Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 321 (2004) 31–37
BBRC
www.elsevier.com/locate/ybbrc

Chaperone characteristics of PDI-related protein A from *Aspergillus niger* ☆

Haiping Zhou, Yuying Zhang *, Cuijuan Jia, Kaiyu Yang

Institute of Microbiology, Chinese Academe of Sciences, Beijing 100080, China

Received 14 June 2004

Abstract

The functional properties of a novel protein, protein disulfide isomerase-related protein A (PRPA) from *Aspergillus niger* T21, have been characterized. (1) PRPA possesses disulfide isomerase activity. (2) In Hepes buffer, at substoichiometric concentrations, PRPA facilitates the formation of inactive lysozyme aggregates associated with PRPA (anti-chaperone activity); while at a high molar excess, PRPA inhibits aggregation by maintaining lysozyme in a soluble, yet inactive, state (chaperone-like activity). However, PRPA only exhibits chaperone-like activity during lysozyme refolding in phosphate buffer. (3) Experiments have indicated that disulfide cross-linkage is not required for the interaction between PRPA and lysozyme, and hydrophobic interaction may be responsible for PRPA effect on lysozyme. (4) Co-expression of PRPA and prochymosin in *Escherichia coli* leads to reduction of inclusion bodies, rendering part of prochymosin molecules soluble yet inactive. The structural and functional characteristics of PRPA suggest that PRPA may play an important role in protein folding, aggregation, and retention in the endoplasmic reticulum.

Keywords: Protein disulfide isomerase-related protein A; Chaperone; Refolding; Solubility

Foldase and molecular chaperone are two kinds of helpers assisting in protein folding [1]. Protein disulfide isomerase (PDI) possesses both foldase and molecular chaperone activities [2]. Therefore, studies on PDI will be helpful to understanding the mechanism of protein folding and improving the renaturation of recombinant protein. PDI was first discovered in 1963 [3]. Since the early 1980s, a number of genes of PDI and PDI-related proteins have been cloned from different organisms. The variety of PDI isoforms differing in structure, in subcellular distribution, in substrate range, and in pattern of induction and expression suggests that there must be some functional differentiation [4,5].

In 2000, a PDI-related gene, *prpA*, was first isolated from *Aspergillus niger* var. *awamori* [6]. There are some other PDI-related proteins in *A. niger* such as tunicamycin inducible gene A polypeptide (TIGA) [7] and PDIA [8], but all of them have not been well investigated. *prpA* encodes a PDI-related protein of 464 amino acids, PRPA. It was reported that the N-terminus 150 amino acids of PRPA contain homology to the protein disulfide isomerase while C-terminal two-thirds of the protein did not match any sequences in the database [6]. Expression of a heterologous protein, bovine prochymosin, in *A. niger* clearly induced expression of the *prpA* gene, while over-expression of *prpA* had little effect on the secretion of chymosin when they were co-expressed in *A. niger* [6]. Apparently, PRPA is a novel PDI-related protein and its function is fully unknown. To address this problem, The cDNA of *prpA* was cloned from *A. niger*

☆ *Abbreviations:* ANS, 8-anilino-1-naphthalene-sulfonic acid; BiP, immunoglobulin heavy chain binding protein; BSA, bovine serum albumin; ER, endoplasmic reticulum; GSH, glutathione; GSSG, glutathione disulfide; IPTG, isopropyl-1-thio-β-D-galactopyranoside; PAGE, polyacrylamide gel electrophoresis; PDI, protein disulfide isomerase; PRPA, PDI-related protein A.

* Correspondence author. Fax: +86-010-62560912.
E-mail address: zhangyy@sun.im.ac.cn (Y. Zhang).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.06.098

T21, inserted into pET23b, and expressed in *Escherichia coli*; the recombinant PRPA was purified to homogeneity and its enzyme and chaperone-like activities were investigated. The functional characteristics of PRPA are reported in this paper.

Materials and methods

Materials. Lysozyme and *Micrococcus lysodeikticus* were from Sigma; Bio-Rex70 was from Bio-Rad; *Taq* and ligase were from Promega; Hepes was from Merck; other enzymes were from Takara, Promega or local companies; and other chemicals were local products of analytical grade. The expression plasmid of bovine prochymosin (pET30a-*prochymosin*) was stored in our laboratory.

*Cloning and purification of PRPA.*Cloning and purification of PRPA were according to [9]. Briefly, the cDNA of PRPA was obtained by RT-PCR from *A. niger* T21 and inserted into pET23b. The expression plasmid with correct sequence and correct direction designated as pET23b-*prpA* was transformed into *BL21*(*DE3*), and the expression of PRPA was induced as described in the handbook of Novagen. Cells were harvested and washed with 50 mM Tris–HCl (pH 7.5), 1 mM EDTA, and then resuspended in the same buffer. The resuspended cells were sonicated. After centrifugation (10,000*g*, 40 min), 0–45% $(NH_4)_2SO_4$ saturated fraction from the soluble extract was prepared and exhaustively dialyzed against 20 mM PBS, pH 6.5 (buffer A). The dialysate was applied to a Bio-Rex 70 column (30 ml) pre-equilibrated with the same buffer. The column was washed with buffer A and then eluted with a linear gradient of buffer A containing 0–0.7 M NaCl. Aliquots of the fractions were analyzed by SDS–PAGE, and the PRPA-containing fractions were diluted with buffer A and then loaded on a smaller Bio-Rex 70 column (10 ml) pre-equilibrated with buffer A containing 0.25 M NaCl, the column was eluted with a linear gradient of buffer A containing 0.25–0.65 M NaCl.

Purification of human PDI. The human *pdi* (*hpdi*) gene in pBV220 was a gift from Dr. Yin Gao. Expression and purification of hPDI were according to Gao et al. [10]. The purified hPDI was shown to have homogeneity by SDS–PAGE (about 95% purity).

Determination of protein concentration. Protein concentration was determined either by the procedure of Lowry or Bradford with bovine serum albumin (BSA) as the standard [11]. As for lysozyme, protein concentrations were determined by measuring A_{280} with following absorbance coefficients ($A^{0.1\%}_{1\,cm}$): 2.63 for native lysozyme and 2.37 for denatured lysozyme [12].

Lysozyme denaturation and renaturation. The methods of denaturation and renaturation of lysozyme were according to [12,13]. Lysozyme (36.4 mg/ml) was reduced and denatured in 25 mM Tris–HCl (pH 8.6) containing 8 M urea, 130 mM 2-mercaptoethanol at 37 °C for 1.5 h. The denaturation and reduction reaction was quenched by dilution of lysozyme with 0.1 M acetic acid (pH 4.0) to a final concentration of 100 μM. Oxidative refolding of denatured and reduced lysozyme (the final concentration is 5 μM) was achieved by diluting it into 0.1 M Hepes buffer (pH 7.0), 2 mM EDTA, 5 mM $MgCl_2$, and 20 mM NaCl, or 0.1 M phosphate buffer (pH 7.5), 2 mM EDTA containing different concentrations of PRPA at 37 °C for 1 h. If not specified otherwise, the refolding buffers contained 2 mM GSH and 1 mM GSSG as suggested in [13]. Refolding reaction was stopped by addition of equal volume of 0.1 M acetic acid (pH 4.0).

Assays for enzyme activities . Lysozyme activity was determined at 30 °C by following the decrease in absorbance at 450 nm of a 0.15 mg/ml *M. lysodeikticus* suspension in 0.05 M potassium phosphate buffer (pH 6.4), using a Beckman DU-7 spectrophotometer [13]. Lysozyme reactivation yield was reported as a percentage of the activity expected for the same molar amount of native lysozyme.

Disulfide isomerase activity was assayed according to [14].

Lysozyme solubility assay. After completion of the folding reaction, insoluble aggregates were pelleted in a microcentrifuge at 10,000*g* for 8 min and separated from soluble material [15]. The PRPA and lysozyme in the supernatant and pellet were separated by SDS–PAGE. The gels were stained with Coomassie blue and scanned; the scanned films were quantified by means of the image analysis and processing software NIH Image*J* (version 1.29X) [16]. This software permits the measurement of the density of a specified area.

Co-expression of PRPA and prochymosin in E. coli. pET23b-*prpA* (ampicillin resistance, T7 promoter) and pET30a-*prochymosin* (kanamycin resistance, T7*lac* promoter) were transformed into *E. coli* strain *BL21*(*DE3*), respectively, or simultaneously. The expression of proteins was induced as described in the handbook of Novagen Company. After 3 h-induction by 1 mM IPTG, cells were harvested, sonicated, and centrifuged at 12,000*g* for 40 min to separate into supernatant and pellet. Samples were analyzed by SDS–PAGE and Western-blotted with chymosin antibody.

Analysis of fluorescence spectra. The ANS fluorescence measurements were made in 0.1 M Hepes buffer (pH 7.0), 2 mM EDTA, 5 mM $MgCl_2$, and 20 mM NaCl at 25 °C with excitation at 380 nm. Before the measurement of ANS spectra, 2 μM PRPA with 300 μM ANS was incubated for 30 min at 25 °C.

Results

Enzymatic activity of PRPA

The purified recombinant PRPA was shown to have about 95% purity by SDS–PAGE. Since the predicted amino acid sequence of PRPA contains a single active thioredoxin-like domain with CGHC motif as in the active site of PDI, it is reasonable to assume that PRPA may be able to catalyze disulfide isomerization. The experiment indicated that PRPA did display disulfide isomerase activity in the reactivation of sRNase (Fig. 1). Compared with hPDI, PRPA is only about 1% as active as hPDI. Considering that thioredoxin has lower than 1% activity of PDI based on the reactivation of sRNase [17], the low enzymatic activity of PRPA is not surprising and may be attributed to the different steric and electrostatic environment of the activity sites.

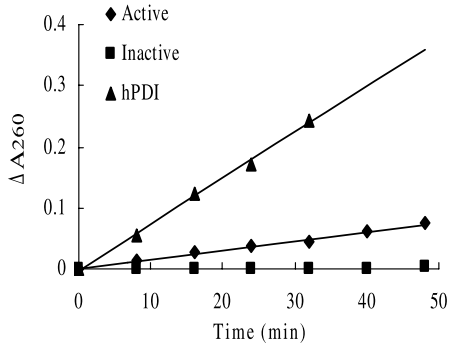


Fig. 1. Disulfide isomerase activities of PRPA. Disulfide isomerase activity was assayed by reactivation of sRNase. The assay mixture contains 20 μM DTT, 6 μg/ml sRNase with 0.066 μM PRPA (active or inactive) or 0.0016 μM hPDI in 50 mM Tris–HCl (pH 7.5) at 30 °C. Active: the purified PRPA; inactive: the purified PRPA incubated at 100 °C for 20 min before the measurement.

The effects of PRPA on lysozyme refolding

To determine whether PRPA can act as a molecular chaperone, purified PRPA was supplemented into lysozyme refolding system to see how PRPA influences the reactivation and solubility of lysozyme. For comparison, hPDI was used as a positive control.

As shown in Fig. 2A, in Hepes buffer (pH 7.0) at 37 °C, the spontaneous refolding yield of lysozyme is 58% after 1 h-incubation. When PRPA was supplemented into the refolding system, the reactivation yield of lysozyme decreased with the increase of PRPA concentration, down to 4% at a stoichiometric concentration and above. When hPDI was supplemented into the refolding system, a dual effect was observed. At a substoichiometric concentration ($\leqslant 0.5\,\mu M$) hPDI suppressed the reactivation yield of lysozyme; while at a higher concentration, hPDI acted as a chaperone, enhancing the reactivation yield. This coincided with the observation on bovine PDI [12,13]. It was demonstrated that the anti-chaperone activity of PDI was attributed to its ability to facilitate lysozyme aggregation, diverting the unfolded lysozyme away from productive folding [12]. According to this point of view, we expected that the suppression of lysozyme reactivation by PRPA might result from the involvement of PRPA in the lysozyme aggregation. To examine this speculation, the effect of PRPA on the solubility of lysozyme during refolding was determined. In the absence of PRPA about 70% of lysozyme is in a soluble from, at lower PRPA concentrations ($\leqslant 2.5\,\mu M$), PRPA does facilitate the formation of lysozyme aggregates; while at higher concentrations ($>2.5\,\mu M$), with the increase of PRPA, the solubility of lysozyme increases (Fig. 2A). The dual effect of PRPA on lysozyme solubility is similar to the dual effect of PDI on lysozyme solubility reported by Gilbert and co-worker [15], although the soluble from of lysozyme in the presence of PRPA is inactive. SDS–PAGE analysis of PRPA and lysozyme in the supernatant and pellet of the reaction mixture after refolding indicated that PRPA alone did not form insoluble aggregates (Figs. 3A and B, lane 5); in the presence of denatured lysozyme, PRPA was observed to be associated with lysozyme in the insoluble fraction, suggesting that PRPA is an integral component of the mixed aggregates (Fig. 3B, lanes 2–4).

Song et al. [13] demonstrated that the PDI anti-chaperone activity was dependent on the refolding

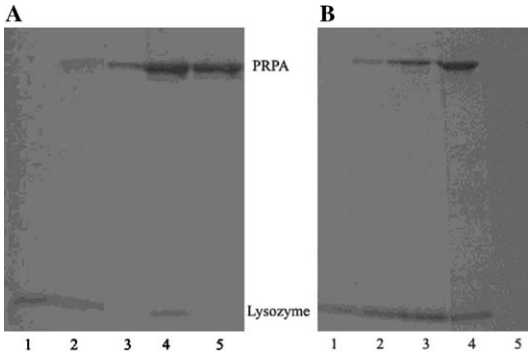


Fig. 3. SDS–PAGE analysis of PRPA and lysozyme in the supernatant and pellet of the reaction mixture after refolding. Refolding of 5 μM denatured and reduced lysozyme in the present of PRPA was performed as described in Fig. 2A. After completion of the refolding reaction, samples were centrifuged and the obtained supernatant and pellet were analyzed by SDS–PAGE. The gel was stained with Coomassie blue. (A) Supernatant and (B) pellet. The PRPA concentrations are 0: (lane 1), 0.5 μM (lane 2), 2.5 μM (lane 3), 10 μM (lane 4), and 5 μM PRPA without lysozyme (lane 5).

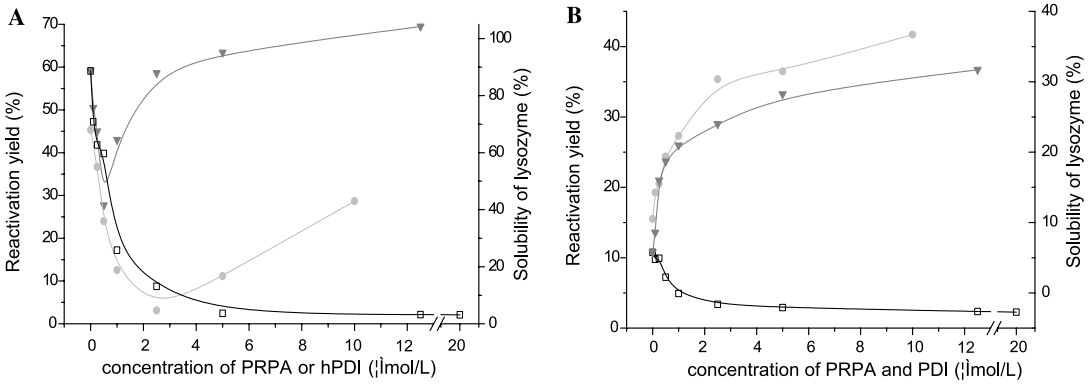


Fig. 2. The effect of PRPA or hPDI on the refolding of lysozyme. Denatured and reduced lysozyme (5 mM final concentration) was added into different refolding buffers [(A) 100 mM Hepes (pH 7.0), 5 mM $MgCl_2$, 20 mM NaCl, 2 mM EDTA, 2 mM GSH, and 1 mM GSSG; (B) 100 mM PBS (pH 7.5), 2 mM EDTA, 2 mM GSH, and 1 mM GSSG] containing indicated concentrations of PRPA or hPDI to cause refolding for 1 h at 37 °C. Activity or solubility measurements were made as described in the text. The reactivation yield of lysozyme (%) was based on the activity of the same amount of native lysozyme measured under the same conditions. The percentage of soluble lysozyme was based on the total amount of lysozyme found in the supernatant and pellet fractions. □, reactivation yield of lysozyme in the presence of PRPA; ▼, reactivation yield of lysozyme in the presence of hPDI; and ●, solubility of lysozyme in the presence of PRPA.

conditions. In phosphate buffer, only chaperone activity was observed. For comparison, the effect of PRPA on the refolding of lysozyme in redox phosphate buffer was investigated. As shown in Fig. 2B, in the absence of PRPA, the solubility of lysozyme in phosphate buffer is much lower than that in Hepes buffer (11% vs. 70%) and the reactivation yields of lysozyme are different from each other coincidently. In the presence of PRPA, the solubility of lysozyme is improved in a PRPA concentration-dependent manner, similar to the hPDI effect on lysozyme reactivation, although PRPA does not promote reactivation yield at all.

In a word, PRPA exhibits both anti-chaperone activity to facilitate lysozyme aggregation and chaperone-like activity to inhibit lysozyme aggregation.

It is worthwhile to mention that BSA has no effect on the solubility and reactivation yield of lysozyme both in Hepes buffer and in phosphate buffer during lysozyme refolding (data not shown), indicating that PRPA effect on lysozyme refolding is a specific function.

Relationship of the hydrophobicity of PRPA and its chaperone-like activity

The interactions between chaperone molecule and its substrate protein are the prerequisite for both chaperone and anti-chaperone activities. As described above, in redox Hepes buffer and in the presence of PRPA, mixed aggregates containing PRPA and lysozyme were observed during lysozyme refolding (Fig. 3). However, what interaction force maintaining this mixed aggregate remained to be unraveled. To address this problem, we first examine if intermolecular disulfides are responsible for the aggregation. From Fig. 4, it is evident that in the presence of 10 mM DTT, the solubility of lysozyme as a function of PRPA concentration is similar to that in the GSH/GSSG buffer, implying that disulfide crossing-linkage is not essential to the interaction between PRPA and lysozyme.

Then, we directed our attention to analyzing the relationship between PRPA hydrophobicity and its effect on lysozyme refolding. It is well known that ANS enhances its fluorescence intensity on binding to hydrophobic sites and has been widely used to probe the hydrophobicity of a molecule. Fig. 5 shows incubation of PRPA with ANS results in enhanced ANS fluorescence intensity, indicating that there are hydrophobic sites on the surface of PRPA molecule. Meanwhile, hPDI can enhance ANS fluorescence intensity on binding to ANS too. During purification we also found that PRPA could strongly bind to hydrophobic phenyl-Sepharose column (data not shown). Fig. 6 indicates that in the absence of PRPA, 5% and 10% ethylene glycol decreases the reactivation yield of lysozyme, while 5% and 10% ethylene glycol can attenuate the PRPA-mediated inhibition of

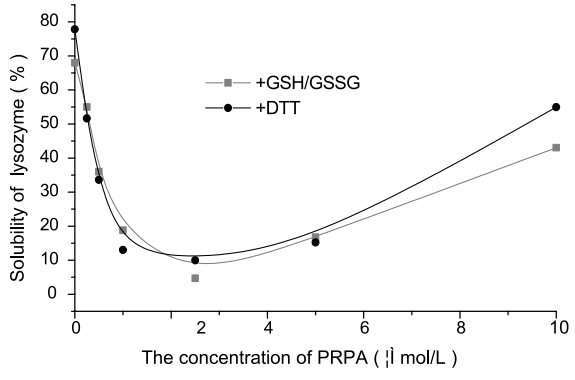


Fig. 4. The effect of redox conditions on PRPA-facilitated aggregation of lysozyme The denatured and reduced lysozyme (5 μM final concentration) was incubated at 37 °C, in 100 mM Hepes buffer (pH 7.0), 5 mM $MgCl_2$, 20 mM NaCl, 2 mM EDTA and indicated concentrations of PRPA in the presence of 2 mM GSH/1 mM GSSG (+GSH/GSSG) or 10 mM DTT (+DTT). After 1 h incubation, the solubility of lysozyme was determined as described in the legend to Fig. 2.

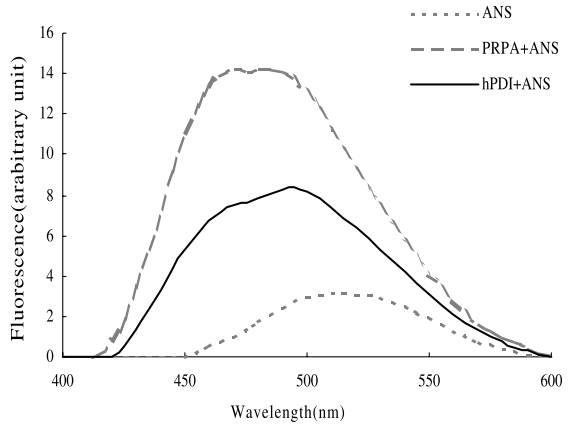


Fig. 5. ANS fluorescence spectra of PRPA and hPDI in Hepes buffer. Before the measurements of ANS fluorescence spectra, 2 μM PRPA or hPDI, and 300 μM ANS were incubated in Hepes buffer for 30 min at 25 °C. The ANS fluorescence spectra were measured at 25 °C with excitation at 380 nm.

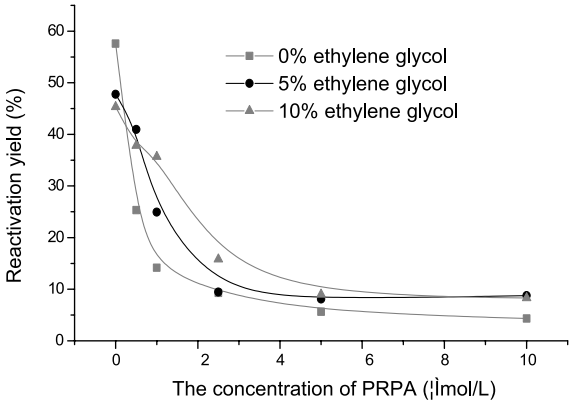


Fig. 6. The effect of ethylene glycol on the PRPA-mediated lysozyme reactivation. The procedure of the refolding was the same as described in the legend to Fig. 2A except containing different concentrations of ethylene glycol in the refolding buffer.

lysozyme reactivation. Most likely, this effect results from that ethylene glycol increases the solution hydrophobicity and in turn decreases the hydrophobic interaction between PRPA and lysozyme folding intermediates, thus more lysozyme can be diverted into productive refolding pathway. Similarly, it was reported that ethylene glycol can decrease the hydrophobic interactions between PDI and lysozyme folding intermediates [13].

All the results described above demonstrate that hydrophobic interaction is responsible for PRPA effect on lysozyme refolding.

Co-expression of prpA and prochymosin in E. coli

It has been reported that co-expression of PDI can selectively increase the solubility of its partner proteins in vivo [18,19]. Therefore, we were interested to see if PRPA can exert similar function to improve the solubility of intracellular prochymosin. The expression plasmids pET23b-*prpA* and pET30a-*prochymosin* were transformed into *E. coli* strain *BL21*(*DE3*), respectively, or simultaneously. After 3 h-induction by IPTG, cells were harvested, sonicated, and centrifuged to separate into supernatant and pellet. As shown in Fig. 7, SDS–PAGE analysis of the expression products reveals that prochymosin expressed alone was exclusively accumulated as inclusion bodies (Figs. 7A and B, lanes 5 and 6), while PRPA expressed alone was mainly as a soluble form (Fig. 7A, lanes 2 and 3). When PRPA and prochymosin were co-expressed, soluble prochymosin appeared (Figs. 7A and B, lane 8). However, the soluble prochymosin could not be transformed into pseudochymosin at pH 2 [20], implying that it is not an active form. In a word, PRPA improves the solubility of prochymosin, although it fails to assist prochymosin to form active conformation.

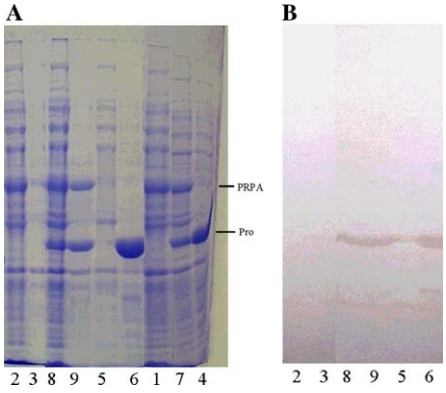


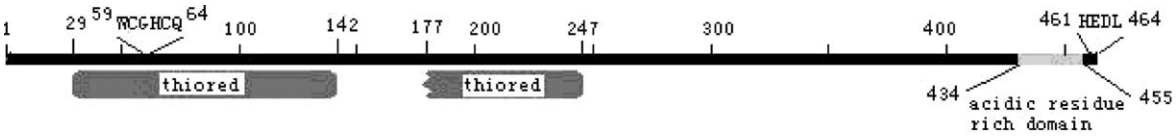
Fig. 7. SDS–PAGE analysis of co-expression of PRPA with prochymosin in BL21(DE3) (A) SDS–PAGE of the expression products; (B) Western blot analysis of the expression products with antibody of chymosin. After 3-h induction by IPTG, cells were harvested, sonicated and centrifuged at 12,000*g* for 40 min to separate supernatant and pellet, and samples were analyzed by SDS–PAGE. 1: total protein of pET23b-prpA; 2: supernatant of pET23b-prpA; 3: pellet of pET23b-prpA; 4: total protein of pET30a-prochymosin; 5: supernatant of pET30a-prochymosin; 6: pellet of pET30a-prochymosin; 7: total protein of co-expression; 8: supernatant of co-expression; 9: pellet of co-expression.

Discussion

It is generally accepted that a chaperone is a protein that binds to the exposed hydrophobic surfaces of a non-native protein, preventing irreversible aggregation and assisting it in reaching its native state [21–23]. This statement can be used as a basis to judge whether a protein is a chaperone or not. In Hepes buffer (Fig. 2A), PRPA facilitates lysozyme aggregation at substoichiometric concentrations and decreases lysozyme aggregation at superstoichiometric concentrations. In phosphate buffer (Fig. 2B), PRPA exclusively improves the solubility of lysozyme at all the concentrations tested. However, PRPA does not promote lysozyme reactivation. From these characteristics, it appears that PRPA does not fully meet the chaperone criteria stated above. Nevertheless, based on the following considerations we prefer to consider PRPA as a molecular chaperone or a chaperone-like protein.

First, the well-defined chaperone, immunoglobulin heavy chain-binding protein, BiP, also behaves similarly during lysozyme refolding as PRPA does in Hepes buffer [24]. When present at substoichiometric concentrations, BiP facilitates the formation of large inactive lysozyme aggregates that are non-covalently associated with BiP; if present at high molar excess, BiP inhibits lysozyme aggregation by maintaining it in a soluble, yet inactive, conformation. Second, chaperone-assisted protein folding is a complicated process. Sometimes, a series of helper proteins are required to form a cooperative, sequential pathway assisting in protein folding [25,26]. For example, BiP can bind stably to heavy chain (HC) of immunoglobulin and keep HC in a non-native conformation until light chain (LC) triggers HC release in vivo [27]. Third, the roles of chaperones are more than helping protein folding [28,23]. Sometimes, chaperone proteins can also participate in the degradation of misfolded protein [21]; some molecular chaperones have been shown to merely stabilize the unfolded structure of newly synthesized proteins [24]. In this context, a protein that functions as a molecular chaperone does not necessarily help its direct substrate protein to acquire an active conformation. Therefore, it is appropriate to pay more attention to solubility of the substrate protein rather than to overemphasize its activity when a new chaperone is under investigation.

It is worthwhile to note that co-expression of PRPA and prochymosin in *E. coli* leads to reduction of inclusion bodies, rendering part of prochymosin molecules soluble yet inactive (Fig. 7). Similarly, it has been



Scheme 1. Modular structure analysis of PRPA. The 29–142 amino sequence of PRPA contains homologue to thioredoxin domain (52%), the 177–246 amino sequence of PRPA is homologous to part of thioredoxin domain (43%), and 434–455 amino sequence of PRPA is acidic residue rich domain. The thioredoxin activity sites of PRPA are WCGCHQ, and PRPA has a HEDL at C-terminal. thiored: thioredoxin domain.

found that co-expression of the α subunit of human proly 4-hydroxylase with BiP polypeptide in insect cells leads to the formation of soluble, yet inactive, α subunit–BiP complexes [29]. The fact that PRPA binds to unfolded protein and improves its solubility, but does not promote correct folding, suggests that PRPA may be functioning as a "holding/stabilizing" protein rather than as a catalyst of productive folding as BiP does [24].

Through NCBI conserved domain search and sequence comparison, it has been found that: (1) the 29–142 amino sequence of PRPA contains 52% homology to thioredoxin domain; (2) the 177–246 amino sequence of PRPA shows 43% homology to part of thioredoxin domain but without active site; (3) 60% of its 434–455 amino acids (EEVVEEKVEELVEEVVEEPVEE) are acidic amino acids, it has been reported that the clusters of pair acidic residues found at the C-terminal of the ER resident proteins such as calreticulin [30], endoplastmin [31], PDI [32], and BiP [33] were required for Ca^{2+} binding; and (4) the ER retention signal (HEDL) is located at the C-terminal end of PRPA suggesting that PRPA is an ER retention protein [6] (Scheme 1).

ER is a specialized cellular compartment where nascent polypeptides cause oxidative folding efficiently [5]; ER is also a major site for the storage of calcium and chaperones such as PDI, BiP, and calreticulin. In addition, ER provides a mechanism to retain the misfolded protein under conditions unfavorable for correct folding and release them upon restoring conditions to permit folding or to some other pathway [34].

Taking the structural features of PRPA and its chaperone characteristics into consideration, it is reasonable to assume that PRPA is an ER resident protein and may act in vivo as a molecular chaperone or chaperone-like protein to recognize and interact with the nascent polypeptides or as a holding protein to retain unfolded proteins.

Acknowledgments

This work was supported by National Natural Science Foundation of China (Grant 3997003 to Yuying Zhang). We thank Prof. Guomin Tang for providing *A. niger* T21 and Dr. Yin Gao for providing pBV220-*hpdi*.

References

[1] M.-J. Gething, J. Sambrook, Protein folding in the cell, Nature 355 (1992) 33–45.
[2] C.-C. Wang, C.-L. Tsou, Protein disulfide isomerase is both an enzyme and a chaperone, FASEB J. 7 (1993) 1515–1517.
[3] R.F. Goldberger, C.J. Epstein, C.B. Anfinsen, Acceleration of reactivation of reduced bovine pancreatic ribonuclease by a microsomal system from rat liver, J. Biol. Chem. 238 (1963) 628–635.
[4] R.B. Freedman, T.R. Hirst, M.F. Tuite, Protein disulphide isomerase: building bridges in protein folding, TiBS 19 (1994) 331–336.
[5] D.M. Ferrari, H.D. Soling, The protein disulphide-isomerase family: unraveling a string of folds, Biochem. J. 329 (1999) 1–10.
[6] H. Wang, M. Ward, Molecular characterization of a PDI-related gene *prpA* in *Aspergillus niger* var. *awamori*, Curr. Genet. 37 (2000) 57–64.
[7] D.J. Jeenes, R. Pfaller, D.B. Archer, Isolation and characterization of a novel stress-inducible PDI-family gene from *Aspergillus niger*, Gene 193 (1997) 151–156.
[8] C. Ngiam, D.J. Jeenes, P.J. Punt, C.A.M.J.J. Van Den Hondel, D.B. Archer, Characterization of a foldase, protein disulfide isomerase A in the protein secretory pathway of *Aspergillus niger*, Appl. Environ. Microbiol. 66 (2000) 775–782.
[9] H.-P. Zhou, C.-J. Jia, Y.-Y. Zhang, Expression and characterization of protein disulfide isomerase-related protein A, Chin. J. Biotech. 20 (2004) 595–600.
[10] Y. Gao, Z. Yang, Z. Hua, H. Gao, P. Wan, H. Li, Cloning and expression of human protein disulfide isomerase cDNA in *Escherichia coli*, Chin. J. Biotech. 15 (1999) 349–354.
[11] G.L. Peterson, Determination of total protein, Methods Enzymol. 91 (1983) 95–121.
[12] A. Puig, H.F. Gilbert, Protein disulfide isomerase exhibits chaperone and anti-chaperone activity in the oxidative refolding of lysozyme, J. Biol. Chem. 269 (1994) 7764–7771.
[13] J. Song, H. Quan, C. Wang, Dependence of the anti-chaperone activity of protein disulfide isomerase on its chaperone activity, Biochem. J. 328 (1997) 841–846.
[14] J. Chen, J. Song, S. Zhang, Y. Wang, D. Cui, C. Wang, Chaperone activity of DsbC, J. Biol. Chem. 274 (1999) 19601–19605.
[15] V. Sideraki, H.F. Gilbert, Mechanism of the antichaperone activity of protein disulfide isomerase: facilitated assembly of large, insoluble aggregates of denatured lysozyme and PDI, Biochemistry 39 (2000) 1180–1188.
[16] E.M. Whiteley, T.-A. Hsu, M.J. Betenbaugh, Thioredoxin domain non-equivalence and anti- chaperone activity of protein disulfide isomerase mutants in vivo, J. Biol. Chem. 272 (1997) 22556–22563.
[17] X. Sun, Y. Dai, H. Liu, C. Wang, S. Chen, Contributions of protein disulfide isomerase domains to its chaperone activity, Biochim. Biophys. Acta 1481 (2000) 45–54.
[18] M. Ostermeier, K.D. Sutter, G. Georgiou, Eukaryotic protein disulfide isomerase complements *Escherichia coli dsbA* mutants

and increases the yield of a heterologous secreted protein with disulfide bonds, J. Biol. Chem. 271 (1996) 10616–10622.
[19] D.P. Humphreys, N. Weir, A. Lawson, A. Mountain, Co-expression of human protein disulphide isomerase (PDI) can increase the yield of an antibody Fab′ fragment expressed in the *Escherichia coli*, FEBS Lett. 380 (1996) 194–197.
[20] V.B. Pederson, K.A. Christenson, B. Foltman, Investigations on the activation of bovine prochymosin, Eur. J. Biochem. 94 (1979) 573–580.
[21] A.M. Slavotinek, L.G. Biesecker, Unfolding the role of chaperones and chaperonins in human disease, Trends Genet. 17 (2001) 528–535.
[22] H.R. Saibil, N.A. Ranson, The chaperonin folding machine, TiBS 27 (2002) 627–632.
[23] J.C. Young, J.M. Barrel, F.U. Hartl, More than folding: location functions of cytosolic chaperone, TiBS 28 (2003) 541–547.
[24] A. Puig, H.F. Gilbert, Anti-chaperone behavior of BiP during the protein disulfide isomerase-catalyzed refolding of reduced and denatured lysozyme, J. Biol. Chem. 269 (1994) 25889–25896.
[25] J. Frydman, Folding of newly translated proteins in vivo: the role of molecular chaperones, Annu. Rev. Biochem. 70 (2001) 603–649.
[26] V.S. Stronge, Y. Saito, Y. Ihara, B.B. Williams, Relationship between calnexin and BiP in suppressing aggregation and promoting refolding of protein and glycoprotein substrates, J. Biol. Chem. 276 (2001) 39779–39787.
[27] M. Vanhove, Y.K. Usherwood, L.M. Hendershot, Ig heavy chains do not cycle from BiP in vivo but require light chains to trigger release, Immunity 15 (2001) 105–114.
[28] H. Saibil, Molecular chaperones: containers and surfaces for folding stabilising or unfolding protein, Curr. Opin. Struct. Biol. 10 (2000) 251–258.
[29] J. Veijola, T. Philajaniemi, K.I. Kivirikko, Co-expression of the α-hydroxylase with BiP polypeptide in insect cells leads to the formation of soluble and insoluble complex; soluble α-subunit-BiP complexes have no proly-4-hydroxylase activity, Biochem. J. 315 (1996) 613–618.
[30] M. Michalak, R.E. Milner, K. Burns, M. Opas, Calreticulin, Biochem. J. 285 (1992) 681–692.
[31] G.L.E. Koch, M. Smith, D. Macer, P. Webster, R. Mortara, Endoplasmic reticulum contains a common, abundant calcium-binding glycoprotein, endoplasmin, J. Cell Sci. 86 (1986) 217–232.
[32] H.A. Lucero, H.A. Lebeche, B. Kaminer, ERcalcistorin/protein disulfide isomerase (PDI). Sequence determination and expression of a cDNA clone encoding a calcium storage protein with PDI activity from endoplasmic reticulum of the sea urchin egg, J. Biol. Chem. 269 (1994) 23112–23119.
[33] J.P. Liévremont, R. Rizzuto, L. Hendershot, J. Meldolesi, BiP, a major chaperone protein of the endoplasmic reticulum lumen, plays a direct and important role in the storage of rapidly exchanging pool of Ca^{2+}, J. Biol. Chem. 272 (1997) 30873–30879.
[34] K.W. Walker, H.F. Gilbert, Protein disulfide isomerase, in: A.L. Fink, Y. Goto (Eds.), Molecular Chaperone in the Life Cycle of Proteins Structure, Function, and Mode of Action, Marcel Dekker Inc., New York, 1998, pp. 331–359.