Published in final edited form as:

Biochim Biophys Acta. 2011 April ; 1814(4): 487–495. doi:10.1016/j.bbapap.2011.01.004.

Both PDI and PDIp Can Attack the Native Disulfide Bonds in Thermally-Unfolded RNase and Form Stable Disulfide-Linked Complexes

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Abstract

Protein disulfide isomerase (PDI) and its pancreatic homolog (PDIp) are folding catalysts for the formation, reduction, and/or isomerization of disulfide bonds in substrate proteins. However, the question as to whether PDI and PDIp can directly attack the native disulfide bonds in substrate proteins is still not answered, which is the subject of the present study. We found that RNase can be thermally unfolded at 65°C under non-reductive conditions while its native disulfide bonds remain intact, and the unfolded RNase can refold and reactivate during cooling. Co-incubation of RNase with PDI or PDIp during thermal unfolding can inactivate RNase in a PDI/PDIP concentration-dependent manner. The alkylated PDI and PDIP, which are devoid of enzymatic activities, cannot inactivate RNase, suggesting that the inactivation of RNase results from the disruption of its native disulfide bonds catalyzed by the enzymatic activities of PDI/PDIP. In support of this suggestion, we show that both PDI and PDIP form stable disulfide-linked complexes only with thermally-unfolded RNase, and RNase in the complexes can be released and reactivated dependently of the redox conditions used. The *N*-terminal active site of PDIP is essential for the inactivation of RNase. These data indicate that PDI and PDIP can perform thiol-disulfide exchange reactions with native disulfide bonds in unfolded RNase via formation of stable disulfide-linked complexes, and from these complexes RNase is further released.

Keywords

protein folding; PDI; PDIP; RNase; disulfide bond; isomerase

1. INTRODUCTION

In eukaryotic cells, secretory and cell-surface proteins are synthesized in the lumen of endoplasmic reticulum (ER) where they are folded to functional native structures (including the formation of disulfide bonds) in the presence of folding catalysts and molecular chaperones [1–2]. Protein disulfide isomerase (PDI, EC 5.3.4.1), a 55-kD protein, is an abundant and efficient catalyst of disulfide bond formation in the ER [3–5]. This protein is comprised of four thioredoxin-like domains, *a*, *b*, *b'* and *a'*, plus a linker region between *b'* and *a'* and a *C*-terminal acidic extension. The two catalytic domains, *a* and *a'*, both

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including a WCGHCK motif, are responsible for their catalytic activities in the formation, reduction, and isomerization of disulfide bonds in substrate proteins [4, 6–12]. Since its identification some 40 years ago [6], many studies using various disulfide bond-containing protein substrates, such as bovine pancreas RNase A (RNase) [13–14], bovine pancreas trypsin inhibitor (BPTI) [13, 15], insulin [16–17] and immunoglobulin [18], have been conducted to probe the mechanism of PDI-mediated protein folding processes (reviewed in ref [5, 19]).

It is generally assumed that a folding catalyst and molecular chaperone like PDI can selectively recognize non-native proteins (usually biologically inactive) for folding or refolding, but not native proteins (usually biologically active) [20]. Here, the question regarding the mechanism of disulfide recognition by PDI, *i.e.*, how it discriminates between non-native and native disulfide bonds in substrate proteins, is still not entirely understood. A number of studies have shown that non-native disulfide bonds or free cysteine residues that are supposed to form native disulfide bonds in substrate proteins, such as reduced RNase (rRNase; containing free cysteine residues) [21–22], scrambled RNase (sRNase; containing randomly-formed disulfide bonds) [21, 23], reduced proinsulin [8], reduced acidic phospholipase A2 [9] and reduced BPTI [7], can be readily recognized by PDI and converted into correct disulfide bonds. Notably, stable disulfide-linked PDI-substrate complexes have also been observed during the refolding of some of these substrate proteins [8, 21, 23–25] and these complexes were suggested to be intermediates during PDI-catalyzed protein folding [26]. Besides non-native disulfide bonds, there are also evidence suggesting that native disulfide bonds, either in native proteins [13, 15–16] or in folding intermediates [7, 14, 27–28], if accessible to PDI, are also subject to its attack. In these cases, although PDI was hypothesized to also form disulfide-linked PDI-substrate complexes [15], there has been no direct evidence presented thus far for the existence of such complexes [7, 14–16, 27–28]. Therefore, whether PDI can really form disulfide-linked complexes with native disulfide bond-containing substrate proteins remains an unanswered question.

In the present study, we sought to investigate the interactions of PDI and its pancreatic homologue PDIp¹⁵ with RNase, which was thermally unfolded under non-reductive conditions (in the absence of reducing agents) and thus still retained the native disulfide bonds. The thermally-unfolded RNase is different from rRNase and sRNase in their disulfide bond status. In addition, the present non-reductive unfolding study is different from some of the earlier reductive unfolding studies [13, 15] because the presence of dithiothreitol (DTT) in these earlier studies would not only reduce the disulfide bonds in substrate proteins but would also reduce the inter-subunit disulfide bonds formed between PDI and substrate proteins, which complicates the interpretation of the effect of disulfide bonds on PDI-substrate interactions. Therefore, the current experimental design may allow us to probe the interactions of PDI/PDIp with unfolded RNase containing the original native disulfide bonds. The results of our present study offer mechanistic insights into the earlier observations that PDI can efficiently catalyze the conversion of kinetically-trapped substrate intermediates containing native disulfide bonds into on-pathway intermediates during the oxidative refolding of RNase and BPTI [7, 13–14, 27, 29].

2. MATERIALS AND METHODS

2.1. Reagents and proteins

DTT, iodoacetamide, GSH and GSSG, bovine pancreatic RNase A, insulin, and lysozyme were purchased from Sigma-Aldrich (St. Louis, MO). Two kinds of BSA were purchased from Sigma-Aldrich and Fisher Scientific. The rabbit anti-PDI antibody was obtained from Sigma-Aldrich (catalog No. P7372, dilution at 1:2000), and the rabbit anti-RNase A

antibody from Abcam (catalog No. ab6611, dilution at 1:6000). The mouse anti-PDIp antiserum was raised in our laboratory using a highly-pure recombinant PDIp protein as described earlier [30], and diluted at 1:2500 for Western blotting analysis. All other commonly-used chemical reagents were of analytical grade or better.

2.2. Construction of plasmids and protein purification

Wild-type PDI and PDIp genes (NCBI database accession No. AAH10859.1 and AAC50401.1, respectively) were cloned into the pET-19b vector as described previously [30–31]. The plasmids for PDIp C4A and C350A mutant proteins were constructed as described previously [32]. The plasmids for Δ C1 (the two cysteine residues in the *N*-terminal active site WCGHC were replaced with serine), Δ C2 (the two cysteine residues in the *C*-terminal active site WCTHC were replaced with serine), and Δ C1 Δ C2 (the four cysteine residues from the two active sites were replaced with serine) were constructed as described previously [33]. PDI and PDIp (wild type proteins plus the C4A, C350A, Δ C1, Δ C2 and Δ C1 Δ C2 mutant proteins) were purified as described previously [31–33] by a combined use of Ni-Sepharose high performance and Mono Q-4.6/100 PE chromatography (both from GE Health, Piscataway, NJ).

2.3. Enzymatic activity assay

Protein mixtures (12 μ l) containing RNase (at a 5- μ M final concentration) and PDI or PDIp (typically at a 2.5- μ M final concentration) were prepared in 10 mM sodium phosphate buffer (pH 7.4) in a PCR tube, thermally treated in a PCR-reactor at 65°C for 30 min, cooled down to 25°C for 30 min, and then transferred to a 384-well plate containing 68 μ l Millipore water in each well for assaying the RNase enzymatic activity.

The enzymatic activity of RNase was determined by monitoring hydrolysis of cCMP (an RNase substrate) at 296 nm on a BioTek μ Quant plate reader. Assays were performed at 25°C and initiated by the addition of 20 μ l 5-fold reaction buffer (0.5 M Tris-HCl buffer, pH 8.0, plus 22.5 mM cCMP and 2 mM EDTA) into each well. The mixture was quickly mixed by pipetting three times yet without generating air bubbles. In some of the experiments (Figure 6), the assay was conducted in the presence of varying concentrations of GSH and GSSG.

The enzymatic activities of PDI/PDIp were measured as described previously [21] using rRNase and sRNase as substrates, respectively. The mixture (at final concentrations) are composed of 0.1 M Tris-HCl (pH 8.0), 4.5 mM cCMP, 1 mM GSH, 0.2 mM GSSG, and 0.5 mM EDTA, plus 1 μ M rRNase (or 1.5 μ M sRNase) and PDI/PDIp proteins (0.1 and 0.4 μ M).

2.4. Analysis of PDI/PDIp-RNase disulfide-linked complexes

RNase (5 μ M) was thermally treated at 65°C for 30 min in the absence or presence PDI (2.5 μ M), cooled down, and then alkylated with iodoacetamide (10 mM) at room temperature for 2 h before non-reducing SDS-PAGE analysis. Protein samples were separated by 15% or 10% acrylamide gels, and then visualized by Coomassie blue staining, or subjected to Western blotting analysis using an anti-PDI antibody or an anti-RNase IgG. For PDIp-RNase complex analysis, PDIp was first treated with 5 mM DTT for 30 min to remove the inter-subunit disulfide bond [32], and then desalted with PD minitrapp-G25 column to remove residual DTT before thermal co-incubating with RNase as did with PDI.

3. RESULTS

3.1. PDI and PDIp can inactivate thermally-unfolded RNase

In this study, we sought to determine whether the native disulfide bonds in non-native RNase can be recognized by PDI and PDIp, and if so, what are the consequences. It was reported earlier that PDI remained as an active chaperone at 60°C [32–33] or even at 80°C [34], while RNase was completely unfolded (yet with native disulfide bonds preserved) at temperature 65°C [35–36]. Here we adopted this thermal treatment (at 65°C for 30 min) to selectively unfold RNase yet without disrupting its native disulfide bonds nor inactivating PDI that needed to be co-present during thermal treatment. The enzymatic activity of PDI is not suitable to be measured at 65°C since the substrate proteins (rRNase and sRNase) are reduced and thus inactive at this temperature in the presence of the reducing agent GSH that is required for activity assay (data not shown).

The preservation of native disulfide bonds in thermally-unfolded RNase was first verified by thermal-unfolding and inactivation analysis (Figure S1 and the Result section of the Supplementary Data File). Then we studied the effect of PDI and PDIp on thermally-unfolded RNase. The purity of the recombinant PDI and PDIp prepared from *E. coli* cells (described in our earlier studies [30–31]) were shown in Figure S1E. The redox state of these purified PDI and PDIp was determined by using DTNB modification (Figure S2). The number of free cysteine residues per subunit was calculated according to the molar absorption coefficient of DTNB ($13600 \text{ M}^{-1}\text{cm}^{-1}$): 4.4 for unmodified PDI and 3.5 for unmodified PDIp.

As shown in Figure 1A, the RNase, when present alone, retained most of its enzymatic activity after going through a sequential thermal treatment first at 65°C for 30 min and then cooling down to room temperature for another 30 min. Thermal treatment of RNase in the presence of PDI, however, completely abolished the enzymatic activity of RNase. PDIp also behaved similarly under these conditions. By contrast, thermal treatment of RNase in the presence of insulin or lysozyme, both containing disulfide bonds, did not have a similar effect on the RNase enzyme activity. The presence of bovine serum albumin (BSA), a protein known to have multiple hydrophobic sites [37] for interaction with unfolded proteins [38–39], had a modest inhibitory effect on the RNase enzyme activity. In addition, the presence of PDI/PDIp did not affect the enzymatic activity of native RNase (without thermal treatment) (data not shown). Lastly, when RNase was thermally treated first and then cooled down alone, subsequent addition of PDI/PDIp to the cooled RNase also did not exhibit any inhibitory effect (data not shown). Hence, the inhibitory effect of PDI/PDIp as observed in Figure 1A was attributable to PDI/PDIp-mediated structural alterations in the RNase during the thermal co-incubation.

Next, we determined the temperature dependence of PDI/PDIp-mediated inactivation of RNase by first co-incubating them at elevated temperatures (from 25 to 90°C) and then cooling the mixture down to room temperature before assaying the enzymatic activity. After normalization with the native RNase activity, PDI and PDIp showed a similar inactivation pattern (Figure 1B). The T_{50} value (thermal treatment temperature that inactivates 50% of the native RNase activity) for RNase alone was $78 \pm 2^\circ\text{C}$, whereas the T_{50} values in the presence of PDI and PDIp were $57.7 \pm 0.3^\circ\text{C}$ and $59.7 \pm 0.3^\circ\text{C}$, respectively.

PDI/PDIp-mediated inactivation of RNase at 65°C also depended on the thermal treatment time. A 5-min treatment showed a significant inhibition. The time course of RNase inactivation (Figure 2A) appeared to follow the exponential decay pattern when either PDI or PDIp was present. The rate constants for RNase inactivation were determined using an

exponential function to fit the data, and they were $0.082 \pm 0.006 \text{ min}^{-1}$ ($R^2 = 0.99045$) and $0.092 \pm 0.006 \text{ min}^{-1}$ ($R^2 = 0.99336$) in the presence of PDI and PDIp, respectively.

PDI/PDIp-mediated inactivation of RNase at 65°C also depended on the concentration of PDI/PDIp. Both PDI and PDIp at low concentrations (0.08–0.5 μM) could efficiently inactivate thermally-treated RNase (5 μM) (Figure 2B). When the concentration of PDI or PDIp was over 2.5 μM , the maximum inhibitory effect was reached. The IC_{50} values (at which concentration a 50% inhibition of the RNase activity is manifested) for PDI and PDIp were $0.46 \pm 0.06 \mu\text{M}$ ($R^2 = 0.99258$) and $0.73 \pm 0.11 \mu\text{M}$ ($R^2 = 0.98971$), respectively, based on regression analysis using a hyperbolar model.

3.2. Alkylated PDI and PDIp cannot inactivate RNase

To probe the mechanism of PDI/PDIp-mediated inactivation of RNase, we first sought to determine whether the inactivation is related to the enzymatic activity of PDI and PDIp. PDI was previously reported to be inactive upon iodoacetamide-mediated alkylation of its cysteine residues in the active sites [17]. Here a similar method was used to alkylate the purified PDI and PDIp proteins. As expected, alkylated PDI and PDIp could not catalyze the reactivation of rRNase whereas unmodified ones could (the insets in Figure 3A and 3B, respectively), suggesting that alkylated PDI and PDIp were devoid of enzymatic activity in catalyzing the formation of disulfide bonds in rRNase. In addition, we also found that they were devoid of enzymatic activity for reactivating sRNase (data not shown). In addition, we determined the redox state of alkylated PDI and PDIp (Figure S2). The numbers of the free cysteine residues per subunit were calculated: 2.2 for alkylated PDI and 2.2 for alkylated PDIp.

Next we determined the ability of alkylated PDI and PDIp to inactivate RNase after they were co-incubated at 65°C. As shown in Figure 3A and 3B, it was evident that alkylated PDI or PDIp even at saturating concentrations (2.5 and 5.0 μM) still could not inactivate RNase; in comparison, unmodified PDI and PDIp at the same concentrations could fully inactivate RNase (Figure 2B or compare with the green curve in Figure 3B).

3.3. PDIp's N-terminal active site plays a principal role in inactivating RNase

Both PDI and PDIp have two active sites located in their *a* and *a'* domains [40], respectively, and each active site has two cysteine residues. In addition, both PDI and PDIp contain two non-active site cysteine residues [32, 41]. It is, therefore, of interest to further determine which cysteine residue(s) is important for the inactivation of RNase. Since far less is known about the functional role of the cysteine residues in PDIp compared to PDI [8, 21, 23, 41–42], here we sought to focus on PDIp by using site-directed mutagenesis. Several PDIp mutant proteins were prepared and purified (Figure S1E).

First, we found that the two PDIp mutants (C4A and C350A) with their non-active site cysteines replaced could still efficiently inactivate RNase (Figure 4A), indicating that the non-active site cysteine residues are not required for RNase inactivation. Next, we examined the inactivating ability of three mutated PDIp proteins, designated as ΔC1 (the two cysteine residues in the *N*-terminal active site WCGHC were replaced with serine), ΔC2 (the two cysteine residues in the *C*-terminal active site WCTHC were replaced with serine), and $\Delta\text{C1}\Delta\text{C2}$ (the four cysteine residues from the two active sites were replaced with serine). As shown in Figure 4B, the mutant protein $\Delta\text{C1}\Delta\text{C2}$ did not exhibit appreciable ability to inactivate RNase. Interestingly, while the ΔC1 mutant protein did not show a significant inactivating ability, the ΔC2 mutant protein retained substantial inactivating activity when present at relatively high concentrations (Figure 4B), although it was not as effective catalytically as the wild-type protein (compare the results to those in Figure 2B).

Collectively, these data suggest that the contribution of the two active sites of PDIp to the inactivation of RNase is non-equivalent, *i.e.*, while the *N*-terminal active site plays a principal role, the *C*-terminal active site is also involved.

3.4. PDI and PDIp form stable disulfide-linked complexes with thermally-unfolded RNase

It is known that PDI can perform thiol-disulfide exchange reaction and form intermediates with rRNase and sRNase [21, 23]. Non-reducing SDS-PAGE was used here to separate the possible intermediates formed between PDI/PDIp and RNase, and Coomassie blue staining and Western blotting analysis were performed to detect these intermediates.

As shown in Figure 5A, thermally-treated RNase when present alone at 65°C existed as a single band between 15 and 20 kD (lane 1). Thermally-treated PDI present alone existed predominantly as a band between 50 and 75 kD (lane 2 in Figure 5A). When PDI and RNase were thermally treated together, new protein bands between the PDI band and the 250-kD band were observed (lane 3 in Figure 5A, as indicated by dark arrows on the left). Meanwhile, the density of the free RNase and PDI bands in lane 3 was significantly decreased compared to the density in lanes 1 and 2, respectively. This observation suggests that PDI and RNase formed complexes during thermal co-incubation. It is apparent that such complexes were cross-linked by inter-subunit disulfide bonds between PDI and RNase because these complexes were completely disappeared in the presence of DTT (lane 5). In addition, no such complexes were formed if PDI and RNase were co-incubated at 50°C (lane 4).

When Western blotting analysis was performed by using the anti-RNase antibody (left part, Figure 5B) or the anti-PDI antibody (data not shown), we confirmed that PDI formed disulfide-linked complexes with thermally-unfolded RNase (lane 3 *vs* 5, Figure 5B), but not with RNase at 50°C (lane 4, Figure 5B). It is of note that the relative reactivity of free RNase with the anti-RNase antibody was much lower than that of RNase in PDI-RNase complexes (compare the relative density of the free RNase band and the complexes in Figure 5B). This is most likely due to the spontaneous refolding of free denatured RNase on PVDF membrane during Western blotting while the disulfide-linked RNase by PDI in complexes cannot refold, and accordingly, the refolded RNase may have a much lower reactivity with the anti-RNase antibody than the unfolded RNase in complexes. This interpretation is supported by the observation that the denatured reduced RNase (lane 5 in Figure 5B) displayed a stronger density than the denatured oxidized RNase (lane 1 in Figure 5B) assuming that the latter was much easier to refold than the former, likely owing to the presence of the preserved disulfide bonds.

Similarly, PDIp could also form disulfide-linked complexes with thermally-unfolded RNase at 65°C (lane 8 *vs* 10, Figure 5A), but not with RNase at 50°C (lane 9 in Figure 5A). Interestingly, three predominant bands of the PDIp-RNase complexes were observed: two are over 250 kD and one is between 75 and 100 kD (lane 8 in Figure 5A, bands are indicated by dark arrows on the left). We confirmed these observations by Western blotting using the anti-RNase antibody (right part in Figure 5B) or the mouse anti-PDIp antiserum (data not shown).

3.5. PDI/PDIp-inactivated RNase can be reactivated in the presence of GSH and GSSG

It was reported earlier that the PDI-catalyzed reactivation efficiency of rRNase strongly depends on the composition of the redox buffer [22]. Hence, we sought to determine whether the conditions of the redox buffer could affect the dynamics of disulfide-linked PDI/PDIp-RNase complexes. First, we found that PDI/PDIp-RNase complexes formed after thermal co-incubation (as observed in Figure 5) were very stable and no reactivation of

RNase was observed even when these complexes were kept over-night at room temperature (data not shown). However, when these complexes were subjected to the RNase enzymatic activity assay in the presence of GSH and GSSG, we observed significant reactivation of RNase which started after the 30-min incubation (PDI-RNase in Figure 6A and PDIp-RNase in Figure 6B). Interestingly, the optimal reactivation of RNase was observed when 1 mM GSH and 0.2 mM GSSG were present, a condition that was found to be also optimal for PDI-catalyzed reactivation of rRNase [22]. Further increasing the ratio of GSH to GSSG did not further improve the reactivation of RNase in its complexes with PDI (Figure 6A), but partially reduced its reactivation when complexed with PDIp (Figure 6B).

In addition, we found that the reactivation of RNase in the presence of GSH and GSSG was due to the release of RNase from PDI/PDIp-RNase complexes. As shown in Figure 6C, the amount of free RNase was significantly increased when PDI/PDIp-RNase complexes were incubated with GSH and GSSG for 4 h (lane 2 vs 1; lane 4 vs 3). Meanwhile, the amount of free PDI and PDIp was also increased while the amount of the PDI-RNase and PDIp-RNase complexes was decreased.

4. DISCUSSION

A large number of earlier studies using various substrate proteins [7–9, 21–25] have clearly shown that the non-native disulfide bonds could be readily recognized by PDI for conversion into correct disulfide bonds. During the refolding of some of these substrate proteins [8, 21, 23–25], stable disulfide-linked PDI-substrate complexes have also been detected. These complexes are generally considered to be intermediates formed during PDI-catalyzed protein folding [26], and their detection provides important experimental evidence for understanding the molecular mechanisms of the protein folding process. Besides non-native disulfide bonds, studies have also suggested that native disulfide bonds, either in native proteins [13, 15–16] or folding intermediates [7, 14, 27–28], are also susceptible to PDI attack. However, no direct evidence has been obtained for the existence of stable complexes between PDI and native disulfide bonds-containing substrate proteins. The results of our present study show that PDI and PDIp can inactivate thermally-unfolded RNase containing the original native disulfide bonds. In addition, we have also demonstrated, for the first time, that PDI and PDIp can form stable disulfide-linked complexes with the native disulfide bonds-containing RNase (Figure 5). These stable complexes could dissociate under physiologically-relevant redox conditions, and the inactive RNase in the PDI/PDIp-RNase complexes can be released and reactivated (Figure 6). The reactivation of RNase from its complexes with PDI (or PDIp) appears to be dependent on the catalytic activity of PDI (or PDIp) since the PDI-catalyzed reactivation of rRNase [22] shares the same optimal redox conditions as the reactivation of PDI/PDIp-inactivated RNase (Figure 6).

It is important to be able to detect that disulfide-linked complexes are formed between PDI and RNase under non-reductive conditions, and that RNase is released and reactivated from the complexes under suitable redox conditions. Such a two-step process of interaction between PDI and native disulfide bond-containing substrate proteins offers mechanistic insights into PDI-catalyzed conversion of kinetically-trapped folding intermediates containing native disulfide bonds into on-pathway intermediates during the oxidative refolding of proteins. For instance, it is known that PDI can accelerate the rate-limiting steps during the refolding of RNase [13–14, 27, 29]. Based on our finding, it is suggested that such acceleration might proceed via a two-step process as follows. First, PDI forms disulfide-linked complexes with kinetically-trapped RNase intermediates *des*-[26–84] and *des*-[58–110], each of which contains three pairs of native disulfide bonds [27]. Second, subsequent release of these trapped intermediates from the complexes coupled with PDI-

catalyzed conversion of them into the on-pathway intermediates like *des*-[40–95] would occur under suitable redox conditions, consequently resulting in an acceleration of the refolding. In addition, the proposed mechanistic explanation can also be used to account for some of the earlier observations on BPTI. It was observed earlier that the refolding of denatured reduced BPTI usually proceeds very slowly due to the formation of two kinetically-trapped intermediates [30–51; 14–38] and [5–55; 14–38], both of which contain two pairs of native disulfide bonds and have to unfold substantially before becoming the final native-like product [30–51; 5–55][43]. PDI was found to accelerate the refolding efficiency of BPTI through increasing the conversion of the trapped intermediates to the native-like product [7, 13]. Here, it is suggested that PDI might perform thiol-disulfide exchange reactions with the native disulfide bonds present in the trapped BPTI intermediates via forming disulfide-linked complexes, and from these complexes the trapped intermediates are released in the presence of GSH/GSSG and converted to the native-like intermediates. This hypothesis can be tested by studying the interactions of PDI with the kinetically-trapped RNase/BPTI folding intermediates.

Based on the observations made in the present study, together with those made by others on sRNase [23] and rRNase [21], it is reasonable to conclude that the nativity (*i.e.*, native *vs* non-native) of the disulfide bonds is not a determining factor in disulfide recognition by PDI/PDIp. Nevertheless, it should be noted that PDI and PDIp can also interact with cysteine-free substrate proteins [44–45], through non-covalent forces (*e.g.*, hydrophobic interactions [46]). It is possible that these interactions might play a role during the formation of the initial non-covalent PDI/PDIp–RNase complexes, which may determine the relative prevalence of various covalent complexes. In the same vein, it is also of note that while the native secondary and tertiary structures of RNase are known to be largely preserved at temperatures below 55°C, the protein undergoes unfolding at 55–65°C [35–36, 47]. This unique biophysical property, together with our observation that PDI/PDIp only inactivates RNase (Figure 1B) and forms stable complexes with it at 65°C but not 50°C (Figure 5), suggests that PDI and PDIp can only form stable complexes with the substantially-unfolded RNase (presumably with more hydrophobic regions exposed), but not with slightly-unfolded or fully-folded RNase. It appears that PDI readily undergoes non-specific thiol-disulfide exchange with unfolded proteins, and any discrimination against disulfide bonds in native states is provided by stable non-covalent structure which shields the native disulfide bonds from attack by PDI. This property very likely stems from the chaperone activities of PDI and PDIp as observed earlier [17, 33], which enable them to preferentially recognize non-native conformations and thus prefer to bind to non-native substrate proteins [15, 20, 25]. This interpretation may be used to explain our observation that BSA can also partially inactivate thermally-unfolded RNase (Figure 1A). BSA is known to be able to act as a chaperone and interact with unfolded proteins through hydrophobic interactions [37–39], and it is possible that BSA may interact with substantially-unfolded RNase during thermal treatment through hydrophobic interactions and thus form complexes, which, in turn, partially inhibit the spontaneous refolding/reactivation of RNase during cooling.

Our study suggests that only certain reduced cysteine residues in PDI/PDIp play a role in their inactivation effect based on the following observations. First, the estimated average numbers of alkylated cysteine residues in each PDI and PDIp are 2.2 and 1.3 per subunit, respectively (based on the data shown in Figure S2). Notably, the value for PDI is consistent with an earlier observation indicating that approximately 2.1 cysteine residues per PDI subunit are alkylated [17]. Second, the alkylated cysteine residues in both PDI and PDIp are essential to their ability to inactivate RNase (Figure 3), which suggests that only certain reduced cysteine residues are involved in this catalytic function. In support of this suggestion, the cysteine residues in the *N*-terminal active site of PDIp appear to play a

principal role in its inactivation of RNase while other cysteine residues only contribute partially, or do not contribute at all, to this function (Figure 4).

It is also of note that the mechanism of RNase inactivation by PDI and PDIp is different from its inactivation by GSH (Figure S1C), on the basis of the following reasons. First, the estimated average 2.2 reduced cysteine residues remaining in each alkylated PDI and PDIp (Figure S2) likely are not capable of inactivating RNase (Figure 3). Second, when PDI was present at a molar ratio of approximately 1:10 to RNase, 50% of RNase proteins were found to be inactivated (Figure 2B). This observation suggests that during inactivation, the PDI-mediated disulfide isomerization likely would entail the release of disulfide-shuffled RNase from the complexes with PDI as well as regeneration of the dithiol active site in PDI. Third, the chaperone activity of PDI/PDIp appeared to be also involved in the inactivation process. Based on these observations, it is thus suggested that PDI and PDIp can serve both as chaperones that can recognize the unfolded RNase and also as enzymes that can attack the native disulfide bonds of RNase. In contrast, GSH would only serve as a chemical agent facilitating the reduction of native disulfide bonds of RNase.

Lastly, we also found that PDI (also PDIp) can form both small complexes and high molecular weight complexes with the thermally-unfolded RNase. The small complexes containing one subunit of PDI (as indicated by the dark arrow between 75 and 100 kD in Figure 5A) were only marginally observed, in line with the earlier observations that PDI formed similar complexes with rRNase [21] or sRNase [23]. The high molecular weight complexes, which presumably contain multiple subunits of PDI and RNase, were clearly observed (between 150 and 250 kD in Figure 5A for PDI, and over 250 kD in Figure 5A for PDIp). Given that the two active sites of PDIp both contribute to the inactivation of RNase (Figure 4), it is hypothesized that each subunit of PDIp may react with two disulfide bonds either from one or two RNase molecule(s), and likewise, one subunit of RNase (containing four pairs of disulfide bonds) may react with up to four active sites from multiple subunits of PDIp. As a result, PDIp and RNase may form a short chain-like complexes linked together by inter-molecular disulfide bonds.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported, in part, by a grant from the NIH (CA097109). Some of the analytical and imaging instruments employed in this study are part of the COBRE core facility that is supported by the NIH Grant P20RR021940 from the National Center for Research Resources.

ABBREVIATIONS USED

PDI	protein disulfide isomerase
PDIp	pancreas-specific PDI homolog
RNase	bovine pancreas RNase A
BPTI	bovine pancreas trypsin inhibitor
BSA	bovine serum albumin
DTT	dithiothreitol
GSH	glutathione

GSSG	oxidized glutathione
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)

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Research Highlights

- Both PDI and PDIp can inactivate thermally-unfolded RNase that preserves native disulfide bonds.
- The N-terminal active site of PDIp plays a principle role in inactivating RNase.
- Stable disulfide-linked complexes are formed between PDI/PDIp and RNase
- RNase can be released and reactivated from the PDI/PDIp-RNase complexes

FIGURE 1. Thermal inactivation of RNase by PDI and PDIp

A: Time course for the absorbance change at 296 nm produced by RNase-catalyzed hydrolysis of cCMP at 25°C, pH 8.5. RNase (5 μ M) was thermally treated at 65°C for 30 min in the absence or presence of PDI (2.5 μ M), PDIp (2.5 μ M), insulin (5 μ M), lysozyme (5 μ M), or BSA (5 μ M, two samples purchased from two different vendors), and then cooled down to 25°C for 30 min before assaying the RNase enzymatic activity as described in Materials and Methods. **B:** Temperature-dependent inactivation of RNase (5 μ M), which was thermally treated at indicated temperature for 30 min in the absence or presence of PDI (2.5 μ M) or PDIp (2.5 μ M) as described in panel A. The curves are the regression analysis results based on the Sigmoidal (Boltzmann) model using the Microcal Origin software. The results in panel A are a representative data set from three independent experiments, and data in panel B are a representative set from two independent experiments.

FIGURE 2. Time- and concentration-dependent thermal inactivation of RNase by PDI and PDIp
A: Time-dependent inactivation of RNase ($5 \mu\text{M}$), which was thermally treated at 65°C for varying length of time (0, 2, 5, 10, 15, 30, or 60 min) in the presence of PDI or PDIp ($2.5 \mu\text{M}$) and then cooled down before assaying the RNase enzymatic activity. The curves are the regression results based on the exponential decay model using the Microcal Origin software. The inset shows the time course for the RNase enzymatic activity. **B:** Concentration-dependent inactivation of RNase ($5 \mu\text{M}$), which was thermally treated at 65°C for 30 min in the presence of increasing concentrations of PDI or PDIp and then cooled down before assaying the RNase enzymatic activity. The curves are the regression analysis results based on the hyperbolic model using the Microcal Origin software. The results in panels **A** and **B** are the representative data sets from two independent experiments.

FIGURE 3. Inability of alkylated PDI and PDIp to inactivate RNase

Time course for the absorbance change at 296 nm produced by RNase-catalyzed hydrolysis of cCMP at 25°C. RNase (5 μ M) was thermally treated at 65°C for 30 min in the absence or presence of PDI (2.5 μ M, panel **A**), alkylated PDI (2.5 or 5 μ M, panel **A**), PDIp (2.5 μ M, panel **B**), or alkylated PDIp (2.5 or 5 μ M, panel **B**) and cooled down before assaying the RNase enzymatic activity. The inset in panel **A** shows PDI- or alkylated PDI-catalyzed reactivation of rRNase as described in Material and Methods, and the insert in panel **B** shows the similar results for PDIp and alkylated PDIp. The results in panels **A** and **B** are representative data sets from three independent experiments.

FIGURE 4. Relative contribution of PDIp's two active sites to the inactivation of RNase
A: Time course for the absorbance change at 296 nm produced by RNase-catalyzed hydrolysis of cCMP at 25°C. RNase (5 μM) was thermally treated at 65°C for 30 min in the presence of PDIp mutant proteins C4A or C350A (1.5 or 2.5 μM) and cooled down before assaying the RNase enzymatic activity. **B:** Concentration-dependent inactivation of RNase by PDIp mutant proteins ΔC1 , ΔC2 or $\Delta\text{C1}\Delta\text{C2}$. Similar experimental conditions as described in Figure 2B were used. The results in panels **A** and **B** are representative data sets from two independent experiments.

FIGURE 5. Disulfide-linked complex formation between PDI/PDIp and RNase

RNase (5 μ M) was thermally treated at 65°C for 30 min in the absence (lane 1 and 6) or presence of PDI (2.5 μ M, lane 3) or PDIp (2.5 μ M, lane 8), cooled down, and then alkylated with 10 mM iodoacetamide for 2 h before non-reducing SDS-PAGE analysis. **A:** For Coomassie blue staining, protein samples were separated by 15% acrylamide gel, in which the density of RNase stained by Coomassie blue reflects its relative abundance. Note that we found that the RNase band separated in the 10% acrylamide gel (data not shown) could not reflect its amount well because of its very narrow band. **B:** For Western blotting analysis, protein samples were separated by 10% acrylamide gel to clearly show the higher molecular complexes. Note that the amount of protein loaded in panel B was one-twentieth of the amount loaded in panel A because of the extremely high sensitivity of the anti-RNase antibody (dilution of 6000 folds) used in the Western blotting analysis. Several controls were analyzed here, including thermal-treated PDI (lane 2) or PDIp (lane 7) alone, thermal-treated mixtures that were analyzed in the reducing SDS-sample loading buffer containing

100 mM DTT (lane 5 and 10), and the mixture of RNase with thermal treatment at 50°C (lane 4 and 9). Additionally, since the inter-subunit disulfide bond of PDIp as observed in our recent study [32] may interfere with the complex detection, PDIp was pre-treated with 5 mM DTT and desalted to remove DTT before the thermal co-incubation with RNase as described in Materials and Methods. The results in panel **A** and **B** are representative data sets from five independent experiments.

FIGURE 6. Reactivation of PDI/PDIp-inactivated RNase in the redox buffer

A and B: Time courses for the absorbance changes at 296 nm produced by RNase-catalyzed hydrolysis of cCMP at 25°C. RNase (5 μ M) was thermally treated at 65°C for 30 min in the presence of PDI (2.5 μ M, panel **A**) or PDIp (2.5 μ M, panel **B**) and cooled down before assaying the RNase enzymatic activity in the redox buffer (0.1 M Tris-HCl, plus varying concentrations GSH and GSSG). **C:** Results from the non-reducing SDS-PAGE analysis (15% acrylamide gel) of the mixtures of thermally treated RNase (5 μ M) with PDI (2.5 μ M, lane 1 and 2) or PDIp (2.5 μ M, lane 3 and 4). The mixtures after thermal treatment was incubated for 4 h in the presence (lane 2 and 4) or absence (lane 1 and 3) of 1 mM GSH and 0.2 mM GSSG, alkylated with 10 mM iodoacetamide for 2 h, and then subjected to non-reducing SDS-PAGE analysis using Coomassie blue staining. The results in panels **A** and **B** are representative data sets from three independent experiments, and the results in panel **C** are representative data from two independent experiments.