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Functional properties of PDIA from *Aspergillus niger* in renaturation of proteins

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Abstract

Functional properties of protein disulfide isomerase A (PDIA) from *Aspergillus niger* were investigated using ribonuclease A, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and prochymosin as substrates. PDIA was shown to function as an isomerase catalyzing the refolding of denatured and reduced ribonuclease A. PDIA also exhibited trx-independent chaperone activity preventing the aggregation of reduced, denatured GAPDH, an enzyme lacking disulfide bonds. Both isomerase activity and chaperone function of PDIA were essential for the efficient refolding of the reduced, denatured prochymosin.

Keywords: Protein disulfide isomerase A; *Aspergillus niger*; Chaperone function; Isomerase activity; Refolding

1. Introduction

The filamentous fungus, *Aspergillus niger*, is an attractive host for the commercial production of heterologous proteins. However, the production levels of heterologous proteins are usually much lower than that of homologous proteins. Previous works show that the main bottlenecks are post-transcriptional [1,2]. One of the most important post-transcriptional processes is folding and assembly of newly synthesized polypeptides by molecular chaperones and foldases in the endoplasmic reticulum (ER). Recent strategies to optimize expression have focused on the manipulation of the levels of chaperones and foldases. The most abundant foldase in the ER is protein disulfide isomerase (PDI, EC 5.3.4.1) [3]. The gene, pdiA, encoding PDIA was isolated from *A. niger* [4] and it was responsive to the overexpression of heterologous proteins and induced by the accumulation of unfolded nascent polypeptides in the ER [5]. However, the functions of PDIA have not been well investigated.

It has been reported that mammalian PDI acts as an isomerase catalyzing the formation, reduction and isomerization of disulfide bonds, and also acts as a molecular chaperone independent of its isomerase activity facilitating the renaturation of denatured proteins by suppressing aggregation [6,7]. However, the functional properties of PDIs from various origins are different, for example, yeast PDI has no independent chaperone activity [8,9]. Here, we investigated the enzyme and chaperone activities of PDIA and demonstrated both activities are required for the efficient refolding of prochymosin.

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2. Materials and methods

2.1. Chemical

Pancreatic ribonuclease A, D-glyceraldehyde-3-phosphate dehydrogenase, bovine serum albumin, cCMP, DTT, GSH and GSSG were from Sigma.

2.2. Cloning of the PDIA gene and generation of PDIA mutants

The total RNA from *A. niger* strain T21 was isolated according to the method of guanidinium-acid-phenol-chloroform [10]. The cDNA of PDIA was amplified by RT-PCR and inserted into the *Nde*I–*Hind*III restriction sites of pET-28a (Novagen). The cDNA was sequenced and assigned the Accession No. AY770494 in GenBank (www.ncbi.nlm.nih.gov/genbank/). Various trx-motif mutations were introduced by site-directed mutagenesis that was performed using the megaprimer method [11]. All mutations were verified by sequencing.

2.3. Expression and purification of PDIA and its mutants

PDIA and its mutants were expressed in *Escherichia coli* strain BL21(DE3). Cells were grown at 37 °C in LB medium supplemented with 30 μg/ml kanamycin to OD_{600} about 0.6 and induced with 1 mM isopropyl-β-D-thiogalactopyranoside. After a further 4 h of cultivation, cells were harvested by centrifugation, washed, suspended in buffer A (20 mM sodium phosphate, pH 7.5, 500 mM NaCl, 50 mM imidazole) and sonicated. The supernatant was applied to a Ni^{2+}-chelating resin column equilibrated with buffer A. After extensive washing with buffer A, the protein was eluted with a linear gradient of 0.05–0.5 M imidazole. Proteins were then further purified by DEAE-Sepharose fast flow column equilibrated with buffer B (20 mM sodium phosphate buffer, 100 mM NaCl, pH 6.3), and eluted with a linear gradient of 0.1–0.6 M NaCl in buffer B. All preparations were >90% homogeneous on SDS–PAGE. All purified proteins were dialyzed against 50 mM NH_4HCO_3, lyophilized and stored at −20 °C. The concentration of purified proteins was determined according to the method of Bradford [12].

2.4. Assay of disulfide isomerase activity

The disulfide isomerase activity was assayed by monitoring the renaturation of 2 μM reduced, denatured ribonuclease A in 0.2 mM GSSG, 1.0 mM GSH, 4.5 mM cCMP, 2 mM EDTA, 100 mM Tris–HCl (pH 8.0) and 1.4 μM PDIA or mutants, at 25 °C as described by Gilbert [13]. The activity was reported as the initial velocity of ribonuclease A renaturation determined from the steady-state slope of the derivative plot after any lag period.

2.5. Assay of chaperone activity

The chaperone activity was assayed by monitoring the renaturation of reduced, denatured GAPDH. Denaturation and assisted reactivation of GAPDH by PDIA were performed as described by Cai et al. [6]. The activity of properly refold GAPDH was measured as the production of NADH from NAD^+ at 340 nm using a Beckman DU 800 spectrophotometer. Aggregation was monitored at 488 nm using a Hitachi F2500 Spectrofluorometer.

2.6. Determination of prochymosin refolding

Prochymosin was prepared as described previously [14] and denatured in 50 mM potassium phosphate, 50 mM NaCl, 1 mM EDTA, 8 M urea, and 130 mM DTT, pH 11, at 30 °C for 2 h then subjected to gel filtration to remove unreacted DTT. Refolding of prochymosin was performed in 50 mM potassium phosphate buffer, 50 mM NaCl, 1 mM EDTA, pH 8, 1 mM GSH, and 2 mM GSSG by 50-fold dilution of the unfolded prochymosin to a final concentration of 1 μM and incubated at 25 °C for 9 h. The renatured prochymosin was acidified and measured by the method of Emtage et al. [15]. The reactivation was expressed as a percentage of active chymosin detected in relation to the prochymosin input in the refolding solution.

3. Results

3.1. Catalytic activity of PDIA

The catalytic activity of PDI was maintained by the reversible reduction and oxidation of an internal disulfide bond between the two cysteine residues in trx-motifs [3]. For a more detailed analysis of the two trx-motifs of PDIA, various trx-motif mutants of PDIA were constructed in which either of the two trx-motifs ($N_{SS}C_{CC}$, $N_{CC}C_{SS}$) or both ($N_{SS}C_{SS}$) were inactivated through the replacement of the cysteine residues by serine residues, or one of the cysteine residues in the trx-motifs was replaced ($N_{CS}C_{CS}$, $N_{SC}C_{SC}$) (Table 1).

Table 1
Alterations introduced into the thioredoxin motifs of mutant proteins

Protein	N-terminal trx-motif	C-terminal trx-motif
Wild-type PDIA	Cys-Gly-His-Cys	Cys-Gly-His-Cys
$N_{SS}C_{CC}$	Ser-Gly-His-Ser	Cys-Gly-His-Cys
$N_{CC}C_{SS}$	Cys-Gly-His-Cys	Ser-Gly-His-Ser
$N_{SS}C_{SS}$	Ser-Gly-His-Ser	Ser-Gly-His-Ser
$N_{CS}C_{CS}$	Cys-Gly-His-Ser	Cys-Gly-His-Ser
$N_{SC}C_{SC}$	Ser-Gly-His-Cys	Ser-Gly-His-Cys

Various trx-motif mutants were constructed through the replacement of the cysteine residues by serine residues.

The isomerase activity was followed through the catalysis of the oxidative folding of reduced ribonuclease A in a glutathione buffer. The mutant $N_{SS}C_{SS}$ had no detectable activity. However, $N_{CC}C_{SS}$ and $N_{SS}C_{CC}$ in which one trx-motif had been left intact still maintained 25% and 16% activity of wild-type PDIA, respectively (Fig. 1). This result demonstrated that the isomerase activity of PDIA required the trx-motifs and the N-terminal motif was more active than the C-terminal motif. The sum of the catalytic activities of the two trx-motif mutants did not add up to the activity of wild-type PDIA, indicating that the two trx-motifs were not completely functionally independent. The mutant $N_{CS}C_{CS}$ still had 3.4% activity while the mutant $N_{SC}C_{SC}$ completely lacked activity, indicating that the first cysteine in each trx-motif was essential for isomerase activity. 100% corresponded to the activity of wild-type PDIA.

3.2. Chaperone function of PDIA

To examine whether PDIA has a chaperone function separable from its isomerase activity, an assay was performed to monitor renaturation of GAPDH, a substrate without disulfide bonds.

The result showed that PDIA had a significant effect on the renaturation of GAPDH increasing the reactivation yield and suppressing spontaneous aggregation (Fig. 2). It was clear that PDIA could act as a molecular chaperone for renaturation of GAPDH.

3.3. Effect of PDIA on the refolding of prochymosin

Mammalian PDI could influence folding of disulfide-containing proteins as an isomerase and/or as a chaperone [6,7,16,17]. Here, we investigated which function of PDIA is required to facilitate efficient refolding of denatured and reduced prochymosin. Prochymosin contains three disulfide bonds and could be converted to the biologically active chymosin after autocleavage in vitro.

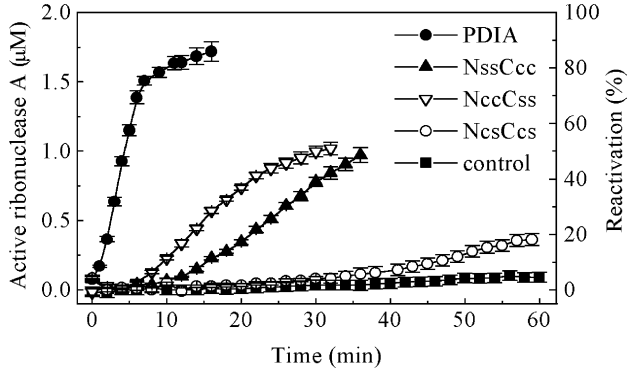

Fig. 1. Isomerase activities of PDIA and its mutants. The assay of isomerase activity was carried out by monitoring the reactivation of reduced, denatured ribonuclease A. The time course of active ribonuclease A was shown for experiments in which 2 μM reduced denatured ribonuclease A was incubated in 4.5 mM cCMP, 0.2 mM GSSG, 1.0 mM GSH in 0.1 M Tris–HCl, pH 8.0, 25 °C, in the presence of 1.4 μM PDIA or the mutants $N_{SS}C_{CC}$, $N_{CC}C_{SS}$, $N_{CS}C_{CS}$ and BSA as negative control (the mutant $N_{SC}C_{SC}$ and $N_{SS}C_{SS}$ were not shown; the data were expressed as means ± SD, $n = 3–5$).

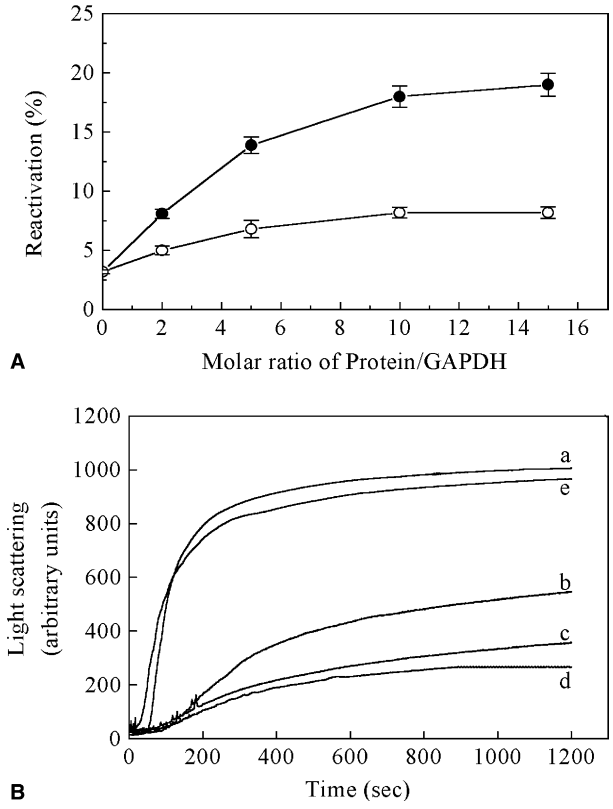

Fig. 2. Effect of PDIA on the renaturation of denatured GAPDH. (A) Refolding was initiated by 50-fold dilution of guanidine hydrochloride-denatured GAPDH to 2.8 μM in 0.1 M phosphate buffer, containing 5 mm DTT, 2.5 mM EDTA in the presence of PDIA (●) or BSA (○) at the molar ratios indicated at 4 °C, after incubation for 30 min the temperature was raised to 25 °C and maintained for 3 h. Activity of refolded GAPDH was measured bases on the production of NADH from NAD^+ monitored at 340 nm. (B) Aggregation was monitored by recording the light scattering at 488 nm at 20 °C immediately after dilution of GAPDH to 2.8 μM in the absence (a) and presence PDIA at the concentrations of 14 μM (b), 28 μM (c) and 42 μM (d). To exclude unspecific protein effects, control experiments in the presence of 5 μM BSA (e) were carried out.

As shown in Fig. 3A, PDIA increased the refolding rate and yield of prochymosin in a concentration-dependent manner. The refolding yield increased from 20% to about 30% once PDIA was present in a molar ratio of PDIA/prochymosin of 0.01. With equimolar amounts of PDIA present the yield was increased to about 40%. The mutant $N_{CC}C_{SS}$ having the C-terminal trx-motif inactivated also increased the folding rate and yield, but not to the same extent as wild-type PDIA (Fig. 3B). The mutant $N_{SS}C_{SS}$ with no isomerase activity was not capable of increasing the folding rate, but it could improve the yield of refolding by about 13% (Fig. 3C). These results indicated that PDIA acted as a

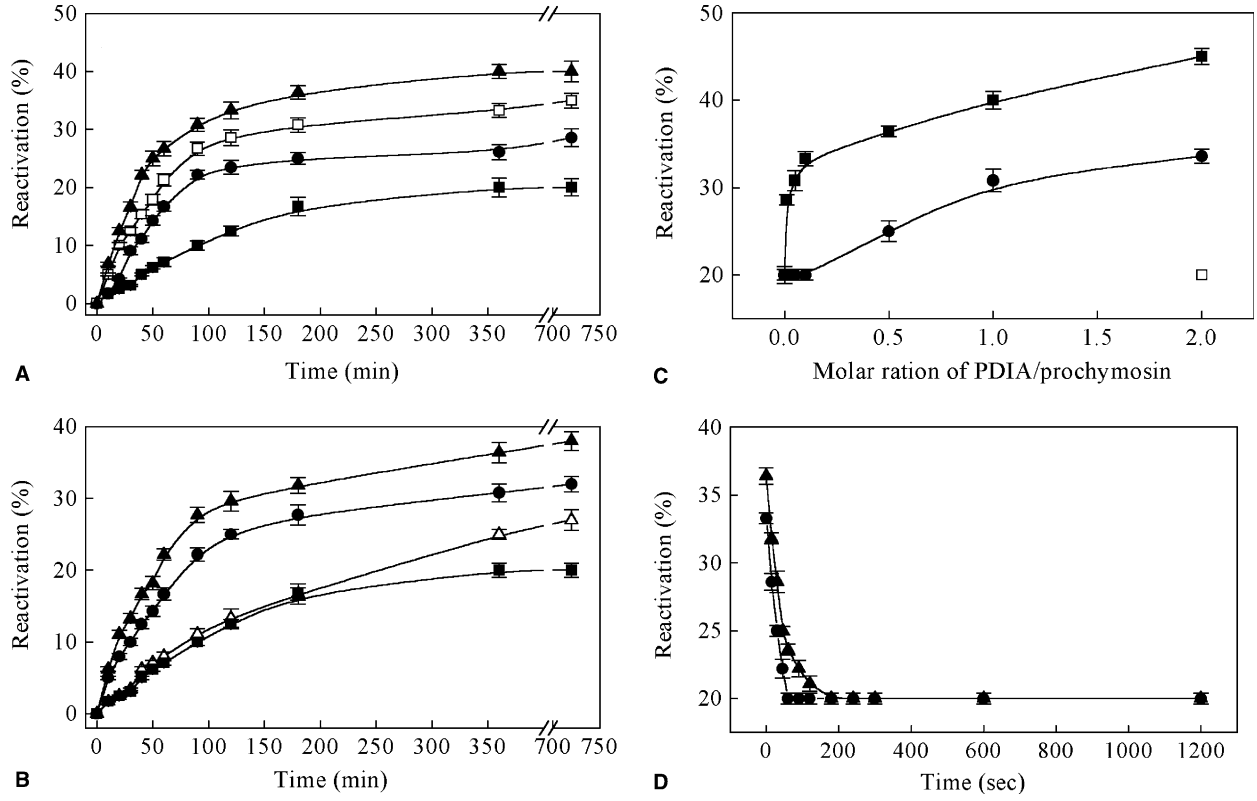

Fig. 3. Effect of PDIA on the refolding of denatured and reduced prochymosin. The refolding was performed in 50 mM K-phasphate, pH 8, 1 mM EDTA, 50 mM NaCl, 1 mM GSH, 2 mM GSSG at 25 °C. The final concentration was 1 μM for prochymosin and 160 mM for urea. At the times indicated an aliquot was withdrawn, acidified and assayed for milk-clotting activity. The reactivation was expressed as a percentage of active chymosin detected in relation to the prochymosin input in the refolding solution. (A) Refolding was performed in the absence of PDIA (■), in the presence of 0.01 (●), 0.1 (□), and 1 (▲) μM PDIA. (B) Effect of PDIA and its variants on the refolding. Refolding was carried out in the absence (■) or presence of 0.5 μM PDIA (▲), the mutants $N_{CC}C_{SS}$ (●) or $N_{SS}C_{SS}$ (Δ). (C) Effect of PDIA and the mutant $N_{SS}C_{SS}$ on the refolding yield. Refolding was preformed in the presence of PDIA (■) or $N_{SS}C_{SS}$ (●) at molar ratios indicated and BSA (□) as negative control. Refolding was analyzed after the renaturation was complete. (D) Delayed addition of PDIA and the mutant $N_{SS}C_{SS}$ to refolding prochymosin. Reactivation was performed as described above except that PDIA (▲) or $N_{SS}C_{SS}$ (●) was added after refolding start at the times indicated. The final concentration was 0.5 μM for PDIA and 2 μM for $N_{SS}C_{SS}$. Refolding was analyzed after the renaturation was complete (The data were expressed as means ± SD, $n = 3–5$).

chaperone and also as an isomerase when present during refolding of prochymosin and both activities of PDIA were required for maximum refolding. Under the same conditions bovine serum albumin did not affect prochymosin folding (Fig. 3C).

To investigate at which stages of the renaturation process the isomerase and the chaperone activities of PDIA were required, wild-type PDIA and the mutant $N_{SS}C_{SS}$ were added at different times of renaturation. The mutant $N_{SS}C_{SS}$ could assist renaturation of prochymosin when present at the beginning of the process, but failed to increase yield after 60 s (Fig. 3D). This result indicated that the chaperone activity was only effective when present during the first seconds of the refolding process. In contrast, wild-type PDIA could improve renaturation even added 60 s after initiation of folding although the improvement was not as efficient as when present from the beginning (Fig. 3D). This result indicated the decrease in the refolding yield due to the absence of the chaperone function in the first seconds of refolding could be partly compensated by the isomerase activity of PDIA.

4. Discussion

PDI contains two conserved trx-motifs (Cys-Gly-His-Cys) related to isomerase activity. While the N-terminal trx-motif of mammalian PDI is more active than the C-terminal motif for isomerase activity [18,19], the reverse is the case for yeast PDI [8,9]. In our study, the characteristic of PDIA is more similar to that of mammalian PDI: the N-terminal trx-motif is more active than the C-terminal trx-motif, and the first cysteine residue in a trx-motif is necessary for isomerase activity. Although the two trx-motifs can function independently, it is also possible that there are synergistic effects between the two motifs in PDIA compared to mammalian PDI, as mutation one of the two motifs reduced the isomerase activity significantly.

In addition to the isomerase activity associated with trx-motifs, it also has been demonstrated that mammalian PDI has a trx-motifs-independent chaperone function [6,7]. GAPDH does not contain disulfide bonds, therefore the PDI-assisted renaturation of GAPDH was considered as the chaperone activity. PDIA could suppress the aggregation of denatured GAPDH and promote the reactivation indicating PDIA has the separable chaperone function. However, no independent chaperone activity of yeast PDI has been found [8,9]. Alignment of the amino acid sequences of PDIs shows that PDIA shares 36% identity with human PDI and 41% with yeast PDI. The disparity in catalytic activities of two trx-motifs and the difference between the general chaperone activities of PDIs were most likely a result of the early evolutionary divergence of PDIs [20].

Prochymosin is characterized by a two-stage renaturation in vitro: dilution of unfolded protein into pH 11 buffer followed by neutralization at pH 8 [21]. This is quite different from other proteins, including its homologous protein, pepsinogen. This unique characteristic makes it a model protein for understanding the refolding mechanism of disulfide-containing proteins. Generally denatured and reduced disulfide-containing proteins have a tendency to aggregate during the refolding due to hydrophobic interactions and incorrect nonnative disulfide bond formation. These nonproductive reactions could be reduced by the chaperone and isomerase functions of PDI. The chaperone function was believed to assist in protein folding by preventing aggregation of the incorrectly folded proteins. The isomerase activity was thought to facilitate protein folding by catalyzing the formation and rearrangement of disulfide bonds, thus releasing the substrate from incorrectly disulfide bonded states and thereby enabling productive protein folding. In spontaneous refolding of prochymosin, the high-pH step is indispensable for efficient renaturation [21]. In our study, the high-pH step can be omitted and prochymosin is able to proceed with refolding at pH 8 alone to achieve one stage renaturation when PDIA was supplemented in to the refolding system. PDIA possesses two separate and distinct functions, isomerase and chaperone. Both functions of PDIA are essential for the efficient refolding of prochymosin at pH 8. The mutant $N_{SS}C_{SS}$ devoid of isomerase activity could increase the refolding yield of prochymosin at pH 8. This favorable effect resulted from the role as a molecular chaperone in decreasing unproductive intra- and intermolecular interactions. However, $N_{SS}C_{SS}$ was not as efficient as wild-type PDIA, indicating that only chaperone activity was not adequate, disulfide isomerization also played an important role in the refolding of prochymosin. The mutant $N_{CC}C_{SS}$ having one trx-motif intact also increased the folding rate and yield suggesting the presence of one active trx-motif was sufficient to accelerate prochymosin refolding. Wild-type PDIA increased the yield of refolding of prochymosin at catalytic concentrations, substoichiometric concentrations and stoichiometric concentrations while the mutant $N_{SS}C_{SS}$ had to be present at stoichiometric concentrations. These results indicated that PDIA acted both as an isomerase and as a chaperone when present during refolding of prochymosin. Maximum rate and yield of prochymosin refolding could only be achieved when both the chaperone and the isomerase activities of PDIA were present. The delayed addition experiments with PDIA showed that in the first seconds of prochymosin refolding the nonproductive reactions play an important role. The delayed addition of PDIA failed to promote the refolding of prochymosin that was already aggregated suggesting the chaperone function of PDIA was important during the initial stage but not at later stages of refolding. Although the formation of native disulfide bonds is the rate-limiting step, chaperone activity is required right from the initiation of refolding to prevent formation of undesirable aggregates.

Heterologous protein production in *A. niger* has been improved through classical mutagenesis and screening, and the sufficient progress has been made to obtain commercial yields of bovine chymosin [22]. Although these approaches can improve protein yield, the mechanism is not well understood. Our results reinforce the role of PDIA as an isomerase and as a chaperone for the folding of proteins. As a part of the strategy of manipulating the secretory pathway of *A. niger*, foldases and chaperones will play a key part in improving heterologous protein production by filamentous fungi.

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