A Mutant Truncated Protein Disulfide Isomerase with No Chaperone Activity*

Yong Dai and Chih-chen Wang†

From the National Laboratory of Biomacromolecules, Institute of Biophysics, Academia Sinica, Beijing 100101, China

A mutant human protein disulfide isomerase with the COOH-terminal 51 amino acid residues deleted (abb’a’) has been expressed in Escherichia coli. Its secondary structures are very similar to those of the native bovine enzyme. The mutant enzyme shows neither peptide binding ability nor chaperone activity in assisting the refolding of denatured D-glyceraldehyde-3-phosphate dehydrogenase but keeps most of the catalytic activities for reduction of insulin and isomerization of scrambled ribonuclease. It assists the reactivation of denatured and reduced proteins containing disulfide bonds, acid phospholipase A2, and lysozyme to different levels, which are significantly lower than those by the native bovine enzyme.

Protein disulfide isomerase (PDI),1 as one of the two foldases thus far characterized (1), has attracted much attention in studies on protein folding. The enzyme catalyzes the formation, reduction, or isomerization of disulfide bonds of proteins depending on the redox potentials in in vitro systems (2) and is responsible for the formation of native disulfide bonds of nascent peptides in vivo (3, 4). Although the three-dimensional structure has not yet been reported, the PDI molecule has been suggested to be constructed of several domains in the order of -α-β-α’-c as inferred from its cDNA deduced sequence (5) and structural studies (6, 7). The sequences of -CGHC- in domain α and α’ and a peptide fragment of 26 amino acid residues in the COOH-terminal c domain have been identified, respectively, to be the active sites (8, 9) and the peptide binding site (10–12). PDI is also a remarkable multifunctional protein (13). In addition to its isomerase activity it is the essential β subunit of prolyl-4-hydroxylase (14, 15) and the small subunit of microsomal triglyceride transfer protein complex (16).

It has recently been suggested that PDI is not only an isomerase but also has chaperone activity (3, 17, 18). This was supported by its assistance in the in vitro refolding of denatured proteins with no disulfide bond, such as D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (19) and ribulose diphosphate carboxylase (20) as well as disulfide-containing proteins such as lysozyme (21) and acid phospholipase A2 (APLA2) (22), although Lilio et al. (23) had reported that PDI showed no chaperone effect on the refolding of denatured immunoglobulin Fab with intact disulfides. A PDI mutant, inactive as an isomerase, has the same function as an essential subunit for the assembly of the fully active tetramer of prolyl-4-hydroxylase α2β2 (24) and the dimer of microsomal triglyceride transfer protein complex (25), suggesting that the role of PDI in the above two proteins is independent of its isomerase activity but related to its chaperone-like peptide binding function. The deletion of the NH2-terminal 3 amino acid residues of the peptide binding region of human PDI indeed prevents prolyl-4-hydroxylase tetramer formation (25, 26). It was shown that a mutant PDI with Ser substituted for Cys at the -CGHC- active sites and devoid of isomerase activity can increase the folding and secretion of lysozyme coexpressed in yeast (27). The yeast bearing a mutant PDI shortened from the COOH terminus and devoid of the putative peptide binding region in the middle part of the molecule can hardly survive (28). It has been shown in our previous work that the chaperone activity of PDI in assisting GAPDH refolding is suppressed by competitive peptide binding (29), which also inhibits its enzymatic activity (12, 29).

In this paper a mutant human PDI, abb’a’, with the deletion of the COOH-terminal 51 amino acid residues responsible for peptide binding, has been expressed in Escherichia coli. The mutant shows neither peptide binding ability nor chaperone activity in assisting the refolding of denatured GAPDH but displays most of the catalytic activities of PDI and assists the reactivation of denatured and reduced APLA2 and lysozyme to an extent significantly lower than those by the native bovine enzyme.

EXPERIMENTAL PROCEDURES

Materials—The plasmid pBR322-PDI, containing the full-length of human PDI (hPDI) cDNA (11), is a generous gift from Prof. K. Kivirikko, University of Oulu, Finland. GAPDH was from rabbit muscle (30). APLA2 was from Agkistrodon b. brevicaudus (Agkistrodon halys Pallas) (31). PDI was prepared from bovine liver (bPDI) essentially according to Lambert and Freedman (32) and showed one band on SDS-polyacrylamide gel electrophoresis with a specific activity of more than 800 units/g; S-Carboxymethylated A-chain of insulin was prepared according to Zheng et al. (33). Modified PDI (mPDI) carboxymethylated at thiol(s) in the -CGHC- sequence of active sites, was prepared as described previously (29).

Restriction endonucleases, T4 DNA ligase, isopropyl 1-thio-β-D-galactopyranoside, and dithiothreitol were from Promega. Vent DNA polymerase and the large (Klenow) fragment of DNA polymerase I were from New England Biolabs, Inc. The prokaryotic gene fusion expression vector pGEX-4T-1, E. coli BL21 strain (F + ompT, Rd + mB + ), the T7 sequencing kit, and glutathione-Sepharose 4B were from Pharmacia Biotech Inc. n35S-DATP was obtained from NEN Life Science Products. 5,5'-Dithio-bis(2-nitrobenzoic acid) was from Fluka, Glutathione (GSH), glutathione disulfide (GSSG), NAD+ (98%), and NADPH (type III) were from Boehringer Mannheim. Thrombin, glutathione reductase (yeast, type III), glyceraldehyde 3-phosphate, 8-anilino-1-naphthalenesulfonic acid (ANS), bovine serum albumin (BSA, 98–99% albumin, fraction V),

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Characterization of the Expressed abb a’—The primer I (5’-CGGGATCCGGCCGCCGAGGAG-3’) was designed to hybridize with the first 15 nucleotides at the 5’-terminus of the hPDI cDNA and contains a BamHI site (underlined) just before the sequence. The reverse primer II (5’-GGTTAGTATACGGATGATCG-3’) with a stop codon (underlined) hybridizes with the sequence between 1309 and 1320 base pairs of the hPDI cDNA. The double-stranded 1.3-kilobase DNA fragment coding for the sequence of the Asp9 to Tyr46 of PDI (abb a’) was generated by polymerase chain reaction using Vent DNA polymerase with the above two primers and pBR322-PDI as a template. The polymerase chain reaction product was elongated to blunt ends in both termini with the large (Klenow) fragment of DNA polymerase I, digested with BamHI at the 5’-terminus, and ligated into pGEX-4T-1 digested with BamHI and Smal in-frame with the glutathione S-transferase fusion codons to construct the expression plasmid pGEX-abb a’. The foreign DNA sequences cloned into the expression plasmid were verified by nucleotide sequencing.

Gene Expression and Purification of abb a’—Protein production was carried out in E. coli strain BL21 containing the pGEX-abb a’ plasmid. Cells grown overnight at 37 °C in LB medium were diluted 100-fold and grown at 28 °C with vigorous shaking to an A660 of 0.8, and then grown for another 4 h after adding 0.1% isopropyl 1-thio-β-D-galactopyranoside. The cell pellet was disrupted by sonication and then mixed gently with 1% Triton X-100 by stirring for 30 min at room temperature. The supernatant of cell lysate was loaded onto a 2-ml glutathione-Sepharose 4B RediPack column, and the fusion protein on the matrix was cleaved with 100 units of thrombin at 22 °C for 16 h. The eluted abb a’ fraction was loaded onto a Mono Q HR (5/5) column equilibrated with 20 mM sodium phosphate, pH 8.0, containing 8 M GdnHCl and 150 mM dithiothreitol at room temperature for 4 h. The reaction mixture was brought to pH 2.0 with 6N HCl, dialyzed first against 0.01N HCl for 3 h and then with 1% Triton X-100 by stirring for 30 min at room temperature. The supernatant was loaded onto a 2-ml glutathione-Sepharose 4B RediPack column, and the fusion protein on the matrix was cleaved.

Circular Dichroism (CD) and ANS Fluorescence Spectra Analysis—CD spectrum determinations in the far ultraviolet region from 200 to 250 nm were carried out with a Jasco J720 spectropolarimeter at 25 °C. ANS fluorescence spectra were measured at 25 °C in a Hitachi F-4010 spectrophotometer with an excitation wavelength of 385 nm. The protein concentrations of both PDI (dotted line) and abb a’ (solid line) were determined spectrophotometrically at 280 nm with the following absorption coefficients: 1.00 for PDI, 0.70 for BSA, and 0.65 for abb a’. The A280 value of abb a’ was determined to be 0.83. For the convenience of comparison, both GAPDH and PDI are considered as protomers in the calculation of molar ratios. The above result is highly suggestive that the molecular weight of abb a’ is about 48,000 as expected.

Secondary Structure of abb a’—As shown in Fig. 2, the CD spectrum of abb a’ is almost the same as that of bPDI, indicating that the COOH-terminal shortened enzyme has secondary structures closely similar to that of bPDI. The same ANS fluorescence spectra of bPDI and abb a’ indicated that the truncation of COOH-terminal 51 residues of PDI has little effect on the surface hydrophobicity of the intact molecule (data not shown).

Enzymatic Activities of abb a’—The mutant abb a’ has about 69 ± 3% of isomerase activity and 80 ± 2% of TOR activity compared with that of bPDI (Table I), suggesting that the truncation of the COOH-terminal 51 amino acid residues of PDI has only a minor effect on these enzymatic activities.

Effects of S-Carboxymethylated A-chain of Insulin on the TPOR Activity of abb a’—As shown in Fig. 3, the presence of S-carboxymethylated A-chain of insulin inhibits the TPOR activity of PDI; in sharp contrast, it has no effect on the TPOR activity of abb a’. The above result is highly suggestive that the S-carboxymethylated A-chain of insulin inhibits the TPOR activity of PDI by binding at its peptide binding site, which is lacking in the mutant abb a’.

RESULTS

Characterization of the Expressed abb a’—The inserted coding sequence for abb a’ in the expression plasmid pGEX-abb a’ was verified by DNA sequence analysis (data not shown). The constructed protein should contain residues 1–440 and an additional Gly-Ser at the NH2 terminus of hPDI. Experimental conditions for sonication, growth temperature, and isopropyl β-D-galactopyranoside concentration were optimized by 10% SDS-polyacrylamide gel electrophoresis analysis (data not shown). Fig. 1 shows the high yield of abb a’ expression and the homogeneity of the purified product. The molecular weight of abb a’ is about 48,000 as expected.

Effects of S-Carboxymethylated A-chain of Insulin on the TPOR Activity of abb a’—As shown in Fig. 3, the presence of S-carboxymethylated A-chain of insulin inhibits the TPOR activity of PDI; in sharp contrast, it has no effect on the TPOR activity of abb a’.
Effects of abb’a on Reactivation of Denatured GAPDH—Fig. 4 shows that the reactivation of GAPDH in the presence of bPDI increases from 4 to 19% with the increase of the molar ratio of PDI to GAPDH from 0 to 10. In contrast, with BSA used for comparison, abb’a at the same range of ratios shows no effect on the reactivation of denatured GAPDH. This result indicates the necessity of the peptide binding site of PDI for its chaperone activity in the reactivation of GAPDH.

Effects of abb’a on the Reactivation of GdnHCl-denatured and Reduced Lysozyme—It has been proposed that the foldase activity of PDI consists of both its isomerase and chaperone activities and the latter activity can be fully replaced by mPDI (22), which is devoid of isomerase activity but nearly as active as native PDI in its chaperone activity in assisting the reactivation of GAPDH (29). The reactivation of GdnHCl-denatured and reduced APLA₂ containing seven disulfide bonds, upon dilution in the presence and absence of abb’a and/or mPDI was determined to examine the foldase activity of abb’a. Fig. 5A shows that the spontaneous reactivation of APLA₂ at 12 μM is only about 1%, and the reactivation in the presence of abb’a takes 10 h to reach completion as in the presence of PDI. In the presence of abb’a the reactivation yield increases with increasing concentrations of abb’a to a maximal level of 15% when the molar ratio of abb’a to APLA₂ approaches 2 as shown in Fig. 5B. Higher ratios of abb’a has little further effect on the reactivation of APLA₂, mPDI alone has no effect on the reactivation of APLA₂; however, the simultaneous presence of both mPDI and abb’a increases markedly the reactivation yield of APLA₂ compared with the same amount of abb’a alone in the refolding buffer. In presence of mPDI at 36 μM together with abb’a at 120 μM the reactivation yield of APLA₂ approaches 48%, about the same as the maximal level obtainable by native bPDI. Further increases in the concentrations of either mPDI or abb’a or both have no further effect on the reactivation of APLA₂.

Effects of abb’a on the Refolding of Denatured and Reduced Lysozyme—As shown in Fig. 6 the very low spontaneous reactivation of denatured and reduced lysozyme in phosphate buffer increases with increasing concentrations of bPDI in the refolding buffer to a maximal level of around 71% at a stoichiometric amount of PDI. However, the maximal reactivation yield of lysozyme in presence of abb’a is reduced to 58% under the same condition.

**FIG. 4. Effects of concentrations of bPDI and abb’a on the reactivation of GAPDH.** The refolding was initiated by a 100-fold dilution of GdnHCl-denatured GAPDH to 2.8 μM in 0.1 M phosphate buffer, pH 7.5, containing 5 mM dithiothreitol, 1 mM EDTA, and PDI (●) or abb’a (○) at different ratios as indicated. BSA was used for comparison (●). The activity recovery was determined 2 h after dilution.

**FIG. 5.** Effect of S-carboxymethylated A-chain of insulin on the TPOR activity of bPDI and abb’a. The coupled reaction mixture contained 2.8 μM insulin, 8 mM GSH, 100 μM NADPH, 1 unit of glutathione reductase, 0.7 μM PDI (●) or 0.83 μM abb’a(○), and different concentrations of S-carboxymethylated A-chain of insulin as indicated. The decrease in absorption at 340 nm of NADPH was followed.

**TABLE I**

| Chaperone | Isomerization | Reduction |
|-----------|---------------|-----------|
| PDI       | +             | 100       | 100       |
| abb’a     | -             | 69 ± 3    | 80 ± 2    |
| mPDI      | +             | -         | -         |

**FIG. 3.** Effect of S-carboxymethylated A-chain of insulin in the reactivation of APLA₂. mPDI alone has no effect on the reactivation of APLA₂. mPDI alone has no effect on the reactivation of APLA₂. In presence of mPDI at 36 μM together with abb’a at 120 μM the reactivation yield of APLA₂ approaches

48%, about the same as the maximal level obtainable by native bPDI. Further increases in the concentrations of either mPDI or abb’a or both have no further effect on the reactivation of APLA₂.

**DISCUSSION**

From the nearly identical CD spectra and ANS binding, in addition to similar enzyme activities between bPDI and abb’a, it appears that the addition of 2 extra residues at the NH₂ terminus and the deletion of the COOH-terminal 51 amino acid residues show little effect on the secondary structures, the surface hydrophobicity, and the enzymatic activity of PDI. The NH₂-terminal sequence of PDI seems to be of little importance as it has also been found that even an extension of 10 residues at the NH₂ terminus showed no deleterious effect on the properties of PDI (38). The mutant abb’a is structurally stable and retains most of the isomerase and oxidoreductase activities of PDI, suggesting that it is more or less independent from the c domain, but the involvement of the c domain in the above activities cannot be excluded, apart from the peptide binding site; it could also contribute to substrate binding. Alternatively, deletion of the c domain could alter somewhat the functional conformation of PDI, leading to decreased enzymatic activities. The peptide inhibition of the TPOR activity of PDI suggests its binding at the peptide binding site of PDI (12, 29, 33). In this respect, it is not surprising that the TPOR activity of abb’a is not inhibited by the presence of an excess of the peptide as abb’a no longer has the c domain, which is the major if not the only peptide binding site (11). The above results indicate that, contrary to the previous suggestion (29), the c domain does not appear to be essential for the enzymatic activities of PDI, and the peptide binding site and the substrate binding site are not identical. However, the above does not exclude the possibility...
that occupation at the peptide binding site could interfere with substrate binding to the substrate binding site, and the c domain could partially contribute to the substrate binding site, thus the peptide and substrate binding sites could be close to and overlapping with each other. This appears to be different from the E. coli trigger factor, in which the active site of peptidyl-prolyl cis/trans-isomerase is separated widely enough from the peptide binding site so that the binding of an unfolded protein does not interfere with the catalysis of prolyl cis/trans-isomerization in a small peptide (39).

As shown previously, the presence of a peptide in the refolding buffer suppresses GAPDH reactivation assisted by PDI, indicating that peptide binding in competition with the GAPDH folding intermediates prevents and suppresses the PDI-assisted refolding of GAPDH (29). In this respect, it is to be expected and is actually found that abb‘a’ is unable to assist the reactivation of denatured GAPDH and provides a straightforward demonstration that the peptide binding site at the GAPDH-assisted refolding of GAPDH (29). In this respect, it is to be expected and is actually found that abb‘a’ is unable to assist the reactivation of denatured GAPDH and thus provides a straightforward demonstration that the peptide binding site at the reactivation of denatured GAPDH and thus provides a straightforward demonstration that the peptide binding site at the reactivation of denatured GAPDH and thus provides a straightforward demonstration that the peptide binding site at the reactivation of denatured GAPDH and thus provides a straightforward demonstration that the peptide binding site at the reactivation of denatured GAPDH and thus provides a straightforward demonstration that the peptide binding site at the reactivation of denatured GAPDH and thus provides a straightforward demonstration that the peptide binding site at the reactivation of denatured GAPDH and thus provides a straightforward demonstration that the peptide binding site at the reactivation of denatured GAPDH and thus provides a straightforward demonstration that the peptide binding site at

FIG. 5. Reactivation of APLA₂ assisted by abb‘a’ and/or mPDI. Reduced and denatured APLA₂ by 4 M GdnHCl was diluted 50-fold to 12 μM in 20 mM Tris buffer containing 1 mM GSSG, 2 mM GSH, and various concentrations of different PDI derivatives. Panel A, the molar ratios of abb‘a’ to APLA₂ were: 0 (●), 0.1 (○), 0.5 (■), 1 (□), 2 (▲), and 5 (○). Panel B, the reactivation of APLA₂ was carried out in the presence of 36 μM abb‘a’ (□), mPDI (●), abb‘a’ (○), or the simultaneous presence of mPDI at 12 μM (■), 36 μM (○), or 60 μM (▲) and abb‘a’ at different ratios as indicated.

FIG. 6. Effects of concentrations of abb‘a’ or bPDI on the reactivation of lysozyme. The conditions for refolding of lysozyme at 10 μM were as described under “Experimental Procedures” with the presence of different concentrations of abb‘a’ (○) or bPDI (●) in phosphate buffer.

the c domain of PDI is directly responsible for its chaperone activity.

It is widely accepted that PDI plays a critical role in nascent peptide folding by catalyzing the formation of native disulfide(s). However, PDI as a foldase not only catalyzes disulfide isomerization but is also intrinsically involved in peptide chain folding through its peptide binding site(s). Therefore PDI most likely binds with folding intermediates at different folding stages (see Scheme 1). The two processes of disulfide formation and peptide folding are intimately interdependent and work in cooperation in the generation of the native conformation of disulfide-containing proteins. The chemically modified PDI alkylated at active site cysteine residues is devoid of isomerase activity but retains its chaperone activity almost fully (29). In this report, the COOH-terminal truncated PDI shows most of the catalytic activity but is devoid of any chaperone activity. Comparisons of the properties and the possible roles of these derivatives in assisting protein folding are summarized in Table I and Scheme 1, respectively.

For spontaneous refolding (the central lines in a, b, and c) the denatured and reduced protein (U) would undergo a fast conformational change upon dilution to form intermediate (I₁), which could fold first to I₂ and finally to native molecule (N) through both folding steps and oxidative disulfide formation. Both intermediates I₁ and I₂ face two alternative folding pathways, correct folding to the native molecule (N) and misfolding leading to aggregation (A). The presence of PDI and/or its derivatives affect the relative proportion between the alternative pathways. As shown in Scheme 1a, PDI, being a chaperone, binds with I₁ at its peptide binding site of domain c and prevents the aggregation of I₁. On the other hand, PDI also probably binds at the substrate binding site with I₂, which is assumed to represent a folding intermediate better folded than I₁ with thios properly paired to be oxidized to the native disulfide(s) and also decrease aggregation and increase reactivation. As shown in Scheme 1b, abb‘a’, devoid of a peptide binding site, no longer binds with I₁ but binds indeed with I₂ through its substrate binding sites and catalyzes disulfide formation, leading to the native molecule (N) with significantly lower efficiency than PDI does. This could also explain the fact that the reactivation of APLA₂ assisted by abb‘a’ at all concentrations shows a lag phase for the first 30 min (Fig. 5A) in
and properties of folding intermediates of the substrates on the one hand and in the specificity of PDI itself as a chaperone or an isomerase on the other. For APLA\textsubscript{2}, the maximal reactivation level decreased from 45 to 15% when PDI was replaced by abb\textsuperscript{a}, whereas for lysozyme, the level decreased from 71 to 58%. It appears that for APLA\textsubscript{2} refolding the chaperone function of PDI is more important compared with the refolding of lysozyme.

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