The EMBO Journal Vol.16 No.3 pp.651–658, 1997

Both the isomerase and chaperone activities of protein disulfide isomerase are required for the reactivation of reduced and denatured acidic phospholipase A_2

Yi Yao, Yuan-cong Zhou[1] and Chih-chen Wang[2]

The National Laboratory of Biomacromolecules, Institute of Biophysics, Academia Sinica, 15 Datun Road, Beijing 100101 and [1]Shanghai Institute of Biochemistry, Academia Sinica, Shanghai, China

[2]Corresponding author

The spontaneous reactivation yield of acidic phospholipase A_2 ($APLA_2$), a protein containing seven disulfide bonds, after reduction and denaturation in guanidine hydrochloride is very low. Protein disulfide isomerase (PDI) markedly increases the reactivation yield and prevents the aggregation of $APLA_2$ during refolding in a redox buffer containing GSH and GSSG. *S*-methylated PDI (mPDI), with no isomerase but as nearly full chaperone activity as native PDI, has no effect on either the reactivation or aggregation of $APLA_2$. However, the simultaneous presence of PDI and mPDI in molar ratios to $APLA_2$ of 0.1 and 0.9 respectively fully reactivates the denatured enzyme, as does PDI alone at a ratio of 1. At ratios of 0.1 and 0.15 respectively, they completely suppress $APLA_2$ aggregation, as does PDI alone at a ratio of 0.25. Moreover, delayed addition of PDI to the refolding buffer greatly diminished the reactivation yield of $APLA_2$, but this deteriorating effect can be alleviated markedly by the presence of mPDI in the refolding buffer. Without GSSG, mPDI prevents the aggregation of $APLA_2$ during refolding. It is proposed that the *in vitro* action of PDI as a foldase consists of both isomerase and chaperone activities, and the latter activity can be fully replaced by mPDI.

Keywords: $APLA_2$/chaperone/isomerase/PDI/protein folding

Introduction

It has been generally agreed in recent years that protein disulfide isomerase (PDI) promotes nascent peptide folding by catalyzing the formation of native disulfide bonds, and thus functions as a foldase (Freedman *et al.*, 1994; Noiva, 1994). PDI stimulates reactivation of denatured and reduced disulfide-containing proteins *in vitro*, such as insulin (Tang *et al.*, 1988), proinsulin (Wang and Tsou, 1991), lysozyme (Puig and Gilbert, 1994), immunoglobulin (Lilie *et al.*, 1994) and riboflavin binding protein (McClelland *et al.*, 1995). PDI has also been used to aid the renaturation of proteins expressed in *Escherichia coli* as inclusion bodies, such as prochymosin (Tang *et al.*, 1994). In *E.coli*, human PDI expressed to the periplasm enhances the yield of pectate lysase C (Humphreys *et al.*, 1995) and a co-expressed antibody Fab′ fragment (Humphreys *et al.*, 1996).

Recently we have put forward a hypothesis that PDI is both an enzyme and a molecular chaperone (Wang and Tsou, 1993). Subsequently, a similar suggestion has been made by Noiva (1994). Experimental data *in vitro* and *in vivo* have accumulated in recent years to support this hypothesis (Wang, 1997). PDI does bind not only peptide with low specificity *in vitro* (Morjana and Gilbert, 1991; Noiva *et al.*, 1991), but also non-native proteins *in vivo* (Roth and Pierce, 1987; Otsu *et al.*, 1994). PDI is necessary for the assembly of some functional multimeric proteins, such as prolyl-4-hydroxylase (John *et al.*, 1993) and microsomal triglyceride transfer protein complex (Wetterau *et al.*, 1991). In yeast, PDI, but not its catalytic activity, is vital for cell viability (La Mantia and Lennarz, 1993). Similarly, the PDI mutant devoid of isomerase activity has the same function for the assembly of fully active prolyl-4-hydroxylase (Vuori *et al.*, 1992). Recently, it was found that co-expression of a mutated PDI lacking its isomerase activity accelerates folding of human lysozyme expressed in yeast, leading to increases both in the amount of intracellular enzyme with the native conformation and in its secretion (Hayano *et al.*, 1995). In addition, PDI is an abundant and highly unusual multifunctional protein and is remarkably capable of non-specific peptide binding, which are important prerequisites for a protein to be a chaperone. However, using genetic and biophysical data in mutant yeast, Raines and his colleagues claimed that any roles ascribed to PDI, other than its catalysis of the formation of native disulfide bonds, are not essential (Laboissiere *et al.*, 1995; Chivers *et al.*, 1996).

It has been demonstrated in this laboratory that PDI shows chaperone-like activity independent of its isomerase activity in the refolding of proteins containing no disulfide bond, D-glyceraldehyde-3-phosphate dehydrogenase (G-APDH) (Cai *et al.*, 1994) and rhodanese (Song and Wang, 1995). The chaperone and anti-chaperone activities of PDI in the refolding of lysozyme have been reported by Puig and Gilbert (1994) and Puig *et al.* (1994). However, on the other hand, Lilie *et al.* (1994) reported that PDI, while promoting the renaturation of denatured and reduced Fab molecules, showed no chaperone activity.

The folding of a protein containing disulfide bond(s) involves two processes: the folding of the peptide chain into a native-like conformation and the oxidative formation of native disulfide bond(s). The above two processes are interconnected intimately and work in conjunction during folding. It is of interest, therefore, to dissect the actions of PDI in assisting refolding and renaturation of denatured and reduced disulfide-containing proteins. In this study, we attempted to distinguish the chaperone and isomerase activities of PDI in the reactivation of denatured and reduced acidic phospholipase A_2 ($APLA_2$), a small protein

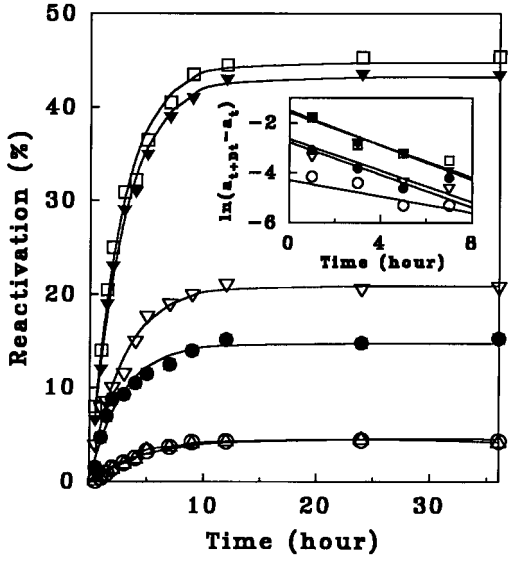


Fig. 1. Effects of PDI on the reactivation of GdnHCl-denatured and reduced $APLA_2$ at 12 μM. Conditions for denaturation and reactivation of $APLA_2$ were as described in the text. The molar ratios of PDI protomer/$APLA_2$ are: 0 (○); 0.1 (●); 0.5 (▽); 1 (▼); and 5 (□). BSA at 12 μM (△) was used for comparison.

of 124 amino acid residues from the venom of *Agkistrodon blomhoffii brevicaudus* (*Agkistrodon halys* Pallas) which contains seven disulfide bonds (Wang *et al.*, 1996). $APLA_2$ refolds spontaneously only to a limited extent due to a strong propensity to aggregation, and can be reactivated only in the presence of low concentrations of denaturants (Thunnissen *et al.*, 1992; Liang *et al.*, 1993). We have now shown that the reactivation yield of $APLA_2$ can be increased greatly by PDI in a redox buffer and this required both the isomerase and chaperone activities of PDI, as a large part of PDI in assisting refolding of $APLA_2$ can be fully replaced by a modified PDI (mPDI) with only chaperone but no isomerase activity (Quan *et al.*, 1995).

Results

Effects of PDI on the reactivation of denatured and reduced $APLA_2$

As PDI is a resident enzyme in endoplasmic reticulum where the redox potential is maintained by GSSG–GSH at a ratio of ~1:2 (Hwang *et al.*, 1992), a refolding buffer containing 1 mM GSSG and 2 mM GSH in 20 mM Tris–HCl buffer, pH 8.0 (referred to hereafter as the redox buffer), was used in this work, although other authors mostly used 1 mM GSSG and 10 mM GSH for a similar purpose (Puig and Gilbert, 1994; McClelland *et al.*, 1995). As shown in Figure 1, the guanidine hydrochloride (GdnHCl)-denatured and reduced $APLA_2$ with 14 sulfhydryl groups shows only a limited extent of reactivation upon dilution in the redox buffer: 4% at the final concentration of 12 μM and 10% at 4 μM. When the refolding of $APLA_2$ was carried out in the presence of PDI, the reactivation yield increases greatly with increasing concentrations of PDI until the molar ratio of PDI protomer to $APLA_2$ approaches 1, and higher ratios of PDI had little further effect on the reactivation yield of $APLA_2$. Hereafter, all ratios refer to molar ratios of PDI or mPDI protomer to $APLA_2$. The reactivation of $APLA_2$ increases from 4 to 45 ± 2% and from 10 to 77 ± 3% at $APLA_2$ final concentrations of 12 and 4 μM respectively. Bovine serum albumin (BSA), commonly used for comparison in the studies of chaperones, does not affect the reactivation of $APLA_2$ at the ratio of 1 (12 μM). As shown in the inset of Figure 1, Guggenheim plots of the time courses of the reaction show that the reactivation of $APLA_2$ follows first order kinetics. The apparent rate constants of reactivation and the final levels of reactivation yield are summarized in Table I. PDI stimulates the spontaneous refolding rate only at a low ratio of 0.1, and a further increase in PDI has very little further effect on the rate. The level of reactivation increases with PDI and approaches a maximal level at a ratio of 1.

Effect of mPDI on the reactivation of $APLA_2$

Modified PDI (mPDI), with both active site Cys residues methylated, is devoid of isomerase activity but is nearly as effective as native PDI in the reactivation of GAPDH (Quan *et al.*, 1995) and, therefore, has close to full chaperone activity. As shown in Figure 2, although mPDI alone does not have any effect on the reactivation of $APLA_2$ in the redox buffer, when present together with PDI it increases the reactivation of $APLA_2$ significantly as compared with the same amount of native PDI alone in the refolding buffer. The increase in reactivation yield by the simultaneous presence of mPDI and PDI can be seen clearly in the inset of Figure 2. PDI at a molar ratio to $APLA_2$ of 0.1 increases the reactivation of $APLA_2$ from 4 to 15%, but the additional presence of mPDI at a molar ratio of 0.9 (which by itself has no effect on the reactivation yield) increases reactivation markedly from 15 to 43% at 12 μM $APLA_2$, which is the saturation level obtainable by native PDI alone at a stoichiometric ratio. Further increases in native PDI have no further effect on the reactivation of $APLA_2$. The above synergistic effect of mPDI cannot be replaced by BSA, the simultaneous presence of which at ratios of 0.95 and 0.9 with PDI at ratios of 0.05 and 0.1 respectively shows no beneficial effect on the reactivation of $APLA_2$ (data not shown). The above results indicate that a stoichiometric amount of PDI, i.e. an equimolar ratio to the substrate in this case, is necessary for the maximal reactivation of $APLA_2$ at 12 μM; however, 90% of PDI can be replaced by mPDI, which is active as a chaperone but devoid of isomerase activity. This suggests that only a small part of PDI (at a ratio of 0.1) acts catalytically as an isomerase and the rest (at a ratio of 0.9) acts as a molecular chaperone, and this part of native PDI can be fully replaced by mPDI. Figure 2 inset also shows that native PDI at a ratio of <0.1 is not enough for the maximal reactivation of $APLA_2$, even when supplemented with mPDI.

The suppression of aggregation by PDI during refolding of denatured and reduced $APLA_2$

As shown in Figure 3A, strong aggregation as monitored by light scattering occurs rapidly upon dilution of denatured and reduced $APLA_2$ to a final concentration of 12 μM in the redox buffer, whereas no light scattering changes can be observed at the lower concentration of 4 μM (data not shown). Concurrent to improving the reactivation yield, increasing concentrations of PDI in the refolding buffer decrease both the rate and the extent of

Table I. Apparent first-order rate constants for the PDI-catalyzed refolding of denatured and reduced $APLA_2$

PDI molar ratio to $APLA_2$	First-order rate constant (per h)		Reactivation (percentage)	
	$APLA_2$ (4 μM)	$APLA_2$ (12 μM)	$APLA_2$ (4 μM)	$APLA_2$ (12 μM)
0	0.19 ± 0.02	0.20 ± 0.02	11 ± 1	4.5 ± 1
0.1	0.35 ± 0.02	0.35 ± 0.01	15 ± 1	15 ± 2
0.5	0.36 ± 0.02	0.34 ± 0.02	53 ± 1	21 ± 2
1.0	0.35 ± 0.02	0.35 ± 0.03	75 ± 2	43 ± 2
5.0	0.36 ± 0.03	0.37 ± 0.03	77 ± 2	44 ± 2

Refolding was initiated by dilution of denatured and reduced $APLA_2$ into 20 mM Tris–HCl refolding buffer, pH 8.0, containing 1 mM GSSG, 2 mM GSH and different ratios of PDI at room temperature. 100% activity refers to the activity of the native $APLA_2$. The rate constants were calculated based on the average of triplicate data.

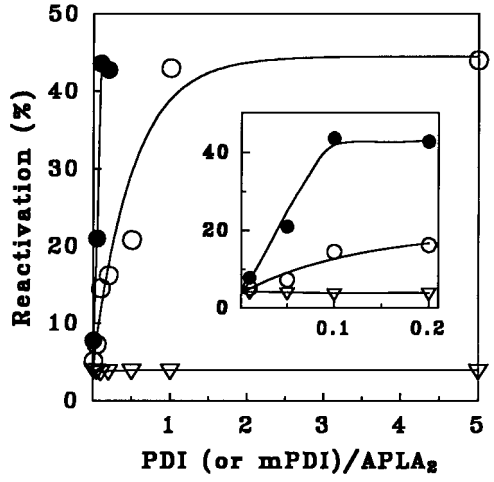


Fig. 2. Effects of concentrations of mPDI and PDI on the reactivation of denatured and reduced $APLA_2$. Experimental conditions were the same as in Figure 1. The reactivations were carried out for 24 h in the presence of mPDI (▽) alone; PDI (○) alone; or in the simultaneous presence of both PDI with the ratios as indicated and mPDI to make a combined ratio of 1 (●). The inset shows the initial portions of the curves at PDI ratios <0.20.

aggregation of $APLA_2$, and the aggregation is almost completely suppressed by PDI at a ratio of 0.25. In contrast, mPDI at molar ratios of 0.1–1.0 even increased the aggregation of $APLA_2$ slightly. In all the above cases, aggregation approaches completion in 15–20 min. However, the simultaneous presence of both PDI and mPDI does result in a further decrease in light scattering as compared with the presence of PDI alone at the same molar ratios as shown in Figure 3B. The efficiency in the suppression of aggregation increases with increasing ratios of PDI and, in agreement with the increase of reactivation of $APLA_2$ in the presence of both PDI and mPDI, PDI at a ratio of 0.1 with mPDI at a ratio of 0.15 suppresses aggregation similarly to PDI alone at a ratio of 0.25.

Refolding of $APLA_2$ in a non-redox or reducing buffer

When the refolding and reactivation of denatured and reduced $APLA_2$ was carried out in a non-redox buffer, i.e. in the absence of GSSG and GSH, aggregation occurred with a rate lower than that in the redox buffer, as shown by a comparison of Figures 3A and 4. The presence of 10 mM dithiothreitol (DTT) decreases the aggregation of $APLA_2$ only slightly, indicating that disulfide cross-linking plays only a minor role in the aggregation of $APLA_2$

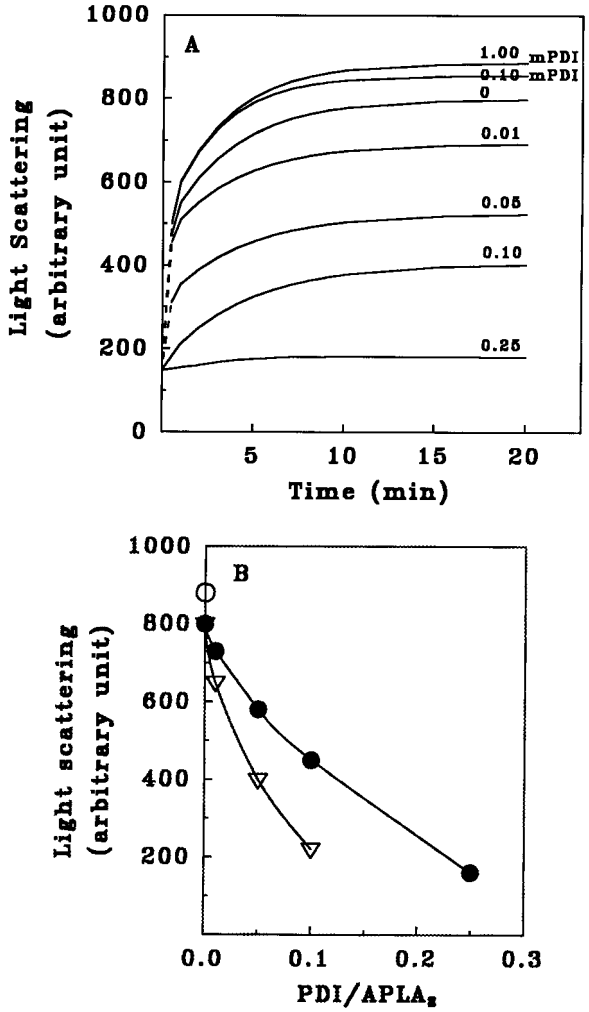


Fig. 3. Effects of concentrations of PDI and mPDI on aggregation of denatured and reduced $APLA_2$ during refolding. Aggregation upon dilution was monitored by light scattering. Experimental conditions were the same as in Figure 1. (**A**) Time courses of light scattering change with ratios of PDI or mPDI to $APLA_2$ as indicated. (**B**) Effects of the simultaneous presence of both PDI and mPDI on the level of aggregation of $APLA_2$ determined in 20 min. PDI (●); mPDI (○); and the simultaneous presence of PDI with the ratios as indicated and mPDI to make a combined ratio of 0.25 (▽).

during refolding without an oxidant. Interestingly, mPDI alone does decrease the aggregation of $APLA_2$ in the absence of an oxidant, and both the rate and extent of aggregation decrease with increasing ratios of mPDI until complete suppression of aggregation occurs at equimolar

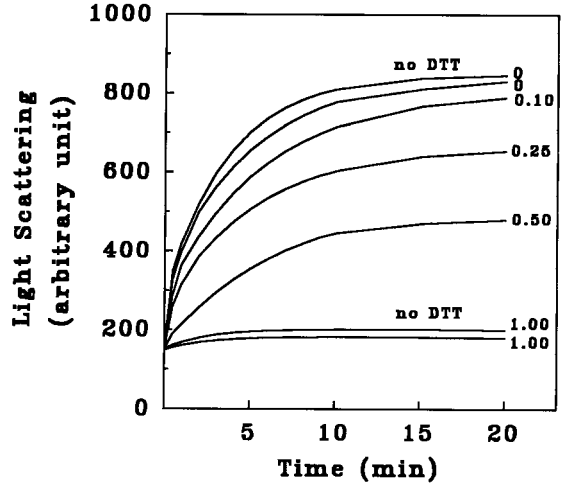


Fig. 4. Effects of mPDI on the aggregation of $APLA_2$ in a reducing buffer. Experimental conditions were as in Figure 3, except that the dilution buffer contained no GSH or GSSG but 10 mM DTT instead, unless otherwise specified. The ratios of mPDI present were as indicated.

mPDI (Figure 4). In addition, mPDI alone increases the reactivation of $APLA_2$ slightly (data not shown). The above indicates that mPDI has no protective effect on $APLA_2$ aggregation caused by disulfide cross-linking in the redox buffer, but it does prevent aggregation by non-covalent, probably hydrophobic interactions at an equimolar ratio in the absence of an oxidant. Moreover, in the reducing buffer, it also requires an equimolar ratio of native PDI for complete suppression of aggregation (data not shown). It is to be remembered that native PDI at a ratio of 0.25 is sufficient for the suppression of $APLA_2$ aggregation in the redox buffer (Figure 3). It appears that the isomerase activity of PDI is necessary for both the reactivation and the suppression of aggregation of $APLA_2$ in the redox buffer.

Results of SDS–PAGE of refolding products shown in Figure 5 provide further support for the dual role of PDI. Spontaneous refolding of $APLA_2$ in either the redox or the reducing buffer results in the formation of precipitates (lanes A1, A2, B1 and B2), and no protein can be detected in the supernatant (A3, A4, B3 and B4). No precipitate was formed in the PDI-assisted refolding in the redox buffer (lanes A9 and A10) and in the PDI- or mPDI-assisted refolding in the reducing buffer (lanes B5, B6, B9 and B10). In the redox buffer, only refolding in the presence of PDI results in the formation of $APLA_2$ (compare lanes A11 and A12 with A7 and A8); with mPDI, the greater part of the protein is precipitated (lanes A5 and A6) with only a little protein in the soluble fraction (lanes A7 and A8). The precipitate formed in the redox buffer can be dissolved in the loading buffer for SDS–PAGE only in the presence of DTT (lanes A1 and A5), suggesting that precipitates are formed mostly by cross-linking of thiols, resulting in non-native disulfide bonds. However, in the absence of an oxidant, the precipitate formed upon spontaneous refolding can be dissolved easily in the loading buffer of SDS–PAGE without DTT (lane B2), suggesting that precipitate formation involves only hydrophobic interactions and no protein can be detected in the supernatant (lanes B3 and B4). The presence of mPDI or PDI at the ratio of 1 suppresses aggregation completely, as no protein band was detected in lanes B5, B6, B9 and B10, which is consistent with the light scattering data in Figure 4. In the reducing buffer, both $APLA_2$ and mPDI or PDI exist in the supernatant (lanes B7, B8, B11 and B12), suggesting the binding of mPDI or PDI with folding intermediates of $APLA_2$ to prevent aggregation by incorrect hydrophobic interactions.

The amount of protein taken from the precipitate fraction is four times that from the supernatant fraction (20 out of 300 μl as compared with 20 out of 1200 μl), which could be one of the reasons why not much $APLA_2$ is recovered as soluble material as shown in the gel profile (lanes A11, A12, B7, B8, B11 and B12) and a certain amount of PDI shows up in the pellet fraction (lanes A5 and A6). As $APLA_2$ aggregates so rapidly and strongly, as shown by the initial jump in light scattering (dotted lines in Figure 3A), it is likely that PDI is occluded in the aggregates. It is also to be noted that the staining strength of the $APLA_2$ band with Coomassie brilliant R250 is not proportional to the amount of protein, as lane A14 (12 μg of $APLA_2$) is much stronger than lane A15 (3 μg of $APLA_2$) and all $APLA_2$ bands in the supernatant are weaker than expected. An attempt to calibrate the staining strength of $APLA_2$ samples at of 1, 2, 3 μg, etc. was unsuccessful. This may have something to do with the fact that $APLA_2$ is a highly disulfide cross-linked small protein (124 residues with seven disulfides). The slight difference in mobility of $APLA_2$ in some lanes is probably due to overloading of the samples as the precipitates were dissolved in a small volume of the loading buffer.

Effects of delayed addition of PDI on the reactivation of $APLA_2$

The effects of delayed addition of PDI at a ratio of 1 after dilution of denatured $APLA_2$ at 12 μM on the reactivation are shown in Figure 6. The activity recovery drops sharply with the increase in the length of delay, significantly for a delay of 2 min after dilution and to a level only slightly higher than the spontaneous refolding level at 5 min delay. However, the presence of mPDI at a ratio of 0.9 in the redox buffer used for dilution results in much higher reactivation as compared with that of delayed addition of PDI alone at ratios of either 0.1 or 1.0, indicating that mPDI present in the refolding buffer binds with $APLA_2$ folding intermediates so as to decrease the aggregation by hydrophobic interaction and thus favor the productive folding pathway. The above suggests the replacement of the chaperone function of PDI by mPDI in the dilution buffer.

Discussion

It has been shown in previous studies from this laboratory that PDI assists in the refolding of proteins containing no disulfide bond, such as GAPDH (Cai *et al.*, 1994) and rhodanese (Song and Wang, 1995), suggesting chaperone activity of PDI independent of its isomerase activity. In the present study, we attempt to provide further evidence for the hypothesis that PDI, as a foldase, has both isomerase and chaperone activities simultaneously assisting in the refolding of a disulfide-containing protein, $APLA_2$. In other words, both activities are necessary for PDI to assist

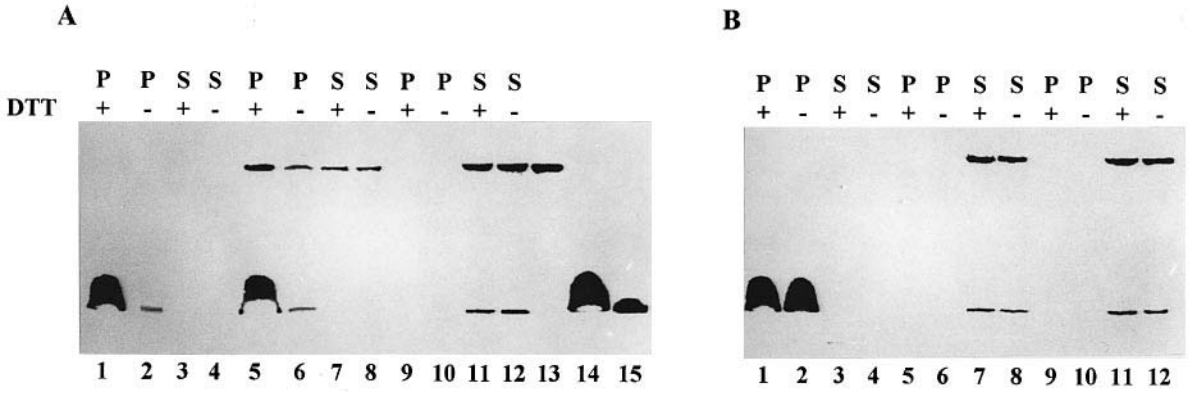


Fig. 5. SDS–PAGE profiles of refolding products of $APLA_2$. Refolding of 12 μM reduced and denatured $APLA_2$ was carried out for 24 h and centrifuged at 13 000 r.p.m. for 60 min. The precipitate (P) and supernatant fractions (S) of samples were prepared as described in the text. The loading buffer either contained 100 mM DTT (DTT, +) or no DTT (DTT, –). (**A**) Refolding of $APLA_2$ in the redox buffer. Lanes 1–4, spontaneous refolding; lanes 5–8, refolding in the presence of mPDI at a ratio of 1; lanes 9–12, refolding in the presence of PDI at a ratio of 1. Lanes 13, mPDI; 14, $APLA_2$ (12 μg); and 15, $APLA_2$ (3 μg). (**B**) The same as in (A) except that the refolding of $APLA_2$ was carried out in the reducing buffer (with 10 mM DTT). Lanes as in (A).

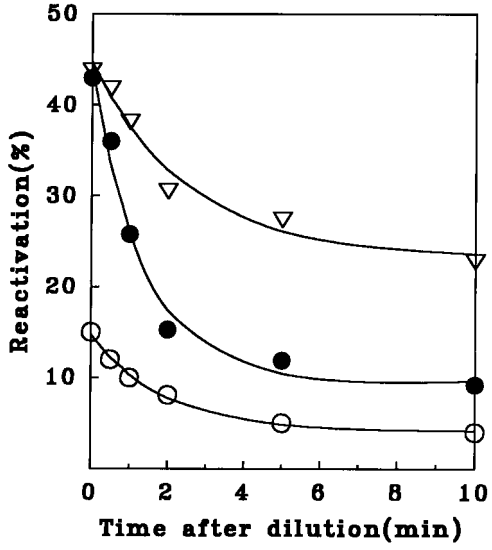


Fig. 6. Effect of delayed addition of PDI on the reactivation of denatured and reduced $APLA_2$. The times of addition of PDI after dilution were as indicated. Other conditions were as for Figure 1. PDI added at a ratio of 0.1 (○); 1 (●); PDI at a ratio of 0.1 in the presence of mPDI at a ratio of 0.9 in the dilution buffer (▽).

in the productive refolding of the peptide chain and catalyzing the formation of native disulfides of the denatured and reduced $APLA_2$.

$APLA_2$ is a small protein of 14 kDa containing seven disulfide bonds. Full reactivation of GdnHCl-denatured enzyme with intact disulfide bonds upon dilution is a very fast process and reaches completion in only a few seconds (data not shown); however, the refolding of the denatured enzyme with all seven disulfide bonds reduced takes at least 10 h, with low activity recovery probably due to strong aggregation upon dilution, especially at high protein concentrations.

In the absence of denaturant at low concentrations, such as 0.9 M GdnHCl (Liang *et al.*, 1993) or 2 M urea (Thunnissen *et al.*, 1992), which has been used to suppress aggregation and to increase the reactivation yield of denatured and reduced $APLA_2$, PDI at stoichiometric concentrations greatly promotes reactivation of $APLA_2$ from 4 to 45% and from 10 to 77% at $APLA_2$ concentrations of 12 and 4 μM respectively. At the same time, PDI efficiently prevents the strong aggregation of $APLA_2$ upon removal of the denaturant. Although the reactivation of denatured and reduced $APLA_2$ is a complex reaction accompanied by non-productive formation of aggregates, the PDI-assisted reactivation of $APLA_2$ apparently follows first-order kinetics. At a molar ratio of 0.1, PDI increases the apparent rate constant significantly over the spontaneous rate, but at higher ratios it increases the reactivation yield without further effect on the reactivation rate. It seems that PDI does assist reactivation of $APLA_2$, not only catalytically in the formation of the correct disulfide bonds as an isomerase, but also non-catalytically in assisting the refolding by preventing misfolding and aggregation of folding intermediates in a way highly suggestive of action as a molecular chaperone. It has been suggested that chaperones generally increase the extents of correct folding without a marked effect on the rate of refolding (Puig and Gilbert, 1994).

In order to provide further support for the chaperone activity of PDI in the refolding of $APLA_2$, we examined the effect of a modified PDI on the refolding of $APLA_2$. PDI alkylated at the Cys residues of its thioredoxin-like active sites -CGHC- (mPDI) is devoid of isomerase activity but is still active as a chaperone in assisting reactivation of a protein containing no disulfide (Quan *et al.*, 1995). It does not show any assisting effect on the reactivation of $APLA_2$, nor does it prevent $APLA_2$ from aggregation in a redox buffer. Aggregation of a denatured and reduced disulfide-containing protein during refolding in the presence of an oxidant could result from either non-productive hydrophobic interactions or incorrect disulfide cross-linking, or both. In the case of $APLA_2$, it appears that both hydrophobic interactions and disulfide cross-linking play some part in its aggregation upon dilution of the GdnHCl-denatured and reduced enzyme in the presence of an oxidant. The aggregation, with a somewhat decreased rate upon dilution of the denatured enzyme in the absence of an oxidant or in the presence of DTT to prevent any possible disulfide formation, very probably involves mostly hydrophobic interactions, which are the dominant

factors for aggregation as in the case of refolding of proteins containing no disulfide bond.

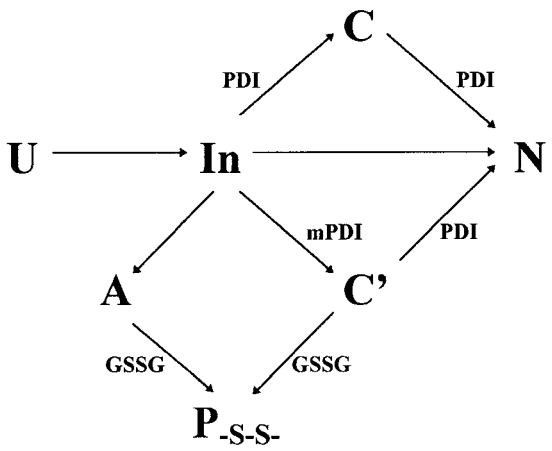
It has been shown that mPDI has nearly the same peptide binding ability and chaperone activity as native PDI in the reactivation of GAPDH containing no disulfide bond (Quan *et al.*, 1995). Either in the absence of an oxidant or in the presence of DTT, mPDI does behave like PDI in suppressing $APLA_2$ aggregation but at a higher ratio of 1 as compared with a ratio of 0.25 for PDI in the redox buffer. However, in the reducing buffer, the same ratio of 1 is required for PDI to suppress aggregation (data not shown). In addition, even in the presence of an oxidant to form disulfide cross-links, mPDI increases the power of native PDI in suppressing $APLA_2$ aggregation, as a mixture of native PDI and mPDI at ratios of 0.1 and 0.15 suppresses $APLA_2$ aggregation to the same extent as does PDI alone at 0.25. It appears that in so far as prevention of $APLA_2$ aggregation is concerned, PDI functions both as a chaperone to prevent non-productive hydrophobic interactions leading to aggregation and as an isomerase to promote native disulfide formation and thus prevents incorrect disulfide cross-linking leading to further aggregation. In this connection, the chaperone-like activity of PDI can be fully replaced by mPDI in the prevention of incorrect hydrophobic interactions of denatured and reduced $APLA_2$.

An equimolar ratio of PDI is enough for maximal reactivation of $APLA_2$ (45%); however, PDI at a ratio of only 0.1 is necessary to function as an isomerase. The major part of PDI (at a ratio of 0.9) actually functions as a molecular chaperone as it can be fully replaced by mPDI, which has nearly the same chaperone activity as native PDI but no isomerase activity. mPDI alone at a ratio of 5 shows no effect on the reactivation of $APLA_2$ in the redox buffer, and a catalytic amount of PDI alone, at a ratio of 0.1, only increased reactivation to a limited extent (from 4 to 15%). It is highly suggestive that for the refolding of $APLA_2$, PDI at a low ratio is sufficient to act as an isomerase but far from sufficient to act as a chaperone in preventing aggregation and promoting correct folding. A stoichiometric ratio of PDI, replaceable by mPDI, is required for the latter activity.

Neither PDI or mPDI showed any anti-chaperone activity in the refolding of $APLA_2$ at 12 and 4 μM at all ratios (0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1) examined and in either Tris–HCl or HEPES buffer.

Delayed addition of PDI decreases the reactivation yield of $APLA_2$ markedly, most probably due to the strong aggregation which takes place very rapidly upon dilution (as shown in Figure 3A), and cannot be rescued by the delayed addition of PDI. However, the pre-existence of mPDI in the dilution buffer at a ratio of 0.9 is indeed able to increase the reactivation yield by delayed addition of PDI at a ratio of 0.1. This probably occurs by binding to the aggregation-prone folding intermediates formed in an early stage upon dilution and alleviating markedly the aggregation of $APLA_2$, thus greatly increasing the reactivation yield (compare curves ▽ and ○, in Figure 6). The pre-existence of mPDI in the dilution buffer can also alleviate the effect of delayed addition of PDI at the ratio of 1 (compare curves ▽ and ●, in Figure 6).

From all of the above, we proposed a simple model for the functions of PDI in the refolding of $APLA_2$ (Scheme 1).



Scheme 1. Simple model for the functions of PDI in refolding of $APLA_2$.

For the spontaneous refolding in redox buffer upon dilution, a very fast conformation change of denatured and reduced $APLA_2$ (U) results in the formation of intermediate(s), In, which has a strong propensity for aggregation. In is faced with two competitive pathways, one is to fold correctly to become a native molecule (N) but with a very low efficiency (only 4% reactivation, Figures 1 and 2; no $APLA_2$ can be detected in lanes 3 and 4 of Figure 5A), and the other, as the major and non-productive pathway, is to misfold to become aggregates (A) by hydrophobic interaction and, in the presence of an oxidant, by further cross-linking to disulfides, P_{-S-S-} (as shown by lanes 1 and 2 in Figure 5A). PDI present in the refolding buffer recognizes and binds to In to form a complex C, preventing the aggregation of In (Figure 3, and Figure 5A, lanes 9 and 10). $APLA_2$ can then be refolded oxidatively, catalyzed by PDI after dissociation from the complex C, to form the native enzyme (N) (Figures 1 and 2, and Figure 5A, lanes 11 and 12). mPDI can also recognize and bind to In; however, devoid of isomerase activity, it is unable to catalyze the formation of correct disulfides and, in the presence of an oxidant, complex C' will then be oxidatively cross-linked to form incorrect disulfides with aggregation and precipitation (P_{-S-S-}) (Figure 3A and Figure 5A, lanes 5–8). In the non-redox buffer or in a reducing buffer with DTT, little or no disulfide cross-linking could occur, and aggregation is prevented by mPDI through formation of complex C' (Figure 4 and Figure 5B, lanes 5 and 6); however, without an active isomerase, native disulfide, and hence native enzyme molecule (N), could not be formed efficiently. The very low increase over spontaneous refolding with mPDI in the absence of an oxidant is probably due to a decrease of aggregation and spontaneous oxidation. Where there is co-existence of mPDI in a stoichiometric amount with PDI in a catalytic amount, mPDI plays the role of chaperone in preventing aggregation and PDI plays the role of isomerase in the formation of native disulfide bonds from the intermediates dissociated from the complexes (Figure 2). This action of mPDI is similar to that of a typical chaperone, GroEL, in its recognition of and binding to reduced and denatured α-lactalbumin (Okazaki *et al.*, 1994; Murai *et al.*, 1995).

Conclusion

PDI promotes reactivation of $APLA_2$, a protein containing seven disulfide bonds, with a maximum yield at stoichiometric concentrations, and functions as both an isomerase and a chaperone. A catalytic amount of PDI assists in reactivation of $APLA_2$ only to a limited level. At 5-fold excess alkylated PDI (mPDI), with only chaperone and no isomerase activity, does not have any effect on the reactivation of $APLA_2$ in a redox buffer.

As a foldase, both the isomerase and chaperone activities of PDI are required for the reactivation of a denatured and reduced disulfide-containing protein, and the chaperone activity of PDI can be fully replaced by mPDI. The isomerase activity is not only involved in the formation of native disulfides, but also in preventing disulfide cross-linking during refolding leading to aggregation.

Materials and methods

Materials

PDI from bovine liver was prepared and assayed essentially according to the method of Lambert and Freedman (1983) and showed one band on SDS–PAGE with a specific activity of ~800 U/g. *S*-Methylated PDI (mPDI) with <4% of the isomerase activity of native PDI was prepared according to Quan *et al.* (1995). Acidic phospholipase A_2 was prepared from the venom of *Agkistrodon blomhoffii brevicaudus* (*Agkistrodon halys* Pallas) according to Wu *et al.* (1984). BSA (98–99% albumin, Fraction V) and GdnHCl were purchased from Sigma. DTT was from Promega and 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) was from Fluka. All other chemicals were local products of analytical grade. In all experiments, 20 mM Tris–HCl buffer, pH 8.0, was employed unless specified otherwise.

Protein concentration determinations

The concentration of denatured $APLA_2$ was determined by the method of Bradford (1976) with BSA as a standard. The concentrations of PDI and $APLA_2$ were determined spectrophotometrically at 280 nm, $A_{0.1\%}$ = 0.9 for PDI (Hu and Wang, 1988) and $A_{0.1\%}$ = 1.3 for $APLA_2$ (Pieterson *et al.*, 1974).

Denaturation and reactivation of $APLA_2$

$APLA_2$ (700 μM) was completely denatured and reduced in 0.1 M Tris buffer with 8 M GdnHCl and 200 mM DTT, pH 8.0, overnight at room temperature. The reaction mixture, after adjustment of the pH to 2.0 with 2 M HCl, was dialyzed thoroughly at room temperature against 4 M GdnHCl, pH 2.0, to remove the excess DTT, as GdnHCl at lower concentrations leads to aggregation and precipitation of the enzyme and at concentrations >0.1 M interferes with the activity assay of $APLA_2$ (data not shown). The number of free thiols of denatured $APLA_2$ was determined with DTNB (Ellman, 1959) in 4 M GdnHCl. Reactivation was carried out by 50- or 150-fold dilution of the denatured enzyme into 20 mM Tris–HCl buffer, containing 1 mM GSSG and 2 mM GSH and various concentrations of PDI and/or mPDI at room temperature, unless otherwise specified. The final concentration of $APLA_2$ was either 12 or 4 μM. Aliquots of the reactivation mixture containing 0.5 μg of $APLA_2$ were taken for activity assay. Aggregation of $APLA_2$ during reactivation was monitored continuously by 90° light scattering at 500 nm in a Hitachi model F-4010 spectofluorometer at 30°C.

Assay for $APLA_2$ activity

The activity of $APLA_2$ was determined at 40°C by following the proton generation during the hydrolysis of L-α-lecithin leading to a color change of brilliant yellow spectrophotometrically, i.e. a decrease in absorbance at 495 nm, pH 8.2. The details of the method will be described elsewhere (Y.Yao, M.H.Wang, K.Y.Zhao and C.C.Wang, submitted). The substrate, soybean L-α-lecithin from Sigma, was freshly prepared before use according to Kawachi *et al.* (1971).

Determination of apparent reaction rate constants

The reactivation of $APLA_2$ catalyzed by PDI displayed first-order kinetics as shown by Guggenheim plots, the first-order rate constants were calculated accordingly.

SDS–PAGE analysis of $APLA_2$ refolding products

Refolding products containing 12 μM $APLA_2$ in the redox buffer (1 mM GSSG and 2 mM GSH) or reducing buffer (10 mM DTT) with or without PDI or mPDI at ratio of 1 were centrifuged at 13 000 r.p.m. for 60 min; both the supernatant and the precipitate with or without treatment with 100 mM DTT were analyzed by SDS–PAGE. The details of experiments are as follows: the precipitate after centrifugation of the refolding product was resuspended in 300 μl of buffer and 20 μl were taken and mixed with an equal volume of 2-fold loading buffer as the precipitate fraction (P). From 1200 μl of supernatant, 20 μl were taken and mixed with an equal volume of 2-fold loading buffer as the supernatant fraction (S). The mixtures were heated in a boiling water bath for 5 min and only supernatants were loaded onto the gel after centrifugation.

Acknowledgements

We sincerely thank Professor C.L.Tsou for his continuous encouragement, helpful advice and critical reading of the manuscript. This work was supported by the Pandeng Project of the Chinese Commission of Science and Technology.

References

Bradford,M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.*, **72**, 248–254.

Cai,H., Wang,C.C. and Tsou,C.L. (1994) Chaperone-like activity of protein disulfide isomerase in the refolding of a protein with no disulfide bonds. *J. Biol. Chem.*, **269**, 24550–24552.

Chivers,P.T., Laboissiere,M.C.A. and Raines,R.T. (1996) The CXXC motif: imperatives for the formation of native disulfide bonds in the cell. *EMBO J.*, **15**, 2659–2667.

Ellman,G.L. (1959) Tissue sulfhydryl groups. *Arch. Biochem. Biophys.*, **82**, 70–78.

Freedman,R.B., Hirst,T.R. and Tuit,M.F. (1994) Protein disulfide isomerase: building bridges in protein folding. *Trends Biochem. Sci.*, **19**, 331–336.

Hayano,T., Hirose,M. and Kikuchi,M. (1995) Protein disulfide isomerase mutant lacking its isomerase activity accelerates protein folding in the cell. *FEBS Lett.*, **377**, 505–511.

Hu,C.H. and Wang,C.C. (1988) Purification and activity assay of protein disulfide-isomerase. *Chin. Biochem. J.*, **4**, 61–66.

Humphreys,D.P., Weir,N., Mountain,A. and Lund,P.A. (1995) Human protein disulfide isomerase functionally complements a dsbA mutation and enhances the yield of pectate lyase C in *Escherichia coli*. *J. Biol. Chem.*, **270**, 28210–28215.

Humphreys,D.P., Weir,N., Lawson,A., Mountain,A. and Lund,P.A. (1996) Co-expression of human protein disulfide isomerase (PDI) can increase the yield of an antibody Fab′ fragment expressed in *Escherichia coli*. *FEBS Lett.*, **380**, 194–197.

Hwang,C., Sinskey,A.J. and Lodish,H.F. (1992) Oxidized redox state of glutathione in the endoplasmic reticulum. *Science*, **257**, 1496–1502.

John,D.C.A., Grant,M.E. and Bullaid,N.J. (1993) Cell-free synthesis and assembly of prolyl 4-hydroxylase: the role of the β-subunit (PDI) in preventing misfolding and aggregation of the α-subunit. *EMBO J.*, **12**, 1587–1595.

Kawauchi,S., Iwanaga,S., Samejima,Y. and Suzuki,T. (1971) Isolation and characterization of two phospholipase As from the venom of *Agkistrodon halys blomhoffii*. *Biochim. Biophys. Acta*, **236**, 142–160.

Laboissiere,M.C.A., Sturley,S.L. and Raines,R.T. (1995) The essential function of protein-disulfide isomerase is to unscramble non-native disulfide bonds. *J. Biol. Chem.*, **270**, 28006–28009.

LaMantia,M. and Lennarz,W.J. (1993) The essential function of yeast protein disulfide isomerase does not reside in its isomerase activity. *Cell*, **74**, 899–908.

Lambert,N. and Freedman,R.B. (1983) Kinetics and specificity of homogeneous protein disulphide-isomerase in protein disulphide isomerization and in thiol-protein-disulphide oxidoreduction. *Biochem. J.*, **213**, 225–234.

Liang,N.S., Pungerčar,J., Križaj,I., Štrukelj,B. and Gubenšek,F. (1993) Expression of fully active ammodytoxin A, a potent presynaptically neurotoxic phospholipase A_2, in *Escherichia coli*. *FEBS Lett.*, **334**, 55–59.

Lilie,H., McLaughlin,S., Freedman,R. and Buchner,J. (1994) Influence of protein disulfide isomerase (PDI) on antibody folding *in vitro*. *J. Biol. Chem.*, **269**, 14290–14296.

McClelland,D.A., McLaughlin,S.H., Freedman,R.B. and Price,N.C. (1995) The refolding of hen egg white riboflavin-binding protein: effect of protein disulfide isomerase on the reoxidation of reduced protein. *Biochem. J.*, **311**, 133–137.

Morjana,N.A. and Gilbert,H.F. (1991) Effect of protein and peptide inhibitors on the activity of protein disulfide isomerase. *Biochemistry*, **30**, 4985–4990.

Murai,N., Taguchi,H. and Yoshida,M. (1995) Kinetic analysis of interactions between GroEL and reduced α-lactalbumin. *J. Biol. Chem.*, **270**, 19957–19963.

Noiva,R. (1994) Enzymatic catalysis of disulfide formation. *Protein Exp. Purif.*, **5**, 1–13.

Noiva,R., Kimura,H., Roos,J. and Lennarz,W.J. (1991) Peptide binding by protein disulfide isomerase, a resident protein of the endoplasmic reticulum lumen. *J. Biol. Chem.*, **266**, 19645–19649.

Okazaki,A., Ikura,T., Nikaido,K. and Kuwajima,K. (1994) The chaperonin GroEL does not recognize apo-α-lactalbumin in the molten globule state. *Nature Struct. Biol.*, **1**, 439–446.

Otsu,M., Omura,F., Yoshimori,T. and Kikuchi,M. (1994) Protein disulfide isomerase associates with misfolded human lysozyme *in vivo*. *J. Biol. Chem.*, **269**, 6874–6877.

Pieterson,W.A., Volwerk,J.J. and de Haas,G.H. (1974) Interaction of phospholipase A_2 and its zymogen with divalent metal ions. *Biochemistry*, **13**, 1439–1445.

Puig,A. and Gilbert,H.F. (1994) Protein disulfide isomerase exhibits chaperone and anti-chaperone activity in the oxidative refolding of lysozyme. *J. Biol. Chem.*, **269**, 7764–7771.

Puig,A., Lyles,M.M., Noiva,R. and Gilbert,H.F. (1994) The role of the thiol/disulfide centers and peptide binding site in the chaperone and anti-chaperone activities of protein disulfide isomerase. *J. Biol. Chem.*, **269**, 19128–19135.

Quan,H., Fan,G.B. and Wang,C.C. (1995) Independence of the chaperone activity of protein disulfide isomerase from its thioredoxin-like active site. *J. Biol. Chem.*, **270**, 17078–17080.

Roth,R. and Pierce,S. (1987) *In vivo* cross-linking of protein disulfide isomerase to immunoglobulins. *Biochemistry*, **26**, 4179–4182.

Song,J.L. and Wang,C.C. (1995) Chaperone-like activity of protein disulfide-isomerase in the refolding of rhodanese. *Eur. J. Biochem.*, **231**, 312–316.

Tang,B., Zhang,S.Z. and Yang,K.Y. (1994) Assisted refolding of recombinant prochymosin with the aid of protein disulfide isomerase. *Biochem. J.*, **301**, 17–20.

Tang,J.G., Wang,C.C. and Tsou,C.L. (1988) Formation of native insulin from the scrambled molecule by protein disulfide-isomerase. *Biochem. J.*, **255**, 451–455.

Thunnissen,M.M.G.M., Franken,P.A., de Haas,G.H., Drenth,J., Kalk,K.H., Verheij,H.M. and Dijkstra,B.W. (1992) Site-directed mutagenesis and X-ray crystallography of two phospholipase A_2 mutants: Y52F and Y73F. *Protein Engng*, **5**, 597–603.

Vuori,K., Pihlajaniemi,T., Myllylyä,R. and Kivirikko,K.I. (1992) Site-directed mutagenesis of human protein disulfide isomerase: effect on the assembly, activity and endoplasmic reticulum retention of human prolyl 4-hydroxylase in *Spodoptera frugiperda* insect cells. *EMBO J.*, **11**, 4213–4217.

Wang,C.C. (1997) Chaperone activity of protein disulfide isomerase. In Guzman,N.A. (ed.), *Prolyl Hydroxylase, Protein Disulfide Isomerase, and Other Structurally Related Proteins*. Marcel Dekker Inc., New York, in press.

Wang,C.C. and Tsou,C.L. (1991) The insulin A and B chains contain sufficient structural information to form the native molecule. *Trends Biochem. Sci.*, **16**, 279–281.

Wang,C.C. and Tsou,C.L. (1993) Protein disulfide isomerase is both an enzyme and a chaperone. *FASEB J.*, **7**, 1515–1517.

Wang,X.Q., Yang,J., Gui,L.L., Lin,Z.J., Chen,Y.C. and Zhou,Y.C. (1996) Crystal structure of an acidic phospholipase A_2 from the venom of *Agkistrodon halys* pallas at 2.0 Å resolution. *J. Mol. Biol.*, **255**, 669–676.

Wetterau,J.R., Aggerbeck,L.P., Laplaud,P.M. and McLean,L.R. (1991) Structural properties of the microsomal triglyceride-transfer protein complex. *Biochemistry*, **30**, 4406–4412.

Wu,X.F., Jiang,Z.P. and Chen,Y.C. (1984) A comparison of three phospholipase A_2 from the venom of *Agkistrodon halys* Pallas. *Acta Biochim. Biophys. Sin.*, **16**, 664–671.

Received on August 14, 1996; revised on October 15, 1996