Catalysis of Creatine Kinase Refolding by Protein Disulfide Isomerase Involves Disulfide Cross-link and Dimer to Tetramer Switch

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Protein disulfide isomerase (PDI) functions as an isomerase to catalyze thio-disulfide exchange, as a chaperone to assist protein folding, and as a subunit of prolyl-4-hydroxylase and microsomal triglyceride transfer protein. At a lower concentration of 0.2 μM, PDI facilitated the aggregation of unfolded rabbit muscle creatine kinase (CK) and exhibited anti-chaperone activity, which was shown to be mainly due to the hydrophobic interactions between PDI and CK and was independent of the cross-linking of disulfide bonds. At concentrations above 1 μM, PDI acted as a protector against aggregation but an inhibitor of reaction during CK refolding. The inhibition effect of PDI on CK refolding was further characterized as due to the formation of PDI-CK complexes through intermolecular disulfide bonds, a process involving Cys-36 and Cys-295 of PDI. Two disulfide-linked complexes containing both PDI and CK were obtained, and the large, soluble aggregates around 400 kDa were composed of 1 molecule of tetrameric PDI and 2 molecules of inactive intermediate dimeric CK, whereas the smaller one, around 200 kDa, was formed by 1 dimeric PDI and 1 dimeric CK. To our knowledge this is the first study revealing that PDI could switch its function from dimer to tetramer in its functions as a foldase. According to the observations in this research and our previous study of the folding pathways of CK, a working model was proposed for the molecular mechanism of CK refolding catalyzed by PDI.

In eukaryotes, all the nascent outer membrane and secreted proteins are synthesized and fold to their native structures in the lumen of endoplasmic reticulum. The specialized compartment provides a favorable environment for native disulfide bond formation, which is often the rate-limiting step in the folding of many proteins. Endoplasmic reticulum is abundant in molecular chaperones and folding catalysts, among which the most abundant and efficient catalyst of disulfide bond formation is protein disulfide isomerase (PDI,1 EC 5.3.4.1) (1–3). PDI is a multidomain and multifunction homodimer. It comprises four thioredoxin-like domains, a, b, b′, and a′, plus a linker region between b′ and a′ and a C-terminal acid extension (2, 4, 5). Each of the two catalytic domains, a and a′, contains a WCGHCK motif, responsible for the isomerase activity (6). In vitro studies showed that the b′ domain provides the principle peptide binding site, whereas the other domains should also be involved when the protein substrates have a substantial secondary structure (7, 8). The latest study (9) demonstrated the existence of a ligand binding site in the b′ domain, which was a small hydrophobic pocket defined as Leu-242, Leu-244, Phe-238, and Ile-272.

Much effort has been devoted to investigating the catalytic mechanism of PDI since the first report published more than 40 years ago (10). As an enzyme, the specific binding to its substrate is a basic property of PDI, and the nature of the interaction between PDI and its substrates plays a crucial role in its activity of accelerating steps in protein folding associated with disulfide formation or isomerization. However, the lack of high resolution structures of PDI or the PDI-substrate complex limits the knowledge of the pathway involved, and thus, the identification of the PDI-substrate complex is the key to elucidating its catalytic mechanism. Unfortunately, only a few reports have addressed this problem directly. Direct evidence of the binding ability of PDI was found in the characterization of a complex (>.500 kDa) of PDI and alcohol dehydrogenase by gel filtration HPLC analysis (11). By chemical cross-linking of peptides or non-native proteins to PDI, Klappa et al. (8, 12) recently proposed that the essential core of the PDI binding site was in its b′ domain, and all four domains might contribute to the binding of large peptides. It, thus, seems likely that PDI provides a specific environment that facilitates large peptide folding through all domains. However, it is not clear yet whether this hypothesis is true or not for large proteins, and it is still unknown how PDI further performs its activity through the cross-linking of disulfide bonds. Particularly, it is still a mystery how PDI acts for very large proteins that might have similar or larger dimensions than PDI.

In this study creatine kinase (CK, EC 2.7.3.2), an 86-kDa

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1. The abbreviations used are: PDI, protein disulfide isomerase; CK, rabbit muscle creatine kinase; GdnHCl, guanidine hydrochloride; SEC, size exclusion chromatography; HPLC, high performance liquid chromatography; DTT, dithiothreitol; WT, wild type.
model protein extensively studied in our laboratory (13–15), was chosen as the protein substrate of PDI. CK is a homodimer with large quantities of secondary structures, and each subunit of CK contains four free cysteines. Because the refolding of CK did not need the oxidation of any free sulphydryls into disulfides, no thiol-disulfide interchange reactions would occur upon the formation of the PDI-CK complexes, and thus, an accumulation of the long-lived PDI-substrate complexes was obtained. These complexes were characterized as being disulfide-linked by comparing the PDI activity of assisting CK refolding in solutions with or without DTT. The mutation analysis further indicated that although the noncovalent hydrophobic interactions were the major driving force, Cys-36 and Cys-295 of PDI were involved in PDI-CK interactions. Moreover, to our knowledge this is the first report that PDI could switch its conformation from dimer to tetramer in its catalysis of CK refolding, which implied that a much more complex mechanism associated with dimer to tetramer transition of PDI might play a crucial role in its assisting large proteins to refold.

EXPERIMENTAL PROCEDURES

Materials—The plasmid pBV220-PDI (5.20 kilobases) containing the human PDI gene without the signal peptide was a generous gift from Dr. Y. Gao, Capital Normal University, People's Republic of China. Ultrapure GdnHCl, Tris, and ATP were from Sigma. DTT was from Prornega. Mouse anti-PDI monoclonal antibody was purchased from XBL International Corp., and horseradish peroxidase-labeled rabbit anti-mouse antibody was from DAKO. N-Ethylmaleimide was from Acros Organics. All the other chemicals were local products of analytical grade.

Protein Purification and Concentration Determinations—PDI was purified according to the published protocol (16). The preparation of rabbit muscle CK was as described earlier (17). Purified PDI and CK were homogeneous on SDS-PAGE. The concentrations of PDI and CK were measured spectrophotometrically at 280 nm, \( A_{280}^{\text{PDI}} = 0.9 \) for PDI (18) and \( A_{280}^{\text{CK}} = 0.88 \) for CK (17).

Construction of PDI Mutants—There are six cysteine residues in the primary sequence of PDI, two located at the N-terminal active site (a domain), two at the C-terminal active site (a' domain), and two in the b domain. According to the requirement of the experiment, some of the cysteines were mutated to serines to give rise to nine mutants. The mutants are constructed and designated as listed in Table I. Purification of these mutants was the same as that of WT PDI.

Biophysical Analysis—Far UV circular dichroism spectra were recorded on a Jasco 725 spectrophotometer using a cell with a path length of 0.1 cm. Fluorescence spectra were collected on an F-2500 spectrofluorometer using a 1-ml cuvette, excitation at 285 nm and emission in the range of 300–400 nm. The concentrations of the PDI species are 10 \( \mu \)M. All resultant spectra were collected at 25 °C as an average of five scans.

Denaturation and Reactivation of CK—CK (with a final concentration of 242 \( \mu \)M) was completely denatured in 3 \( \text{m} \) GdnHCl at 25 °C overnight. Reactivation was carried out by a 121-fold dilution of the denatured enzyme into 30 \( \text{m} \) Tris-HCl buffer (pH 8.0) containing various PDI species at different concentrations. The final concentration of CK was 2 \( \mu \)M. At suitable intervals 10 \( \mu \)l of the sample was used for activity assay according to the pH colorimetry method (17). After refolding for 4 h, DTT was added to a final concentration of 10 \( \mu \)M for an incubation period of 2 h, and then CK activity was remeasured. All the reactions were conducted at 25 °C, and all the measurements were repeated at least 3 times.

Non-reduced SDS-PAGE and Western Blot Analysis—Samples containing PDI and CK at a molar ratio of 5 were used for non-reduced SDS-PAGE and Western blot analysis. Before non-reduced SDS-PAGE was carried out, N-ethylmaleimide was added to a final concentration of 5 \( \mu \)M to stop the disulfide exchange. Western blot was carried out after 10% non-reduced SDS-PAGE with mouse anti-PDI monoclonal antibody as the primary antibody, and horseradish peroxidase-labeled rabbit anti-mouse antibody as the secondary antibody.

Aggregation of Denatured CK in the Presence of Various PDI Species—3 \( \text{m} \) GdnHCl denatured CK was diluted into 30 \( \text{m} \) Tris-HCl (pH 8.0) buffer with or without 10 \( \text{m} \) DTT in the presence of various PDI species at different concentrations. Aggregation was followed by monitoring the turbidity at 400 nm with a PerkinElmer Life Sciences Lambda Bio UV spectrophotometer at 25 °C. All the measurements were repeated at least three times.

RESULTS

CK Refolding in the Presence of WT PDI—To characterize the effect of PDI on CK refolding, the reactivation and aggregation of CK without or with various amounts of PDI was investigated first. Similar to the results reported previously (19), GdnHCl-denatured CK could refold to about 45% of the activity of native CK after being diluted into 30 \( \text{m} \) Tris-HCl buffer (pH 8.0). The final concentration of CK was 2 \( \mu \)M. The molar ratios of PDI and CK were 0 (■), 0.1 (●), 0.5 (▲), 1.0 (▲), 2.0 (○), and 5.0 (△), respectively.

Size Exclusion Chromatography (SEC) Analysis—Samples containing PDI and CK at a molar ratio of 5 were chosen for SEC analysis with a Superdex HR200 column. Then samples were combined with 10 \( \text{m} \) DTT for 2 h at 25 °C and used for SEC analysis. All the samples were centrifuged at 13,000 \( \times \) g for 10 min before loading. The SEC was recorded with an AKTA purifier. Each peak was collected for further analysis by reduced SDS-PAGE.

FIG. 1. Effect of WT PDI on the reactivation (A) and aggregation (B) of GdnHCl denatured CK. CK was completely denatured in 3 \( \text{m} \) GdnHCl at 25 °C overnight and refolded by dilution into 30 \( \text{m} \) Tris-HCl buffer (pH 8.0). The final concentration of CK was 2 \( \mu \)M. The molar ratios of PDI and CK were 0 (■), 0.1 (●), 0.5 (▲), 1.0 (▲), 2.0 (○), and 5.0 (△), respectively.

Cys-36 and Cys-295 of PDI Play a Central Role in the Inhibition Effect on CK Reactivation—Mutational analysis were...
used to characterize the central cysteine(s) in PDI-CK complex formation. Besides the six cysteines in the N- and C-terminal active site, the two cysteines in the b’ domain were also considered. First of all WT PDI and the nine mutants (Table I) were constructed for biophysical analysis. CD spectra of the PDI species showed only little differences (Fig. 3A), whereas the intrinsic fluorescence spectra were undistinguishable between the WT PDI and mutants (Fig. 3B). These results indicated that both the secondary and the tertiary structures of the PDI mutants were unaffected by mutation. The reactivation of CK in the presence of different PDI mutants at different molar ratios revealed quite different behaviors when compared with WT PDI (Fig. 2). According to the changes in CK activities before and after the addition of DTT, the mutants can be classified into three classes, and the changes of typical species in each class are shown in Fig. 2, A–C, respectively. NCS:BCC:CSS and NCS:BCC:CSS demonstrated a significant activity-enhancing effect (several times enhancing when [PDI]/[CK] was larger than 2) after the addition of DTT, which was similar to WT PDI (Fig. 2A). NSS:BCC:CSC, NSS:BCC:CSC, NSS:BCC:CSC, and NSS:BCC:CSC had less inhibition effect on CK reactivation and exhibited less enhancing effect (Fig. 2B), whereas no significant changes could be detected for NSS:BCC:CSC and NSS:BCC:CSC (Fig. 2C). A clearer result can be seen in Fig. 2D in the plot of the logarithm of the recovered CK activity before and after the addition of DTT in the presence of various PDI species. A close inspection of Fig. 2D indicated that mutations of Cys-36, the cysteine in the N-terminal active site (a domain), and Cys-295, the non-active site cysteine in the b’ domain of PDI, could effectively eliminate the inhibition effect of WT PDI on CK reactivation in DTT-free conditions. This result suggested that Cys-36 and Cys-295 of PDI might be involved in the formation of intermolecular disulfide bonds with CK and were responsible for the inhibition effect of PDI on CK.

The Existence of the Disulfide-linked Complex and the Dimer to Tetramer Switch of PDI—More direct evidence of the formation of intermolecular disulfide bonds between PDI and CK came from the characterization of the PDI-CK complexes. The solutions of CK refolding in the presence of various PDI species in DTT-free conditions were first analyzed by non-reduced SDS-PAGE and Western blotting. As a control, all the PDI species except NSS:BCC:CSS had only one band, whereas additional bands with a higher molecular weight were found for NSS:BCC:CSS (Fig. 2), which disappeared after the addition of DTT and was characterized to be a PDI tetramer linked by disulfide bonds by SEC analysis (see Supplemental Fig. 8B). This unusual tetramer peak might have been due to the cross-linking of Cys-36 between NSS:BCC:CSS dimers. For the samples of GdnHCl-denatured CK refolding in the presence of various PDI species containing Cys-36 or Cys-295, the formation of PDI-CK complexes could be identified by the appearance of some new high molecular weight bands. In contrast, no new bands could be found for NSS:BSC:CSS or NSS:SSS:CSS (Fig. 4). The results here confirmed the existence of the disulfide-linked complexes between PDI and CK, and once more they revealed that Cys-36 and Cys-295 were responsible for the formation of the complexes.

A further characterization of the complexes was carried out using samples of CK refolding in the presence of various PDI species with or without DTT by SEC analysis. Samples containing PDI and CK at a molar ratio of five were chosen for SEC analysis. As a control, no complex peaks could be observed in the SEC spectra of samples prepared by directly mixing solutions of WT PDI and native CK either with or without DTT (data not shown). As shown in Fig. 5A, in addition to the peaks from WT PDI and CK, two new peaks appeared in the SEC spectrum of the CK refolding sample assisted by WT PDI. One peak appeared at the void volume (±400 kDa), and another one appeared at a molecular mass of about 200 kDa. The two new

![Fig. 2. CK reactivation in the presence of different PDI species before ( ) and after ( ) the addition of DTT. A, CK reactivation in the presence of WT PDI. The results in the presence of NCS:BCC:CSC and NCS:BCC:CSC were similar to those of WT PDI. B, CK reactivation in the presence of NCS:BCC:CSC. The results in the presence of NCS:BCC:CSC, NCS:BCC:CSC, and NCS:BCC:CSC were similar to those of NCS:BCC:CSC. C, CK reactivation in the presence of NCS:BCC:CSC. The results in the presence of NCS:BCC:CSC were similar to those of NCS:BCC:CSC. D, the logarithm of CK activities before (A) and after DTT (A) was added in the presence of WT PDI ( ), NCS:BCC:CSC ( ), NCS:BCC:CSC ( ), NCS:BCC:CSC ( ), NCS:BCC:CSC ( ), NCS:BCC:CSC ( ), and NCS:BCC:CSC ( ), respectively. For DTT-containing samples, DTT was added with a final concentration of 10 mM after CK refolding for 4 h, and the activity of CK was re-measured after 2 h of equilibrium. The activities of self-refolding CK were taken as 1.0. All other conditions of CK reactivation were the same as those in Fig. 1.](image)

![Table I](image)

**Cys to Ser mutants of PDI and their designation**

The nomenclature for describing mutations of the six cysteines of PDI uses C to represent cysteine at a given position and S (in bold type) to represent serine at this position. The N-terminal active site in a domain, C-terminal active site in a’ domain, and b’ domain are designated N, C, and B, respectively. The domain boundaries are according to Freedman et al. (3).

| N-terminal active site | b’ domain | C-terminal active site | Designation |
|-----------------------|-----------|-----------------------|-------------|
| CGHC                  | Cys       | Cys                   | CGHC        |
| CGHC                  | Cys       | Cys                   | SGHS        |
| SGHC                  | Cys       | Cys                   | SGHS        |
| SGHS                  | Cys       | Cys                   | SGHS        |
| SGHS                  | Ser       | Cys                   | SGHS        |
| SGHS                  | Cys       | Ser                   | SGHS        |
| SGHS                  | Cys       | Ser                   | SGHS        |
| SGHS                  | Ser       | Ser                   | SGHS        |
The concentrations of the PDI mutants used for analysis were 10 μM. B, intrinsic fluorescence analysis of the PDI species. The spectra of various mutants were indistinguishable from the spectrum of WT PDI, and thus, no labels were presented in this figure.

When DTT was added to the above CK refolding sample, the amount of the 400-kDa peak was found to decrease gradually (Fig. 5B). Reduced SDS-PAGE showed that the remainder of this peak contained both WT PDI and CK, which suggested that the addition of DTT destabilized the 400-kDa peak. Surprisingly, the peak around 200 kDa still existed, and it only contained WT PDI, which suggested that it might be tetrameric PDI according to the molecular mass. At the same time, the peak of refolded CK increased to an amount similar to the peak of refolded CK increased to an amount similar to the 200-kDa peak of refolded CK. The precipitation was analyzed by reduced SDS-PAGE (Fig. 6). It was found that the precipitation contained both PDI and CK for all samples in DTT-free (Fig. 6A) or DTT-containing buffer (Fig. 6B). Thus, the anti-chaperone activity of PDI at low concentrations resulted from noncovalent cross-linking rather than the cross-linking of the disulfide bonds. These observations were quite consistent with those in previous studies (11, 20).

At higher concentrations (≥1 μM), PDI could efficiently inhibit the aggregation of CK in a concentration-dependent manner (Fig. 1B). Thus, PDI should still be taken as a chaperone at higher concentrations (≥1 μM), although WT PDI gradually inhibited the reactivation of CK in DTT-free conditions. However, this inhibition effect could be reversed by the addition of DTT and resulted in a slight increase in the recovered activity relative to self-refolding CK (Fig. 2A). Moreover, NSS:BSS:CSS, a mutant of PDI without any cysteines, did assist the reactivation of unfolded CK (Fig. 2C).

The abilities of various PDI species to inhibit aggregation behaved slightly differently when the buffer contained 10 mM DTT (Fig. 7). A more direct comparison of the changes in the abilities of the PDI mutants to depress CK aggregation with or without DTT was presented in Fig. 7D. Compared with that in DTT-free buffer, a relatively large decrease in the abilities to depress CK aggregation was found for WT PDI, NCC:BCC:CSS, and NSS:BSS:CSS in DTT-containing buffer (Fig. 7A), a slight decrease was found for Nss:BSS:CSS, Nss:BCC:CSS, Nss:BCC:CSS, and NSS:BSS:CSS (Fig. 7B), whereas no significant change
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FIG. 6. Reduced SDS-PAGE analysis of the refolding products of CK in the DTT-free (A) or DTT-containing (B) buffer. The final concentrations of PDI species and CK were 0.2 and 2 μM, respectively. The samples were centrifuged at 13,000 g for 10 min, and then the supernatant (S) and precipitate fractions (P) were used for SDS-PAGE analysis. Std denotes molecular mass standard. The PDI species are as indicated in Fig. 3.

FIG. 7. CK aggregation in the presence of various PDI species at different concentrations in 30 mM Tris-HCl (pH 8.0) with (●) or without DTT (○). A, CK aggregation in the presence of WT PDI. The results in the presence of N_{CC}:B_{CC}:C_{SS} and N_{CS}:B_{CC}:C_{SS} were similar to those of WT PDI. B, CK aggregation in the presence of N_{SS}:B_{CC}:C_{SS}. The results in the presence of N_{SS}:B_{CC}:C_{SS}, N_{NC}:B_{CC}:C_{SS}, and N_{NC}:B_{CC}:C_{SS} were similar to those of N_{SS}:B_{CC}:C_{SS}. C, CK aggregation in the presence of N_{SS}:B_{CC}:C_{SS}. The results in the presence of N_{SS}:B_{CC}:C_{SS} were similar to those of N_{SS}:B_{CC}:C_{SS}. D, the logarithm of the turbidity of CK aggregation in the DTT-containing (T_{DTT}) and DTT-free (T_{DFT}) buffer. ▲, WT PDI; ●, N_{CC}:B_{CC}:C_{SS}; ○, N_{SS}:B_{CC}:C_{SS}; □, N_{SS}:B_{CC}:C_{SS}; △, N_{SS}:B_{CC}:C_{SS}; ◆, N_{SS}:B_{CC}:C_{SS}; ◆, N_{SS}:B_{CC}:C_{SS}. The experimental conditions were the same as those in Fig. 1. The turbidities of self-refolding CK were taken as 1.0.

was found for N_{SS}:B_{SC}:C_{SS} or N_{SS}:B_{SC}:C_{SS} (Fig. 7C). It should also be noted that all the PDI mutants could strongly inhibit the aggregation of CK whether in the DTT-free or DTT-containing buffer (Fig. 7A–C), although the abilities of some of the PDI species to depress CK aggregation could be affected by DTT (Fig. 7, A and B). These results suggested that the formation of disulfide bonds between PDI and CK contributed to the suppression of CK aggregation during refolding but was not the major driving force.

DISCUSSION

PDI has been demonstrated to act as a molecular chaperone independent of its isomerase activity (21–25), and it can exploit the synergy between these functions, especially when disulfide reshuffling is needed in the refolding of the substrates (3, 26). Our results showed that WT PDI at high concentrations could act as a protector against aggregation but acted as an inhibitor of reactivation during CK refolding. The inhibition effect of PDI on CK reactivation was further characterized as being due to the formation of PDI-CK complexes through intermolecular disulfide bonds. In general, the hydrophobic interaction between PDI and its substrates is the major driving force of the binding (12). Since no disulfide bond is required for CK reactivation, the catalysis of thiol-disulfide interchange by PDI will not happen. Thus, the protection effect of PDI on CK aggregation could be attributed to its chaperone activity mainly through hydrophobic interactions, whereas the inhibition effect on CK reactivation could be attributed to its uncompleted isomerase activity through the cross-linking of intermolecular disulfide bonds. This hypothesis was further confirmed by site-directed mutagenesis (Figs. 2 and 7), which indicated that PDI without any cysteines could increase the reactivation of CK and gradually depressed the formation of aggregates during CK refolding in either DTT-free or DTT-containing conditions.

The more N-terminal cysteine, Cys-36 or Cys-380, in each active site of PDI has been characterized as playing a central role in its isomerase activity (27–29), and the C-terminal active site was considered to contribute more to the whole isomerase activity of PDI (30). The non-active site cysteines in the b' domain were reported not to be required for the catalytic activity in human PDI (7) and unlikely to participate in substrate binding (9). However, our results indicated that Cys-36 and Cys-295 were involved in the cross-linking of intermolecular disulfide bonds, whereas Cys-380 was not required. A possible explanation was that the binding of unfolded CK to PDI might have sequence specificity, as that had been demonstrated in pancreas-specific PDI (31, 32). The specific recognition made the binding of PDI to unfolded CK directional, and this resulted in the exposure of a certain cysteine of CK that was conformationally close to Cys-36 but not to Cys-380. The participation of Cys-295 in the formation of disulfide bonds with CK might also result from the specific binding of PDI to CK. Moreover, it was noteworthy that a recent study reported that Cys-295 is located near the ligand binding site of PDI (9), which further suggested that the ligand binding site near Cys-295 of PDI might also be one of the specific binding sites of large proteins like CK.

It has been widely accepted that PDI is a dimer, although it had been shown to be a mixture of dimer and tetramer at 4 °C and pH 7.5 in a previous study (33). The changes from dimer to oligomer, however, have never been reported to be required for PDI to function as a chaperone or foldase. In our research, two disulfide-linked PDI-CK complexes, a 400-kDa one and a 200-kDa one, which appeared during CK refolding in DTT-free conditions, were characterized by SEC and reduced SDS-PAGE analysis (Fig. 5). Our previous work indicated that unfolded monomeric CK could rapidly form inactive dimeric folding intermediates upon dilution to a refolding buffer and that the dimerization of CK was not the rate-limiting step during the refolding process (34). Moreover, it was shown that GroEL could strongly bind an inactive dimeric intermediate of CK (14). According to all these previous observations, CK in the 400- or 200-kDa complexes was more likely to be an inactive dimeric folding intermediate. Thus the 400-kDa peak was assigned to complexes containing 2 dimeric PDIs and 2 dimeric CKs, whereas the 200-kDa peak was assigned to complexes containing 1 dimeric PDI and 1 dimeric CK. The conformational state of the 400-kDa peak was further characterized by comparing the SEC results of DTT-free (Fig. 5A) and DTT-containing conditions (Fig. 5B). The 200-kDa complex in the DTT-containing conditions was found to be formed by PDI without CK, which suggested that this complex was a PDI tetramer. At the same time, the 400-kDa peak in Fig. 5B gradually decreased, and the peak of CK increased in comparison to Fig. 5A, which suggested that the existence of DTT destabilized the 400- and 200-kDa complexes and released the...
cross-linked CK from the complexes. Further identification of the PDI tetramer was obtained by SEC analysis of CK refolding in the presence of various mutants constructed in this study (see Supplemental Fig. 8). Although a rather different behavior could be observed for different mutants, PDI tetramer was found in all samples. Similar to the previous report (33), the PDI tetramer that occurred in the CK refolding process was not formed by cross-linking of disulfides between PDI dimers but rather by specific oligomerization interfaces. The effect of NCC:BCC:CSS on CK refolding was almost the same as that of WT PDI, which suggested that the mutations of Cys in the a’ domain did not affect the function of PDI in its assisting CK refolding. The decrease of the 400-kDa peak in profiles of CK refolding in the presence of NSS:BCC:CSC, NSS:BCC:CSS, NSS:BCC:CSS, and NSS:BCC:CSS might be due to the slightly decreased stability of the PDI-CK complex for these species. These results by SEC analysis further suggested that the transition from dimer to tetramer might be an essential step in the process of PDI-assisted CK refolding.

Our research strongly suggested that the transition from dimer to tetramer was involved in PDI activities in assisting the folding of large proteins. However, several important issues still remain to be answered. (i) Is there another inverse transition from tetramer to dimer in its assisting the refolding of large proteins requiring the formation of native disulfide bonds? Since the major component of PDI in native conditions is a dimer, such an inverse process might exist although it could not be distinguished in this research. (ii) Is this transition from dimer to tetramer specific for the substrate CK? As described in the introduction, the PDI tetramer might not accumulate for substrates containing native disulfide bonds due to the thiol-disulfide interchange and the low stability of both the complex and the tetramer. However, such an accumulation might be achieved by using a substrate containing Cys but no native disulfide bonds. Primm et al. (11) had reported a large soluble complex (>500 kDa) of PDI and ADH by gel filtration HPLC analysis. This PDI-ADH complex was quite similar to the 400-kDa PDI-CK complex in this research. Tetrameric PDI might also be involved in the PDI-ADH complex (>500 kDa), although the model suggested that the complex might be formed between two dimeric PDI and two unfolded protein substrate (11). (iii) It is also not clear yet whether the changes in oligomerization of PDI can take place in vivo or not. Nevertheless, the widely reported examples for such a transition of many chaperones, such as small heat shock proteins (Hsp) (35), Hsp90 (36), and some other chaperones (37, 38), suggested that such a switch from dimer to tetramer might be involved in the functions of PDI.

According to the unusual experimental observations in this research and our previous work on the folding pathways of CK (14, 15, 34), a working model was proposed for the molecular mechanism of CK refolding catalyzed by PDI (Scheme I). Previous work showed that the native dimeric CK (N) dissociated to monomers (U) upon denaturation (kdissN). When diluted into the refolding buffer, the monomers can form inactive dimeric folding intermediate (U2) in a very short time (kdissU). Two fates of U2 were found; one was the formation of large insoluble aggregates through hydrophobic interaction (kadsU). In the other, U2 undergoes a fast phase (k1) to the partially active dimer N2'. Then N2' refolds to the active native-like form N2 through a slow phase (k2) and finally refolds to the native state after a very slow adjustment (kan1U). When PDI is present in the buffer, it can bind the inactive dimeric intermediate U2, which further forms a soluble complex (U2P) (k3). If PDI is at a low concentration (0.2 μM), the excessive U2 binds with U2P through hydrophobic interaction, which results in the formation of large insoluble aggregates. In this way PDI facilitates the aggregation of CK and exhibits anti-chaperone activity (k1,a-chap). If PDI is at a higher concentration (≥1 μM), U2P further forms large disulfide-linked soluble complexes (PU2U2P, k4). The intermolecular disulfide bonds between U2 and PDI prevent U2 from further refolding, and an inhibition effect is observed for CK reactivation. With the addition of DTT, the inactive dimeric intermediate CK (U2), dimeric PDI, or tetrameric PDI are released from the large complexes. Then U2 undergoes a fast phase and a slow phase and finally refolds to the native state.

In conclusion, PDI was found to act as a protector against aggregation, which resulted from its chaperone function, and an inhibitor of reactivation, which arose partially from its incompletely isomerase function, in CK refolding. The unusual observations in this research suggested that the two major functions of PDI were interrelated in its assisting CK refolding and further suggested that the formation of intermolecular disulfide bonds through Cys-36 and Cys-295 and the switch of dimer to tetramer of PDI plays a crucial role in its activities. In our system the large PDI-CK complex (400 kDa) for WT PDI was strongly protected by the intermolecular disulfide bonds, whereas the mutation species had less stability. This implied the PDI tetramer might be short-lived in assisting the folding of proteins containing disulfide bonds due to the thiol-disulfide interchange and the potential hidden transition from tetramer to dimer. However, the consistence of our working model (Scheme I) with the previous findings in PDI activity as well as those in many chaperones suggested that this model might also work in other in vitro systems as well as in vivo conditions. A further investigation of the nature of the transition in oligomerization and the structures of both the dimer and tetramer forms of PDI should elucidate how PDI meets the specific requirements for large proteins.

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