The Time Course of Light-scattering Changes of abb′a′ Incubated at 80 °C—Conditions were the same as stated above, except that abb′a′ alone (0.2, 0.8, or 2 μM) was incubated in phosphate buffer at 80 °C, and the light-scattering intensity was measured.

Measurements of Emission Fluorescence of abb′a′ and PDI during GdnHCl Denaturation—PDI or abb′a′ (final concentration, 0.3 μM) was incubated in different concentrations of GdnHCl for 12 h to reach equilibrium, and then intrinsic fluorescence was measured by excitation at 295 nm (slit widths: $E_x = E_m = 5$ nm) at 37 °C. The fluorescence intensities of PDI or abb′a′ alone at 338 nm, measured under the same conditions, were taken as 100%. Kinetic measurements were carried out at 37 °C. PDI or abb′a′ was added into 0.5 M GdnHCl solution, and the intrinsic fluorescence was measured at different time intervals.

Absorbance of abb′a′ or PDI at Different Temperatures—PDI or abb′a′ (final concentration, 1 μM) was incubated in phosphate buffer at different temperatures, and the absorbance at 280 nm was measured until no further changes were observed. The difference absorbance value was obtained by subtracting the value measured at 25 °C.

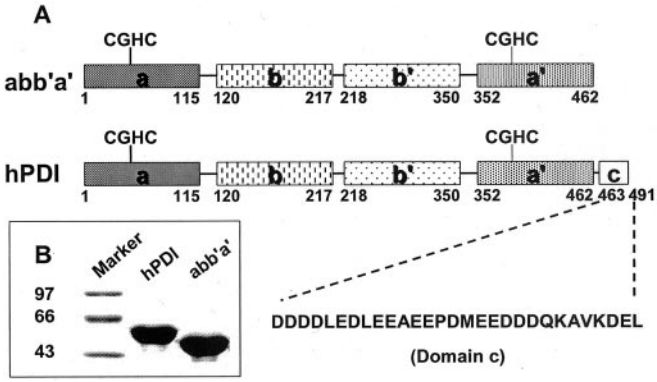

FIG. 1. **Profile of mutant abb′a′ by SDS-PAGE after purification.** *A*, the linear arrangement of domains in human PDI (*hPDI*) and abb′a′, according to the domain boundaries reported by Kemmink *et al.* (9). The *solid lines* represent the linkage between the domains. *B*, SDS-PAGE (12%) profiles of purified PDI and abb′a′.

RESULTS

Effects of abb′a′ and PDI on Reactivation and Aggregation of GdnHCl-denatured LDH—The protein abb′a′ is a mutant of PDI, which lacks domain c, as illustrated in Fig. 1*A*. Domain c has 29 residues and is rich in negatively charged amino acids, including 8 Glu and 10 Asp residues. After expression and purification, PDI and abb′a′ each appear as a single band by SDS-PAGE (Fig. 1*B*). The concentrations of PDI and abb′a′ were determined as described by Bradford (21). The CD spectra of wild-type PDI and abb′a′ are essentially identical, as reported (10).

Fig. 2*A* shows that the effects of PDI and abb′a′ at different molar ratios ([chaperone]/[LDH]) on the reactivation of GdnHCl-denatured LDH after dilution in the presence of 10 mM DTT. The presence of wild-type PDI or abb′a′ results in an increase in the reactivation yield. The reactivation yield of LDH with wild-type PDI (at a molar ratio [chaperone]:[LDH] of 30) is ~41%, and that with abb′a′ is slightly higher (~46%). The spontaneous reactivation yield of LDH alone, or in the presence of BSA, is significantly lower (~19%). The intensity of intrinsic fluorescence of abb′a′ at 350 nm is slightly (10.3 ± 1.8%) higher than that of wild-type PDI under the same conditions (see Fig. 6, *A* and *B*). The higher level of intrinsic fluorescence suggests a slight difference in the microenvironment around Trp residues, implying that the lack of the acidic domain c causes slight alteration of the hydrophobic packing as compared with wild-type PDI. Furthermore, the theoretical pI of abb′a′ (5.03) is slightly higher than that of PDI (4.69). The slightly higher yield of reactivation for abb′a′ as compared with wild-type PDI may result from the difference in packing and/or from the reduced charge of the mutant. Under the same conditions, BSA shows only a slight effect on the LDH reactivation yield, much lower than either abb′a′ or PDI. Thus, abb′a′ maintains chaperone function toward LDH, although domain c is truncated.

Protein aggregation can be detected by the increase in the light scattering. Fig. 2, *B* and *C*, show changes in the light-scattering intensity of GdnHCl-denatured LDH in the presence of PDI and abb′a′, respectively. For LDH alone, the intensity increases markedly within 5 min after dilution and reaches a plateau after about 25 min. LDH in the presence of wild-type PDI (Fig. 2*B*) or abb′a′ (Fig. 2*C*) displays a clear decrease in the light-scattering intensity under the same conditions. With in-

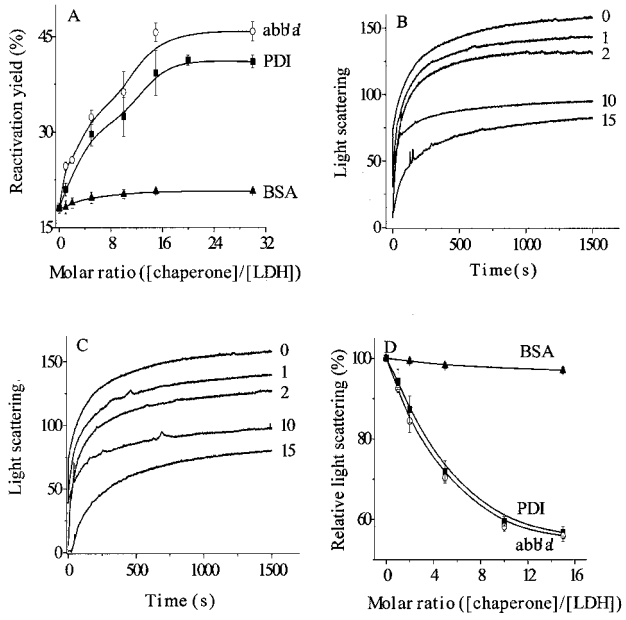

FIG. 2. **Effects of PDI or abb′a′ at different concentrations on the reactivation and aggregation of GdnHCl-denatured LDH.** 50 μM LDH was incubated in 3 M GdnHCl overnight at 4 °C to reach the completely denatured state. The denatured enzyme was diluted 50-fold with 0.1 M phosphate buffer (pH 7.4) containing PDI or abb′a′ at different molar ratios ([chaperone]/[LDH]) as indicated and 10 mM DTT. The reactivation mixture was kept at 4 °C for 30 min and then at 25 °C for 2 h before a 50-μl aliquot was taken for an activity assay at 25 °C. *A*, effects of PDI and abb′a′ concentrations on reactivation of GdnHCl-denatured LDH. BSA, under the same conditions, was used as control. *B* and *C*, the time courses of the light-scattering intensity of LDH during refolding in the presence of wild-type PDI or abb′a′. *D*, the effects of abb′a′ or PDI concentration on the eventual levels of aggregation. The eventual light-scattering intensity of LDH alone, under the same conditions, was taken as 100%.

creasing concentrations of abb′a′ or PDI (Fig. 2*D*), both the rates and the extents of aggregation decrease markedly until the molar ratio of [chaperone]/[LDH] reaches about 15. BSA, as a control, causes no significant difference in the scattering intensity, as compared with LDH alone. This suggests that aggregation of LDH molecules competes with correct folding and thus leads to the low spontaneous reactivation yield. PDI and abb′a′ repress self-aggregation of LDH and promote its reactivation during refolding in reducing buffer. This suggests that PDI and abb′a′ exert this effect by binding to the exposed hydrophobic regions of non-native LDH, preventing self-aggregation of the GdnHCl-denatured enzyme, rather than by involvement in formation or breaking of covalent bonds.

Effects of Delayed Addition of abb′a′ or PDI on GdnHCl-denatured LDH Reactivation—The effects of the delayed addition of PDI or abb′a′ on enzymatic reactivation after the dilution of denatured LDH are shown in Fig. 3. The reactivation of LDH (final concentration, 1 μM) drops sharply when the delay in the addition of abb′a′ (or PDI) after dilution is increased, reaching the spontaneous reactivation yield after a delay of around 1 h. This suggests that both abb′a′ and PDI assist the refolding of LDH to the active state by interacting with the aggregation-prone species of LDH and thus suppress its self-aggregation. This then strongly supports that abb′a′ and PDI act as chaperones toward LDH *in vitro*. As the effect of PDI and abb′a′ is similar, this implies that domain c-(463–491) is dispensable to the chaperone function of PDI during reactivation of GdnHCl-denatured LDH at room temperature.

Effects of abb′a′ or PDI on Aggregation of Thermally Denatured LDH—To further investigate the role of domain c in PDI structure and function, we investigated the ability of PDI

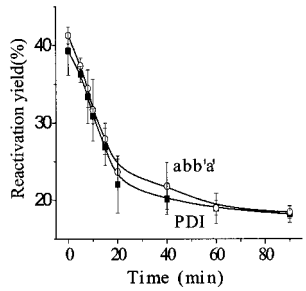

FIG. 3. **Effects of delayed addition of PDI or abb′a′ on GdnHCl-denatured LDH refolding.** Conditions were the same as in Fig. 2, except that PDI (10 μM) or abb′a′ (10 μM) was added at different time intervals after the dilution of the denatured LDH (1.0 μM).

and abb′a′ to prevent aggregation of LDH during thermal denaturation. 80 °C is a commonly used temperature to study non-native LDH aggregation (17). Fig. 4*A* shows changes in the light scattering of thermally denatured LDH in the presence of wild-type PDI at different molar ratios. For LDH alone, the scattering intensity rapidly increases with time and approaches a constant value within 4 min. Wild-type PDI, however, markedly reduces the light-scattering intensity of LDH when the molar ratio of [PDI]/[LDH] is increased to 16, indicating that LDH aggregation can be almost completely blocked under these conditions. In contrast, for abb′a′ (Fig. 4*B*), the intensity of light scattering of LDH increases markedly as the concentration of abb′a′ is increased, and the rate of the light-scattering change also increases with the abb′a′ concentration. At a molar ratio [abb′a′]/[LDH] of 4 or above, the light-scattering intensity reaches a maximum, decreases, and then reaches a plateau. The eventual decrease in the light-scattering intensity can be accounted for by the precipitation of aggregated-LDH and abb′a′ from solution. Thus, abb′a′ actually promotes thermally induced aggregation of the substrate protein. The higher the concentration of abb′a′, the more the LDH molecules tend to aggregate. Furthermore, the lag time becomes shorter and the rate of change in the light-scattering intensity becomes faster as the abb′a′ concentration is increased (Table I and Fig. 4, *C* and *D*). For LDH alone, the aggregation rate is slower than for LDH with abb′a′ but faster than in the presence of PDI. In contrast, Fig. 4*C* shows clearly that the intensity of light scattering decreases as the concentration of PDI increases. BSA as a control has no noticeable effect on the aggregation of LDH. This indicates that wild-type PDI binds to LDH under thermally denaturing conditions and suppresses aggregation; thus, it maintains its function as a chaperone under these conditions. In contrast, abb′a′ loses its chaperone function under thermally denaturing conditions.

The light-scattering intensity of abb′a′ alone increases with increasing concentration under these conditions (Fig. 5, *A* and *B*), suggesting that abb′a′ is denatured and aggregated at temperatures at which PDI is still stable. It appears that aggregated abb′a′ induces more aggregation of LDH, leading to the increase in the light scattering. Eventually, the deposits of LDH and abb′a′ precipitate, which results in the decrease of the light scattering after reaching the maximum value. It is clear that abb′a′ loses the protective effect toward LDH and promotes its aggregation under thermally denaturing conditions. This suggests that domain c plays an important role in preventing PDI from self-aggregation during thermal denaturation.

Changes in Conformations of abb′a′ and PDI during GdnHCl and Thermal Denaturation—Intrinsic fluorescence of abb′a′ and PDI is mainly due to the contribution of Trp residues (Trp-35, Trp-111, Trp-341, Trp-379, and Trp-390) (22). As seen in Fig. 6, *A–D*, the emission intensities of both PDI and

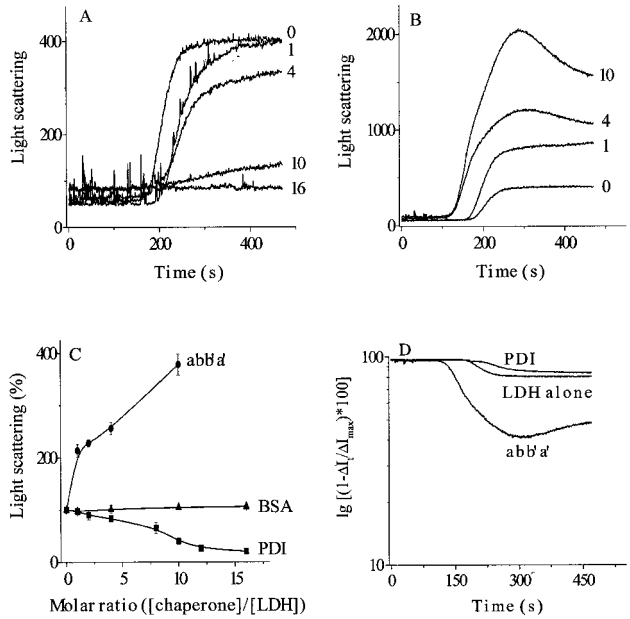

FIG. 4. **Effects of PDI or abb′a′ on aggregation of thermally denatured LDH monitored by light scattering.** LDH (0.2 μM) in the presence of PDI or abb′a′ at different molar ratios was incubated at 80 °C in phosphate buffer containing 10 mM DTT, and the 90° light scattering was monitored (slit widths: $E_x = E_m = 500$ nm). BSA was used as a control. *A* and *B*, the changes in the light-scattering intensities of LDH in the presence of wild-type PDI or abb′a′. The molar ratios ([chaperone]/[LDH]) are as indicated in the figures. The light-scattering intensities of LDH in the presence of abb′a′ were subtracted from those of abb′a′ alone at each molar ratio (Fig. 5*A*). *C*, the eventual light-scattering intensities of LDH in the presence of PDI or abb′a′ at different molar ratios. The eventual light-scattering intensity of LDH alone was taken as 100%, and BSA was used as a control in place of chaperone. *D*, the changes in the light-scattering intensities of LDH with 4-fold excess of PDI or abb′a′, plotted semilogarithmically as $((1 - \Delta I_t/\Delta I_{max})*100)$, where I_t is the light-scattering change with time and ΔI_{max} is the maximal light-scattering change when the aggregation reaches a plateau (30).

TABLE I
First order rate constants for lag time and changes in light scattering of LDH in the presence of PDI or abb′a′ during 80 °C thermal denaturation

Molar ratio ([chaperone]/[LDH])	PDI		abb′a′1	
	Lag time	Fast phase	Lag time	Fast phase
	sec	$\times 10^{-3}$ *s*$^{-1}$	*sec*	$\times 10^{-3}$ *s*$^{-1}$
0	170 ± 12	2.63 ± 0.99	170 ± 12	2.63 ± 0.99
1	192 ± 10	1.67 ± 0.61	152 ± 18	5.25 ± 2.11
4	202 ± 8.0	1.21 ± 0.30	106 ± 15	7.28 ± 2.38
10	210 ± 12	0.08 ± 0.02	105 ± 21	19.2 ± 5.18

abb′a′ show a clear red shift as reported previously (23). The red shift in the emission maximum for abb′a′ is slightly larger than for PDI (Fig. 6*D*). As seen in Fig. 6, *E* and *F*, the kinetics of unfolding is significantly faster for abb′a′ than for PDI. The decrease in fluorescence for PDI is a biphasic process, showing a fast and a slow phase (Table II), whereas for abb′a′, the decrease in fluorescence is on a single fast phase that can be detected. The first order rate constant for the single phase observed for abb′a′ is over five times larger than that of the fast phase for PDI. This indicates that abb′a′ is significantly more labile than PDI in 0.5 M GdnHCl. Furthermore, changes in the difference absorbance at 280 nm of abb′a′ as compared with the value at ambient temperature are marked when the temperature is increased above 60 °C, in contrast to PDI (Fig. 7). This indicates that abb′a′ is likewise much less stable than PDI to thermal denaturation.

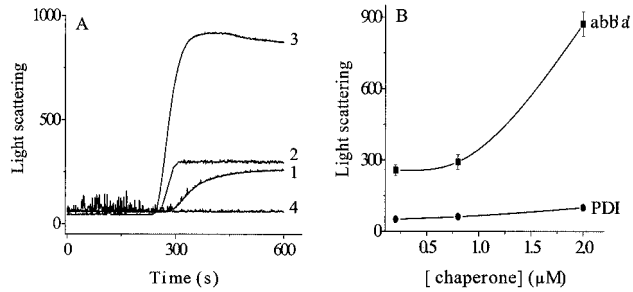

FIG. 5. **Kinetics of light-scattering intensities of abb′a′ incubated at 80 °C.** Conditions were the same as in Fig. 4, except that abb′a′ alone was incubated in phosphate buffer at 80 °C followed by measurement of the light-scattering changes. *A*, *curves 1–3* represent abb′a′ at 0.2, 0.8, and 2 μM, respectively. *Curve 4* represents 0.8 μM PDI under the same conditions. *B*, final intensities of PDI or abb′a′.

Fig. 8 illustrates the effects of GdnHCl-incubated abb′a′ or PDI on reactivation of LDH. Spontaneous reactivation of LDH is only about 10% under these conditions. After GdnHCl incubation (0–1 M), PDI still exhibits chaperone function toward LDH. The maximum reactivation yield of LDH is ~2.5-fold higher than the spontaneous reactivation yield. Under the same conditions, however, the chaperone activity of abb′a′ decreases sharply when the denaturant concentration is higher than 0.3 M and is completely lost in 1 M GdnHCl solution. This further demonstrates that the structure required for chaperone function toward LDH is more labile in the mutant abb′a′, as compared with PDI. This indicates a role of domain c in stabilization of the functional conformation of PDI.

DISCUSSION

Protein disulfide isomerase is an abundant protein in the endoplasmic reticulum (24); the concentration of PDI in the endoplasmic reticulum lumen is estimated to be 100–200 μM, a level comparable (or in excess of) that of newly synthesized proteins (24–26). Coexpression of PDI with human lysozyme in yeast results in an increase in the yield of native lysozyme produced (27). This indicates that PDI is a catalyst of intracellular protein folding. In this case, the secretion of lysozyme increases even when the isomerase activity of PDI is disrupted, suggesting that the effect is related to a function of PDI other than catalysis of disulfide bond formation. This finding demonstrates that PDI lacking isomerase activity assists protein folding *in vivo*. LDH is an important cytoplasmic enzyme in carbohydrate metabolism (28) and is commonly used as a substrate for chaperone-assisted folding *in vitro*.

The role of domain c in the chaperone activity of PDI has been a matter of debate for some years. In 1997, Noiva *et al.* (2) hypothesized that the C-terminal region could be important for the chaperone activity of PDI. Two years later, Koivunen *et al.* (10) constructed 17 PDI mutants, including the mutant with residues 463–491 truncated (abb′a′). They found this mutant to be fully active as a chaperone and a disulfide isomerase, suggesting that domain c did not have a significant role in the chaperone or isomerase activities of PDI. To further investigate the function of the C-terminal region, Wang and co-workers (4, 29) constructed a mutant of PDI (ABB′A′), truncated after residue 440. The mutant ABB′A′ displays isomerase activity similar to that of the intact molecule but does not show chaperone activity in assisting refolding of denatured D-glyceraldehyde-3-phosphate dehydrogenase. The mutant abb′a′ differs from ABB′A′ in containing an additional stretch of 22 amino acid residues in the C-terminal region (441–462, Fig. 1). The mutant abb′a′ has both the chaperone and the isomerase activities of wild-type PDI, suggesting that this 22-residue region is involved in the chaperone function of PDI.

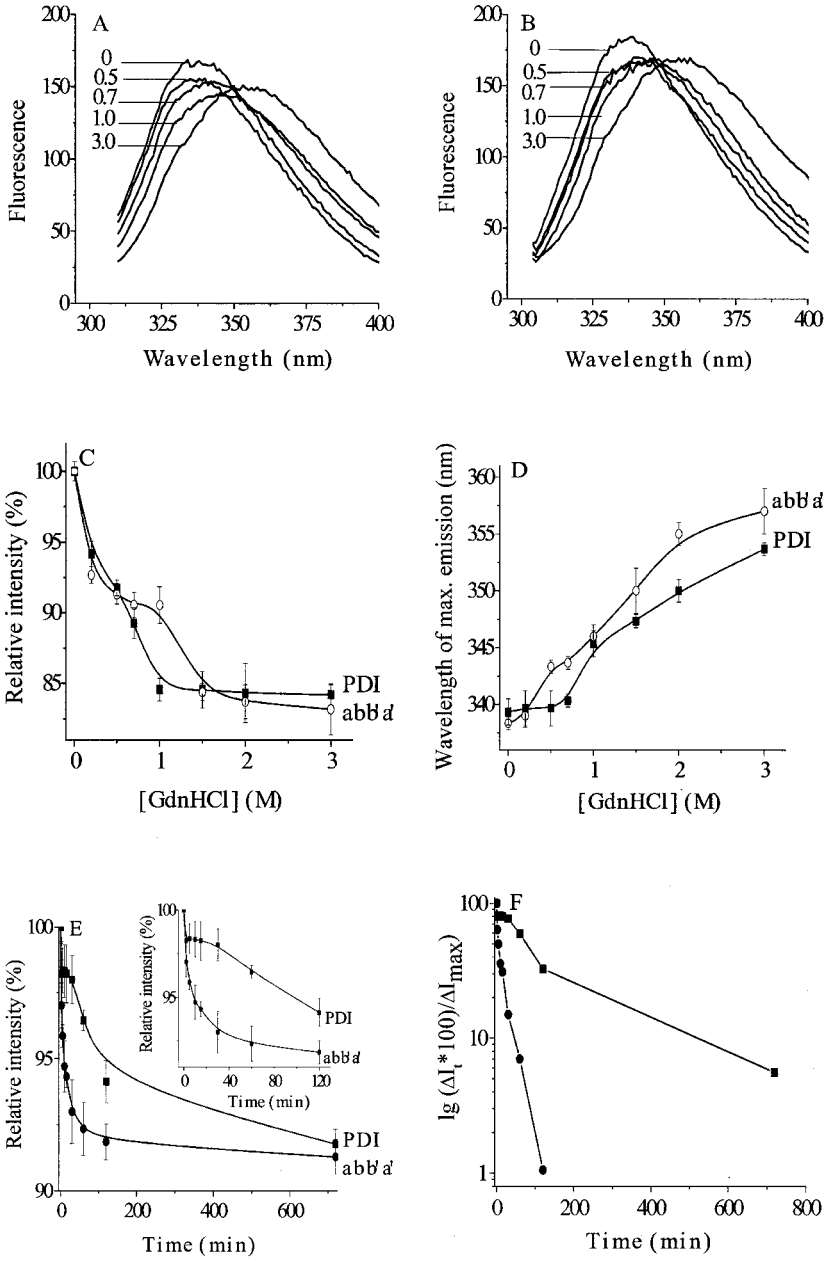

FIG. 6. **Effects of GdnHCl on the fluorescence of PDI or abb′a′.** PDI or abb′a′ (final concentration, 0.3 μM) was incubated in the desired concentration of GdnHCl for 12 h to reach equilibrium followed by measurement of intrinsic fluorescence by excitation at 295 nm (slit widths: $E_x = E_m = 5$ nm) at 37 °C. *A* and *B*, the fluorescence spectra of PDI or abb′a′. The GdnHCl concentrations were as indicated in the figures. *C*, the changes in the fluorescence intensities. The maximum emission of PDI or abb′a′ without denaturant was taken as 100%. *D*, the changes in the maximal emission wavelength. *E*, the kinetics of the change in fluorescence intensity of PDI or abb′a′ upon the addition of 0.5 M GdnHCl. The *inset* is the same data over a shorter time scale. *F*, the same data as in *panel C* plotted semilogarithmically as $((\Delta I_t/\Delta I_{max})*100)$, where ΔI_t represents changes in the intrinsic fluorescence intensity with time, and ΔI_{max} represents the maximal fluorescence change (30).

TABLE II
First order rate constants for changes in intrinsic fluorescence of PDI or abb′a′ in 0.5 M GdnHCl solution

	Intrinsic fluorescence	
	Fast phase	Slow phase
	$\times 10^{-5}\ s^{-1}$	$\times 10^{-5}\ s^{-1}$
PDI	14.4 ± 2.02	4.46 ± 0.92
abb′a′	82.7 ± 9.57	

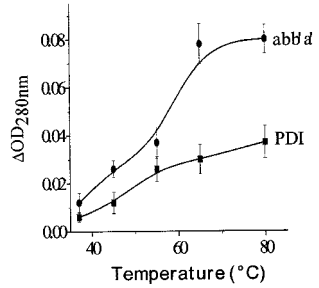

FIG. 7. **Difference absorbance of PDI or abb′a′ at different temperatures.** PDI or abb′a′ (final concentration, 1 μM) was incubated in phosphate buffer at different temperatures followed by measurement of the difference absorbance at 280 nm until the absorbance showed no further observable changes. The difference absorbance is plotted for each temperature, relative to the value at 25 °C.

Koivunen *et al.* (10) reported that abb′a′ has chaperone activity of about 91% as compared with PDI. In our experiments, similar results were obtained for the reactivation and aggregation of GdnHCl-denatured LDH. Under these conditions, the functional conformation of both PDI and abb′a′ is maintained. These results then support the conclusion of Koivunen *et al.* (10) that the acidic domain c is not critical for chaperone function of PDI. However, the results we obtained from thermal denaturation support the hypothesis proposed by Noiva *et al.* (2). The mutant abb′a′ is prone to self-aggregation and causes increased aggregation of LDH during thermal denaturation, whereas PDI is stable. It is possible that the self-aggregation of abb′a′ induces LDH molecules to aggregate by noncovalent

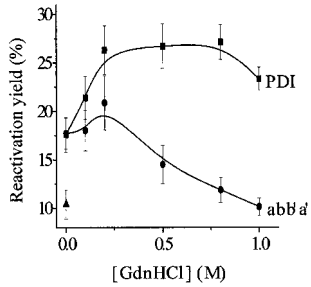

FIG. 8. **Effects of GdnHCl on the chaperone activity of PDI or abb′a′.** PDI or abb′a′ (final concentration, 100 μM) was incubated in phosphate buffer containing GdnHCl at different concentrations at 37 °C overnight. The LDH reactivation process and conditions were as described in the legend for Fig. 2, except that the GdnHCl-incubated PDI and abb′a′ were added at a molar ratio ([chaperone]/[LDH]) of 10 and the reactivation mixtures were kept at 25 °C for 2 h before the assay.

interaction of the aggregates, which then co-precipitate. The rapid formation of aggregates followed by sedimentation of the precipitates leads to the observed increase in the intensity of the light scattering followed by a decrease with time (Fig. 4*B*). This then suggests that domain c is indispensable for maintenance of the native state of PDI under thermal denaturation conditions, and this native structure must be maintained to preserve the chaperone activity of PDI toward LDH. The increased stability of wild-type PDI as compared with abb′a′ toward denaturation by GdnHCl (Fig. 6) or temperature (Fig. 7) supports this conclusion. The increased effect of GdnHCl on abb′a′ as compared with PDI when monitoring reactivation of LDH (Fig. 8) further demonstrates this point. Thus, the data presented here suggest that domain c is critical for PDI chaperone function, due to its role in stabilization of the functional conformation under extreme conditions.

Acknowledgments—We are sincerely grateful to Dr. Chih-Chen Wang for the valuable suggestions regarding this work and to Dr. S. Perrett for editing this study. We thank Ya-Qun Zhang for technical assistance.

REFERENCES

1. Wang, C. C., and Tsou, C. L. (1993) *FASEB J.* **7,** 1515–1517
2. Noiva, R., Onodera, S., Schwaller, M. D., and Irwin, W. A. (1997) in *Prolyl 4-Hydroxylase, Protein Disulfide Isomerase and Other Structurally Related Proteins* (Guzman, N. A., ed) pp. 369–397, Marcel Dekker, Inc., New York
3. Cai, H., Wang, C. C., and Tsou, C. L. (1994) *J. Biol. Chem.* **269,** 24550–24552
4. Song, J. L., and Wang, C. C. (1995) *Eur. J. Biochem.* **231,** 312–316
5. Puig, A., and Gilbert, H. F. (1994) *J. Biol. Chem.* **269,** 7764–7771
6. Yao, Y., Zhou, Y. C., and Wang, C. C. (1997) *EMBO J.* **16,** 651–658
7. Song, J. L., Quan, H., and Wang, C. C. (1997) *Biochem. J.* **328,** 841–846
8. Edman, J. C., Ellis, L., Blacher, R. W., Roth, R. A., and Rutter, W. J. (1985) *Nature* **317,** 267–270
9. Kemmink, J., Darby, N. J., Dijkstra, K., Nilges, M., and Creighton, T. E. (1997) *Curr. Biol.* **7,** 239–245
10. Koivunen, P., Pirneskoski, A., Karvonen, P., Ljung, J., Helaakoski, T., Notbohm, H., and Kivirikko, K. I. (1999) *EMBO J.* **18,** 65–74
11. Freedman, R. B., Hirst, T. R., and Tuite, M. F. (1994) *Trends Biochem. Sci.* **19,** 331–336
12. Pelham, H. R. B. (1990) *Trends Biochem. Sci.* **15,** 483–486
13. Jaenicke, R., Koberstein, R., and Teuscher, B. (1971) *Eur. J. Biochem.* **23,** 150–159
14. Ruoppolo, M., Orrù, S., Talamo, F., Ljung, J., Pirneskoski, A., Kivirikko, K. I., Marino, G., and Koivunen, P. (2003) *Protein Sci.* **12,** 939–952
15. Yu, X. C., Wang, C. C., and Tsou, C. L. (1994) *Biochim. Biophys. Acta* **1207,** 109–113
16. Ma, Y. Z., and Tsou, C. L. (1991) *Biochem. J.* **277,** 207–211
17. Rudolph, R., Zettlmeissl, G., and Jaenicke, R. (1979) *Biochemistry* **18,** 5572–5575
18. Dams, T., Ostendorp, R., Ott, M., Rultkat, K., and Jaenicke, R. (1996) *Eur. J. Biochem.* **240,** 274–279
19. Hana, T. B., and Alexander, L. (1976) *Biochim. Biophys. Acta* **445,** 261–279
20. Chen, Y. H., He, R. Q., Liu, Y., and Xue, Z. G. (2000) *Biochem. J.* **351,** 233–240
21. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
22. Pihlajaniemi, T., Helaakoski, T., Tasanen, K., Myllylä, R., Huhtala, M. L., Koivu, J., and Kivirikko, K. I. (1987) *EMBO J.* **6,** 643–649
23. Churchich, J. E. (1999) *Biochem. Biophys. Res. Com.* **261,** 41–45
24. Noiva, R. (1999) *Semin. Cell Dev. Biol.* **10,** 481–493
25. Freedman, R. B., Bulleid, N. J., Hawkins, H. C., and Paver, J. L. (1989) *Biochem. Soc. Symp.* **55,** 167–192
26. Lyles, M. M., and Gilbert, H. F. (1991) *Biochemistry* **30,** 613–619
27. Hayano, T., Hirose, M., and Kikuchi, M. (1996) *FEBS Lett.* **377,** 505–511
28. Jaenicke, R., and Rudolph, R. (1990) in *Protein Structure* (Creighton, T. E., ed) pp. 191–223, IRL Press, Oxford
29. Dai, Y., and Wang, C. C. (1997) *J. Biol. Chem.* **272,** 27572–27576
30. Tsou, C. L. (1965) *Acta Biochim. Biophys. Sin.* **5,** 398–417