

# Dependence of the anti-chaperone activity of protein disulphide isomerase on its chaperone activity

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Protein disulphide isomerase (PDI) shows chaperone and anti-chaperone activities in assisting refolding of denatured and reduced lysozyme in redox Hepes buffer, but only chaperone activity in phosphate buffer and redox Hepes buffer containing 0.1 M NaCl. In non-redox Hepes buffer its anti-chaperone activity is very weak. PDI displays its anti-chaperone activity only for those substrates showing relatively low aggregation during refolding, and is strongly dependent on refolding conditions, of which ionic strength appears to be an important factor.

The S-methylated PDI, fully active as a chaperone but devoid of isomerase activity, by itself shows only anti-chaperone activity, but reinforces rather than suppresses the chaperone activity of native PDI in the refolding of lysozyme. A fragment of PDI with the C-terminal peptide-binding sequence removed and devoid of chaperone activity does not show anti-chaperone activity in lysozyme refolding. It appears that the anti-chaperone activity of PDI is dependent on its chaperone activity.

## INTRODUCTION

Protein disulphide isomerase (PDI; EC 5.3.4.1), an abundant protein in the lumen of the endoplasmic reticulum, has been identified as the physiological catalyst for the formation of native disulphide bonds of nascent peptides in cells [1]. In recent years because of the discovery of a new family of functional proteins, molecular chaperones, the classical 'self-assembly' principle of nascent peptide folding has been replaced by the new concept of 'assisted folding'. The proteins involved are classified as molecular chaperones and foldases [2]. PDI, which facilitates the folding of disulphide-containing peptides by catalysing the formation of correct disulphide bond(s) [1,3], has been recognized as one of the two foldases so far characterized. PDI has also been found to be a remarkable multifunctional protein [4]. It is the  $\beta$ -subunit of prolyl-4-hydroxylase [5] and the small subunit of a microsomal triglyceride-transfer protein complex [6]. It is a peptide-binding protein with low specificity [7] and has many other properties.

Many experimental data obtained *in vivo* imply that the isomerase activity might not be the only, or even the major, function of PDI in cells. A mutant PDI with no isomerase activity can increase the folding and secretion of lysozyme co-expressed in yeast [8]. PDI was found to be associated with misfolded but not native proteins in cells [9]. PDI mutated at its active sites with no isomerase activity is still functional as the  $\beta$ -subunit for the assembly of the fully active tetramer of prolyl-4-hydroxylase  $\alpha_2\beta_2$  [10] and the small subunit of the dimer of the microsomal triglyceride-transfer protein complex [11].

It has been explicitly stated by Ellis [12] that PDI is not a chaperone according to the definition of molecular chaperones, i.e. chaperones assist protein folding that does not involve the formation or breaking of covalent bonds. However, we [13,14] and others [3] have put forward a hypothesis that PDI is both an

enzyme and a molecular chaperone. The isomerase-independent chaperone activity of PDI has been shown by increasing re-activation yield and decreasing aggregation during refolding of denatured D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and rhodanese on dilution, both of which contain no disulphide bonds [15,16]. The chaperone activity of PDI has also been shown in the refolding of disulphide-containing proteins, such as lysozyme [17] and acidic phospholipase A<sub>2</sub> (APLA<sub>2</sub>) [18]. For maximal re-activation of proteins containing disulphide bond(s), both isomerase and chaperone activities of PDI are required [18].

On the other hand, PDI had no effect on refolding of immunoglobulin with intact disulphide bonds but promoted oxidative re-activation of the reduced molecule and thus was suggested by Lilie et al. [19] to act as an isomerase but not as a chaperone.

Puig et al. [20] reported that, in addition to its isomerase and chaperone activities, PDI at low concentrations and a mutant PDI (N<sub>m</sub>C<sub>m</sub>-PDI) with Cys replaced by Ser in the sequence -CGHC- of both active sites at all concentrations decreased the refolding yield of lysozyme and considered this an unusual anti-chaperone activity. In a recent publication, Primm et al. [21] proposed that the anti-chaperone activity of PDI was due to multivalent binding of the partially aggregated substrate intermediates to the PDI molecule.

We did not find any anti-chaperone activity of PDI in the refolding of GAPDH [15] and rhodanese [16], neither did Primm et al. [21] for citrate synthase. In the present study we examined the chaperone and anti-chaperone activities of PDI in the refolding of lysozyme under different experimental conditions and found that the anti-chaperone activity of PDI in Hepes buffer disappeared if NaCl was added to the buffer or phosphate buffer was used instead of Hepes. In the presence of small amounts of native PDI the reduced re-activation of lysozyme can

Abbreviations used: PDI, protein disulphide isomerase; mPDI, S-methylated PDI; abb'a', mutant PDI with the C-terminal 51 amino acid residues deleted; GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase; DTT, dithiothreitol; N<sub>m</sub>C<sub>m</sub>-PDI, mutant PDI with all four cysteines in two active sites replaced by serines; APLA<sub>2</sub>, acidic phospholipase A<sub>2</sub>.

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be increased considerably rather than further suppressed by the presence of increasing concentrations of S-methylated PDI (mPDI), which is devoid of isomerase activity but fully active as a chaperone [22] and by itself behaves as an anti-chaperone for lysozyme refolding in redox Hepes buffer. It appears that the anti-chaperone activity of PDI is an alternative behaviour of the intrinsic chaperone activity of PDI under specified conditions.

## EXPERIMENTAL

### Materials

Hen's egg white lysozyme, dithiothreitol (DTT) and GSSG were purchased from Serva; BSA, *Micrococcus lysodeikticus* dried cells, guanidinium chloride and GSH were from Sigma; Hepes was from Merck. All other chemicals were local products of analytical grade.

PDI was prepared from bovine liver by the method of Lambert and Freedman [23] and showed one band on SDS/PAGE with a specific activity of more than 800 units/g. mPDI, methylated at the thiol groups of active sites with the sequence -CGHC- and with less than 4% of the isomerase activity of native PDI, was prepared as described by Quan et al. [22]. Mutant PDI with the C-terminal 51 amino acid residues deleted (abb'a') was a gift from Mr. Y. Dai of this group.

### Denaturation and reduction of lysozyme

Lysozyme at 20 mg/ml was completely denatured and reduced in 0.1 M sodium phosphate buffer, pH 8.0, containing 8 M guanidinium chloride and 0.15 M DTT at room temperature for 4 h. The reaction mixture was brought to pH 2.0 with 6 M HCl, and thoroughly dialysed first against 0.01 M HCl for 3 h and then against 0.1 M acetic acid at 4 °C. The denatured and reduced lysozyme was divided into aliquots and stored at -20 °C.

### Refolding of lysozyme

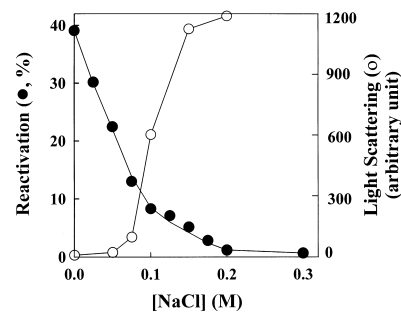
Oxidative refolding of reduced and denatured lysozyme was achieved by dilution in various buffers as specified with or without different concentrations of PDI and/or mPDI at room temperature. The Hepes buffer, 0.1 M, pH 7.0, contained 2 mM EDTA, 5 mM MgCl<sub>2</sub> and 20 mM NaCl, and the phosphate buffer, 0.1 M, pH 7.5, contained 2 mM EDTA. If not specified otherwise, the refolding buffer contained 1 mM GSSG and 2 mM GSH (as the ratio of GSH to GSSG has been determined to be 2 in the endoplasmic reticulum [24]), and the final concentration of lysozyme for refolding was 10 μM. When GSSG and GSH were not present, the system was referred to as the non-redox buffer. Recovery of activity was complete 2 h after dilution, at which point it was determined. Lysozyme activity was determined at 30 °C by following the lysis of *Micrococcus lysodeikticus* [17,25]. The decrease in  $A_{450}$  of a 0.25 mg/ml cell suspension in 67 mM sodium phosphate buffer, pH 6.2, containing 0.1 M NaCl was measured in a Shimadzu UV-250 spectrometer. The full scale for  $\Delta A_{450}$  was set at 0.2 and the amount of enzyme was selected to give a  $\Delta A_{450}$  of less than 0.1 per minute. Protein concentrations were determined by measuring  $A_{280}$  with the following absorption coefficients ( $A_{1\text{cm}}^{0.1\%}$ ): 0.9 for PDI, 0.66 for BSA, 2.63 for native lysozyme and 2.37 for denatured lysozyme [17]. Lysozyme aggregation during refolding was followed continuously by measuring 90° light scattering at 500 nm in an Hitachi model F-4010 spectrofluorimeter at 20 °C.

## RESULTS

The re-activation yield of denatured and reduced lysozyme on dilution into phosphate buffer decreases markedly with increasing protein concentration. The recovery of activity of 22% at 2.1 μM falls to about 1% with strong aggregation as monitored by light scattering when the concentration of denatured lysozyme increases to above 8 μM. However, in Hepes buffer the spontaneous re-activation of lysozyme in the concentration range 1–10 μM remains at a fairly high and constant level of about 42% with little aggregation (results not shown).

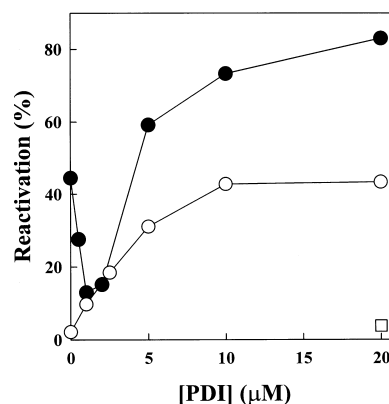
### Effect of NaCl concentration on spontaneous refolding of denatured and reduced lysozyme in Hepes buffer

Phosphate and Hepes buffers differ greatly in ionic strength, and the effect of increasing concentrations of NaCl in Hepes buffer was therefore examined. Figure 1 shows that the spontaneous re-



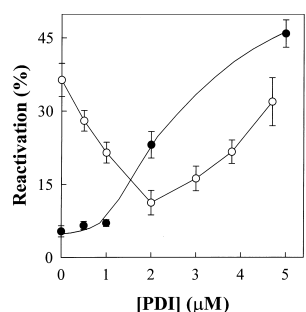
**Figure 1** Effects of NaCl concentration on the re-activation and aggregation of lysozyme during refolding in Hepes buffer

The refolding buffer contained different concentrations of NaCl as indicated. Re-activation was assayed 2 h after dilution. The eventual level of aggregation was recorded 10 min after dilution as measured by light scattering at 500 nm. The experimental details for re-activation of lysozyme were as described in the text.  $\Delta A_{450}/\text{min}$  for 0.1 nmol of native lysozyme is 0.084 corresponding to 100% re-activation.



**Figure 2** Effects of PDI concentration on lysozyme re-activation in phosphate buffer and Hepes buffer

The conditions for refolding of lysozyme at 10 μM were as described in the text with the presence of different concentrations of PDI (○) or BSA (□) in phosphate buffer and PDI (●) in Hepes buffer.  $\Delta A_{450}/\text{min}$  for 0.1 nmol of native lysozyme is 0.084 corresponding to 100% re-activation.



**Figure 3** Effect of PDI on the lysozyme re-activation in Hepes buffer containing 0.1 M NaCl or 5% ethylene glycol

The experiments were carried out as described in the legend to Figure 2 except that the refolding system contained 0.1 M NaCl (●) or 5% ethylene glycol (○).  $\Delta A_{450}/\text{min}$  for 0.1 nmol of native lysozyme is 0.078 corresponding to 100% re-activation. Data are expressed as means  $\pm$  S.D. ( $n = 2$ ).

activation of lysozyme in Hepes buffer decreases with added NaCl from 40% to about 1% at 0.2 M NaCl. Concurrently, the slight aggregation as monitored by light scattering at 0.05 M NaCl increases to its maximal level at 0.2 M NaCl.

#### PDI-assisted refolding of denatured and reduced lysozyme

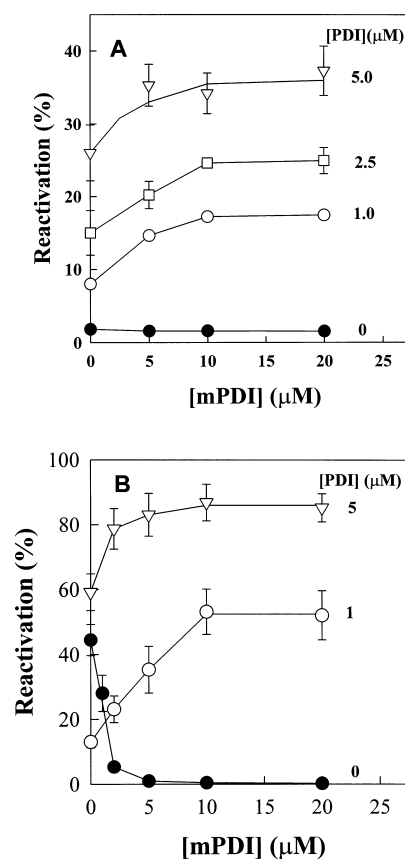
As shown in Figure 2, in Hepes buffer, the presence of low concentrations of PDI decreases the re-activation yield of lysozyme from 44% to a minimum of 13% at a PDI concentration of 1  $\mu\text{M}$ , and with further increases in PDI concentration the re-activation increases to a maximal level of 83% with PDI at 20  $\mu\text{M}$ . The above results are similar to those reported by Puig and Gilbert [17] and denoted as the anti-chaperone and chaperone activity of PDI. However, the effect of PDI on the re-activation of lysozyme in phosphate buffer is very different in that the very low spontaneous re-activation of lysozyme increases with increasing concentrations of PDI in the refolding buffer up to a maximal level of around 43% at a stoichiometric amount of PDI (equimolar ratio of PDI protomer to lysozyme). BSA at 20  $\mu\text{M}$  has no effect on the re-activation of lysozyme.

#### Effects of NaCl and ethylene glycol on the PDI-assisted refolding of lysozyme in Hepes buffer

As shown in Figure 3, in the presence of 0.1 M NaCl, PDI increases the re-activation of lysozyme with increasing concentrations without showing any anti-chaperone effect at all the concentrations examined. When 5% ethylene glycol was present in the refolding system, lysozyme re-activation first decreases from 37 to 11% and then increases with increasing PDI concentrations, showing the strongest anti-chaperone effect at 2  $\mu\text{M}$  PDI as compared with a concentration of 1  $\mu\text{M}$  in Hepes buffer with no ethylene glycol. As mentioned above, during spontaneous refolding in Hepes buffer, lysozyme shows little aggregation which decreases further in the presence of 5% ethylene glycol (results not shown).

#### Effect of mPDI on the re-activation of lysozyme

As shown in our previous work [22], mPDI is inactive as an isomerase but retains almost full chaperone activity in assisting re-activation of GAPDH, a protein containing no disulphide bonds. For the disulphide-containing lysozyme, mPDI does not

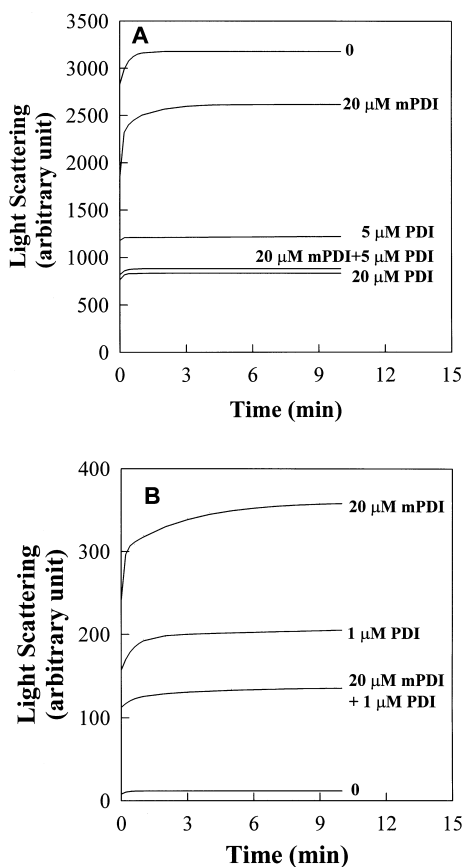


**Figure 4** Effects of mPDI concentration on lysozyme re-activation in phosphate buffer (A) and Hepes buffer (B) containing different concentrations of PDI

Conditions were as described in the legend to Figure 2 except that the refolding buffer contained different concentrations of mPDI and PDI as indicated.  $\Delta A_{450}/\text{min}$  for 0.1 nmol of native lysozyme is 0.087 corresponding to 100% re-activation. Data are expressed as means  $\pm$  S.D. ( $n = 3$ ).

promote its re-activation in phosphate buffer (Figure 4A), undoubtedly because of its lack of isomerase activity for disulphide formation, as in the case of its effect on APLA<sub>2</sub> re-activation [18]. However, in the presence of native PDI at different concentrations the simultaneous presence of increasing concentrations of mPDI always increases the extent of lysozyme re-activation, reaching a constant level at a stoichiometric concentration of mPDI. With PDI at 5  $\mu\text{M}$  and the simultaneous presence of 10  $\mu\text{M}$  mPDI, lysozyme re-activation reaches the same maximal level as produced by PDI alone at 10  $\mu\text{M}$ .

In Hepes buffer, mPDI alone decreases the re-activation of denatured and reduced lysozyme (Figure 4B) and suppresses the re-activation completely at concentrations higher than 5  $\mu\text{M}$ ; it thus behaves as an anti-chaperone in a similar way to N<sub>m</sub>C<sub>m</sub>-PDI, as reported by Puig et al. [20]. However, interestingly, with native PDI at the low concentration of 1  $\mu\text{M}$ , the concentration at which it functions most strongly as an anti-chaperone (Figure 2), the simultaneous presence of mPDI, instead of further suppressing the extent of lysozyme re-activation, actually increases it with increasing concentrations of mPDI. In the presence of PDI at 5  $\mu\text{M}$ , mPDI at 10  $\mu\text{M}$  increases lysozyme re-activation to above 80% which is the maximal level that can be reached with native PDI alone.



**Figure 5** Effects of PDI and/or mPDI on lysozyme aggregation during refolding in phosphate buffer (A) and Hepes buffer (B)

The conditions for refolding of lysozyme at 10  $\mu\text{M}$  were as described in the legend to Figure 2 except for the presence of PDI and/or mPDI at concentrations as indicated. The aggregation was followed by recording light scattering at 500 nm immediately after dilution.

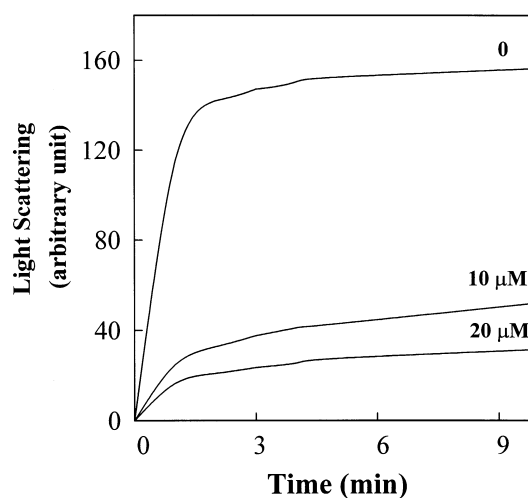
#### Effect of PDI and mPDI on lysozyme aggregation during refolding

As shown in Figure 5(A), during spontaneous refolding, lysozyme at 10  $\mu\text{M}$  aggregates strongly in phosphate buffer. The aggregation is decreased markedly by 5  $\mu\text{M}$  PDI and further by 20  $\mu\text{M}$  PDI, but only slightly by the same concentration of mPDI. Concurrent to the re-activation, the simultaneous presence of 5  $\mu\text{M}$  PDI and 20  $\mu\text{M}$  mPDI decreases aggregation to an extent similar to that caused by PDI alone at 20  $\mu\text{M}$ .

Different from the results obtained in phosphate buffer shown in Figure 5(A) and compatible with the fairly high spontaneous re-activation, lysozyme at 10  $\mu\text{M}$  shows little light scattering during spontaneous refolding in Hepes buffer (Figure 5B). Concurrent with the decrease in re-activation yield of lysozyme, both mPDI at 20  $\mu\text{M}$  and PDI at 1  $\mu\text{M}$  greatly increase aggregation, but the simultaneous presence of both mPDI at 20  $\mu\text{M}$  and PDI at 1  $\mu\text{M}$  decreases it, in accord with the increase in lysozyme re-activation in the simultaneous presence of both PDI and mPDI as shown above.

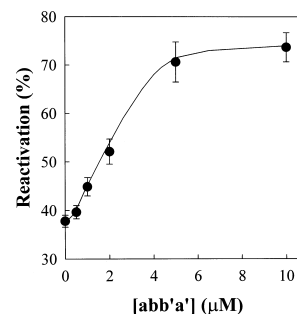
#### Refolding of denatured and reduced lysozyme in a non-redox or reduced Hepes buffer

The spontaneous refolding of denatured and reduced lysozyme in Hepes buffer with no redox component took 5–6 h (results not shown) to reach a constant re-activation level of only 5% as



**Figure 6** Effects of mPDI on lysozyme aggregation during refolding in reduced Hepes buffer

The conditions for lysozyme refolding were as described in the legend to figure 5 except that the Hepes buffer contained 2 mM DTT. mPDI at the indicated concentrations was preincubated in the buffer for 2 min before the addition of lysozyme.



**Figure 7** Effect of abb'a' on lysozyme re-activation in Hepes buffer

Experimental conditions were as in the legend to Figure 2 except that PDI was replaced by abb'a'.  $\Delta A_{450}/\text{min}$  for 0.1 nmol of native lysozyme is 0.083 corresponding to 100% re-activation. Data are expressed as means  $\pm$  S.D. ( $n = 2$ ).

compared with 2 h and 40% in the presence of 1 mM GSSG and 2 mM GSH. The presence of PDI at 1  $\mu\text{M}$  shows only a weak anti-chaperone effect and at 10  $\mu\text{M}$  increases re-activation to only 11%. The presence of 20  $\mu\text{M}$  mPDI alone in the non-redox buffer produces slight suppression of lysozyme re-activation compared with that of spontaneous re-activation, but when added together with PDI always increases the re-activation produced by PDI alone at concentrations lower than 10  $\mu\text{M}$  (results not shown).

Denatured and reduced lysozyme shows no spontaneous re-activation in Hepes buffer containing 2 mM DTT, as the formation of disulphide bonds is not possible, but shows extensive aggregation (Figure 6). In contrast with the case with the redox buffer (Figure 5B), the presence of mPDI at 10 or 20  $\mu\text{M}$  decreases aggregation markedly.

#### Effect of abb'a' on lysozyme refolding in Hepes buffer

The C-terminal 51 amino acid residues of PDI have been shown to contain a peptide-binding site [26], and abb'a', which has these

residues removed, retains most of the isomerase activity of PDI but shows no chaperone activity in GAPDH refolding [26a]. In contrast with native PDI, abb'a' shows no anti-chaperone activity in the refolding of lysozyme in Hepes buffer under the same conditions, but functions as an isomerase in increasing the re-activation yield to 78% similar to the maximal re-activation of lysozyme assisted by native PDI (Figure 7).

## DISCUSSION

The oxidative re-activation and aggregation of denatured and reduced lysozyme on dilution in phosphate and Hepes buffers are considerably different. The refolding of a protein is affected not only by its concentration, but also by experimental conditions, of which the ionic strength of the solution is of great importance. In phosphate buffer or Hepes buffer with NaCl, the aggregation-prone intermediates could be highly populated probably by decreased ionic interaction between intermediates less prone to aggregation. The addition of ethylene glycol increases the hydrophobicity of the solution, thereby decreasing the hydrophobic interaction of the folding intermediates and resulting in an increase in free intermediates and lower aggregation. The nature of the redox reagents is also of importance. In the absence of GSSG, the spontaneous re-activation of lysozyme in Hepes buffer becomes very low because only air oxygen can be used as an oxidant, and PDI has only a weak assisting effect on re-activation and little anti-chaperone activity. In reduced Hepes buffer, extensive aggregation of refolding intermediates of lysozyme still occurs even though no re-activation can be detected. Folding intermediates of denatured and reduced lysozyme under different redox systems have been studied [27], and different redox reagents show different abilities to stabilize mixed disulphides, thus affecting various folding pathways differently [28].

Puig and Gilbert [17] suggested that the anti-chaperone activity of PDI resulted from the combination of insolubility of the protein substrate and an intrinsic activity of PDI. That PDI exhibited no anti-chaperone activity during refolding of ribonuclease was ascribed to the high solubility of the denatured ribonuclease [17]. However, we found that PDI showed anti-chaperone activity for lysozyme refolding in Hepes buffer but only chaperone activity in phosphate buffer, although, during refolding, lysozyme at 10  $\mu$ M aggregates strongly in phosphate but only weakly in Hepes buffer, indicating lower solubility of folding intermediates in the former than in the latter. To examine carefully any possible anti-chaperone activity of PDI in phosphate buffer, lysozyme refolding at 3  $\mu$ M has also been studied. In this case, lysozyme shows much higher re-activation (11%) than that at 10  $\mu$ M (1%) and still aggregates significantly. Nevertheless the presence of PDI from 0.15  $\mu$ M (a molar ratio of 0.05) to 3  $\mu$ M has no anti-chaperone effect at all, only a positive chaperone effect (results not shown). Moreover, PDI in the concentration range 0.5–5  $\mu$ M shows only a positively assisting effect and no longer any anti-chaperone activity if 0.1 M NaCl is added to the Hepes buffer, leading to a marked decrease in re-activation and a significant increase in aggregation of lysozyme at 10  $\mu$ M. In contrast, the presence of 5% ethylene glycol decreases aggregation and leads to a widening of the PDI concentrations required to show anti-chaperone activity up to a concentration of PDI of 2  $\mu$ M as compared with 1  $\mu$ M in the absence of ethylene glycol. Our previous work indicated that PDI does not show any anti-chaperone activity for APLA<sub>2</sub> refolding in either Hepes or Tris buffer, at either high or low concentrations of APLA<sub>2</sub>, despite the strong propensity of APLA<sub>2</sub> folding intermediates to aggregate at high concentrations

[18]. Similarly, PDI behaves only as a chaperone with citrate synthase [21], GAPDH [15] and rhodanese [16], all of which aggregate strongly during folding. Therefore it is questionable whether the anti-chaperone activity is in any way dependent on the insolubility of the folding intermediates of the protein substrate. It appears that PDI displays anti-chaperone activity only when its substrate shows relatively high spontaneous re-activation and low aggregation.

It is very interesting to note that at 1  $\mu$ M, the concentration at which PDI exhibits its strongest anti-chaperone activity, the simultaneous presence of mPDI at high concentrations increases rather than decreases lysozyme re-activation, whereas mPDI alone shows only anti-chaperone activity at all concentrations and is therefore expected to suppress lysozyme refolding even further if added to the refolding system as the result of intermolecular competition with the chaperone activity of PDI, as suggested by Puig et al. [20]. It should be noted that, in phosphate buffer, mPDI alone shows no positive effect on the recovery of lysozyme activity, but definitely promotes lysozyme re-activation in the presence of native PDI, and the promoting effect increases with increasing mPDI concentration. The presence of mPDI alone markedly increases and slightly decreases lysozyme aggregation in Hepes and phosphate buffers respectively, but, concurrent with the lysozyme re-activation, the simultaneous presence of mPDI and native PDI decreases aggregation synergistically. The synergistic but not competitive effect of mPDI on lysozyme re-activation in the presence of PDI has also been observed in a non-redox buffer. mPDI, like PDI, decreased aggregation of lysozyme during refolding in a reducing buffer. All the above imply that the chaperone activity, rather than the anti-chaperone activity, of mPDI is its intrinsic property, and the latter is probably an alternative expression of the former under special conditions. It has been shown that, in assisting refolding of proteins containing no disulphide, PDI functions only as a chaperone [15,16]; however, in assisting refolding of proteins containing disulphide, as a foldase, PDI functions both as a disulphide isomerase and a chaperone [18]. Therefore the maximal re-activation produced by PDI is an integral result of both chaperone and isomerase actions exerted by PDI simultaneously during the whole refolding process, but usually not the result of its chaperone or isomerase function alone. Consequently, a lack of increased lysozyme re-activation could be the result of a defect of either one or the other activity of PDI, and cannot necessarily be accounted for by the lack of the chaperone activity only. In this respect therefore, the -CGHC-active site of PDI is necessary for its isomerase activity only and not for its chaperone activity. mPDI does not have a positive effect on the re-activation of either lysozyme or APLA<sub>2</sub> because, without isomerase activity, it is unable to catalyse the formation of the disulphide bond(s) necessary for the re-activation of these two enzymes. However, mPDI does facilitate re-activation of GAPDH through its chaperone activity because the re-activation of GAPDH does not involve disulphide bond formation.

It was concluded that the peptide-binding site is not involved in the anti-chaperone activity and that PDI has a distinct site responsible for the anti-chaperone activity that is independent of its isomerase active site and the peptide-binding site from the fact that a photoactivatable tripeptide affinity-labelled PDI showed significantly reduced isomerase and chaperone activities and behaved only as an anti-chaperone [20]. However, the mutant PDI, abb'a', with the C-terminal 51 amino acid residues that contain the peptide-binding site responsible for the chaperone activity deleted, displays no chaperone activity in GAPDH refolding and much lower foldase activity in APLA<sub>2</sub> re-activation [26a], and does not show anti-chaperone activity in lysozyme re-

folding under conditions in which PDI does at low concentrations. On the other hand, abb'a' assists lysozyme re-activation to almost the same extent as PDI, because it is an almost fully active isomerase, and little aggregation was detected during spontaneous refolding of lysozyme in Hepes buffer. We would like to emphasize that the more strongly the substrate aggregates during refolding, the more intensive is the chaperone effect of PDI required for its re-activation. It seems that the anti-chaperone activity is closely dependent on its chaperone activity, as the critical step for anti-chaperone activity is the binding of partially folded intermediates, which is the same as is required for its chaperone activity. mPDI has nearly full chaperone activity and some anti-chaperone activity, whereas abb'a', without the peptide-binding site, lacks both chaperone and anti-chaperone activity. In this respect, covalent binding to the non-specific peptide-binding site by a small molecule such as a tripeptide may be not sufficient to cover the peptide-binding site, thus it retains its anti-chaperone behaviour.

The critical factor for PDI to show anti-chaperone activity probably depends on the folding pathways of the substrate, and the stability and relative populations of different intermediates, both of which could be considerably different under different conditions, including the ionic strength and redox state of the solution. PDI and mPDI, by complexing with non-native structures, could also affect the stability and relative population of different intermediates. The intermediates could be different in either conformation and/or their redox states and thus differ in their propensity to aggregate. In Hepes buffer, PDI at low concentrations could favour the formation of the aggregation-prone intermediates more than in its absence, showing anti-chaperone activity, but at high concentrations, PDI would decrease greatly the concentration of the aggregation-prone intermediates and catalyse the rapid formation of native disulphides, preventing aggregation of lysozyme. The so-called anti-chaperone activity of PDI is the same as its chaperone activity in its ability to bind non-native conformations of folding intermediates. It is not an intrinsic activity in addition to its isomerase and chaperone activities but dependent on the chaperone activity, and its appearance depends on the folding properties of the substrates under specified conditions.

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