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Covalent Association of Protein Disulfide Isomerase with Recombinant Human Interleukin 2 *in Vitro*

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Protein disulfide isomerase has broad specificity in the catalysis of the formation and rearrangement of native disulfide bonds in proteins. This enzyme has two independent thioredoxin-like active sites (-CGHC-) and a peptide binding site. However, the mechanisms involving the catalytic processes are not clearly understood. It was reported that the enzyme associates with scrambled pancreatic ribonuclease A *in vitro*, and with misfolded human lysozyme *in vivo.* In the present study, recombinant human interleukin 2 has been chosen to probe the reaction intermediate in the reaction with the enzyme. We have identified and characterized a covalent associate formed *in vitro* by SDS-PAGE and Western blot analysis. This associate has a molecular weight of 71–72 kDa, the approximate sum of the molecular weights of the enzyme and the substrate. Western blot analysis confirmed that it formed via an intermolecular disulfide bond. Upon treatment with 2-mercaptoethanol, this bond was cleaved.

Protein disulfide isomerase (PDI) catalyzes the in vitro isomerization of intramolecular disulfide bridges (1). It exhibits a broad specificity and has been used in vitro to catalyze the folding of a variety of proteins (2). This broad specificity of PDI has been suggested to be due to the recognition of the peptide backbone, rather than recognition of the individual amino acid residues (3). However, PDI does recognize the cysteine side chain (3). PDI was first isolated from the liver (4), and it is now clear that the enzyme is present in many organs (5) and its primary structure is highly conserved between species. The presence of copious amounts of PDI in the rough endoplasmic reticulum (ER) (0.4% of total cellular protein) (6) is significant because the ER represents the key junction between the initiation of translation of proteins and the routing of these proteins through the endomembrane system, and the ER is also a key in subjecting the nascent polypeptides to co- and post-translational modifications (7).

PDI appears to function both as an enzyme and as a chaperone. As an enzyme, it does not alter the pathway of folding but does increase the overall rate of disulfide bond formation (8). PDI reduces incorrect disulfide bonds of scrambled ribonuclease A (RNase) so that it can ultimately form the correct disulfide bonds present in native RNase (9). It has also been demonstrated that *E. coli* PDI (DsbA) mediates the conversion of misfolded insulin-like growth factor I (IGF-1) to correctly-folded IGF-1 by rearranging the scrambled disulfide bonds (10). In addition, PDI appears to function as a chaperone because it mediates major conformational changes in polypeptide chains and has broad specificity. Recently, it has been demonstrated in vitro that PDI increases reactivation and decreases aggregation of denatured D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (11) and rhodanese (12), proteins that have no disulfide bonds and have a high propensity to aggregate in the denatured state. The mechanism of proper GAPDH folding is believed to be due to the binding of the protein with PDI, thus preventing incorrect association leading to the formation of aggregates (and therefore favoring the correct folding of the protein).

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Abbreviations: PDI, protein disulfide isomerase; ER, endoplasmic reticulum; RNase, ribonuclease A; IGF-1, insulin-like growth factor I; GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase; rhIL-2, recombinant human interleukin 2; ELC, enhanced-chemiluminescence; amino acid abbreviations are standard 1- or 3-letter abbreviations.

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It was demonstrated by SDS-PAGE that during a reaction of scrambled ribonuclease A (RNase) with PDI, a covalent intermediate formed that had a molecular mass corresponding to the sum of the masses of the two proteins (13). An in vivo study by Otsu, et al. showed that PDI associates with misfolded human lysozyme but not the wild-type protein (14). However, the mechanisms of the catalysis of native disulfide bond formation and rearrangement of incorrect disulfide bridges by PDI are not clearly understood.

rhIL-2 is a potent pleiotropic immunomodulator. Its effects include (a) initiating and maintaining T-cell proliferation (15), (b) stimulating the generation of cytotoxic T lymphocytes (16), and (c) activating natural killer cells (17) and lymphokine-activated killer cells (18). rhIL-2 serves as an excellent protein to examine PDI-assisted folding of proteins because rhIL-2 has only three possible disulfide isomers that can form, the simplest case for disulfide scrambling. It is known that the redox potential and the composition of the redox system affects the function of PDI as an enzyme (19–21). By studying the interactions between PDI and the protein substrate by characterizing the intermediate formed, it will be possible to better understand the mechanisms of action of PDI. We report the formation of a covalent associate in the reaction of PDI with scrambled rhIL-2 under reducing conditions. This heterodimer was separated from the reaction mixture by SDS-PAGE and characterized by western blot analysis.

MATERIALS AND METHODS

Materials

Recombinant human interleukin 2 (rhIL-2, 0.4 mg/mL in water) was generously provided by Amgen, Inc. as the mixture of three disulfide isomers (scrambled rhIL-2). Native rhIL-2 standard and anti-human interleukin 2 (anti-rhIL-2, monoclonal, from mouse) were purchased from R & D Systems, Inc. (Minneapolis, MN). Protein disulfide isomerase (PDI, from bovine liver) and anti-mouse IgG heavy and light chain (from rabbit) peroxidase conjugate were purchased from Calbiochem-Novabiochem Corp. (San Diego, CA). Anti-protein disulfide isomerase (anti-PDI, monoclonal, from mouse) was purchased from StressGen Biotechnologies Corp. (Victoria, Canada). Polyacrylamide Tris-glycine pre-cast mini-gels, buffer solutions, Mark 12, and nitrocellulose membranes were obtained from Novex (San Diego, CA). ECL (enhanced-chemiluminescence) western blot reagents, molecular weight markers, streptavidin for ECL marker, and ECL-films were purchased from Amersham Life Science Inc. (Arlington Heights, IL). Buffer salts and magnesium chloride were obtained from the standard suppliers. HPLC-grade solvents were purchased from J. T. Baker.

Reaction of PDI with rhIL-2

Reaction 1: Reduced rhIL-2 and reduced PDI. PDI (30 μM, 60 μL) and rhIL-2 (20 μM, 60 μL) were each individually preincubated with 0.5 mM DTT in the degassed folding buffer (100 mM HEPES, 20 mM EDTA, and 5 mM $MgCl_2$, pH 7.0) at 37°C for 30 min, respectively. The reaction was initiated by adding the reduced rhIL-2 to the reduced PDI and warming at 37°C. Aliquots of the reaction mixture (10 μL) were withdrawn at 0, 5, 15, 30, 45, 60, 75, 90, 105, and 120 min, respectively. Each sample was quenched with SDS sample buffer (10μL) and 2 mM DTNB containing 0.5 M acetic acid, and immediately heated in boiling water for 30 seconds to stop the reaction. As a control experiment, the reaction was performed by incubating rhIL-2 using the same conditions, but in the absence of PDI.

Reaction 2: rhIL-2 and reduced PDI. This reaction was performed in the same manner as Reaction 1 except that rhIL-2 (20 μM, 60 μL) in the folding buffer was added directly to PDI (30 μM, 60 μL) that had been reduced with 0.5 mM DTT, as described above. The rhIL-2 was not reduced prior to initiation of this reaction. The aliquots were withdrawn and quenched similarly.

SDS-PAGE and Silver-Staining

Aliquots from Reaction 1 were loaded on to a 4–12% 15-well Tris-glycine gel with Mark 12 as the molecular weight standards and PDI and rhIL-2 as control samples. Electrophoresis was performed at 125 volts and 400 mA for 1.5 hour using a Novex mini-cell and SDS Tris-glycine running buffer according to the protocol provided by Novex supplier. The resolved gel was silver-stained (22) and photographed.

Detection of PDI-rhIL-2 Associate by Western Blot

Each aliquot from Reaction 1 was loaded on one 15-well 4–12% gel (10 μL in each well) and one 15-well 8% gel (15 μL in each well), respectively. PDI and rhIL-2 controls and ECL molecular weight markers were also loaded onto each gel. Electrophoresis was performed as described above. Western blot analysis was then performed according to the ECL protocol

provided by Amsham supplier. Monoclonal anti-rhIL-2 (2 μg diluted in 1 mL T-PBS) was used to specifically detect rhIL-2 transferred from the 4–12% gel onto one membrane. Monoclonal anti-PDI (1 μg diluted in 1 mL T-PBS) was used to specifically detect PDI transferred from the 8% gel onto the other membrane. Rabbit anti-mouse IgG coupled with horseradish peroxidase (1 μg diluted in 1 mL T-PBS) was used as the secondary antibody together with streptavidin solution for the ECL markers (used as indicated by the ECL protocol). ECL film was then exposed to the membrane for 20 sec to 2 min until satisfactory results were obtained.

The aliquots from reaction 2 were analyzed in the same manner.

Since the ECL molecular weight markers could only be used as the reduced forms and the reaction mixtures had to be analyzed under non-reducing conditions, there were deviations between the actual molecular weights of the proteins and the calculated molecular weights based on the mobility of the ECL markers. Both PDI and rhIL-2 control samples had lower calculated molecular weights, based on the ECL markers, than their actual molecular weights. Therefore, the reported molecular weight of each protein was based upon the results from the silver-stained gels, rather than upon the results of the western blot analyses.

Verification of the Covalent Nature of PDI-rhIL-2 Associate

A reaction was performed under the conditions described for Reaction 1, except that duplicate aliquots were withdrawn at 10, 30, 45, 60, and 90 min, respectively. One aliquot was quenched with 10 μL SDS sample buffer and 10 μL 0.5 M acetic acid and 2 mM DTNB and boiled for 2 min; the other aliquot was quenched with SDS sample buffer containing 4% 2-mercaptoethanol and boiled for 4 min. SDS-PAGE was performed on one 4–12% gel and one 8% Tris-glycine gel. PDI and rhIL-2 controls and ECL molecular weight markers were used as described above. Reduced and non-reduced samples were loaded onto each gel side by side. Following electrophoresis, the bands were analyzed by western blotting to detect rhIL-2 and PDI, respectively, as described previously.

RESULTS

The SDS-PAGE results of Reaction 1 are shown in Figure 1. A well-defined band is seen above the PDI band which starts to appear after 5 min of the reaction and increases progressively with the reaction time, as indicated by the arrow in Figure 1. The molecular weight of the protein at this band was calculated as approximately 71–72 kDa according to the molecular weight references and it corresponds to sum of the molecular weights of PDI (57 kDa) and rhIL-2 (14.5 kDa). The same band was also observed when reduced PDI reacted with non-reduced rhIL-2 (result not shown). The western blot analysis results are shown in Figure 2. Figure 2a has bands corresponding to molecular weights of 14.5, 29, 43.5, 58 and 71–72 kDa, respectively, which bind to anti-rhIL-2 antibody. The four lower molecular weight bands correspond to rhIL-2 monomer, dimer, trimer, and tetramer, respectively, and appear in the control experiment as well, in which the reaction was performed in the absence of PDI (data not shown). The intensities of the monomer and dimer do not change over the course of the reaction, but those of the trimer and tetramer increase and reach a maximum by 75 min. The fifth band (identified with an arrow) has a molecular weight of 71–72 kDa, as measured on the SDS-PAGE gel in Figure 1, and is absent in the rhIL-2 control (Lane 4) and the

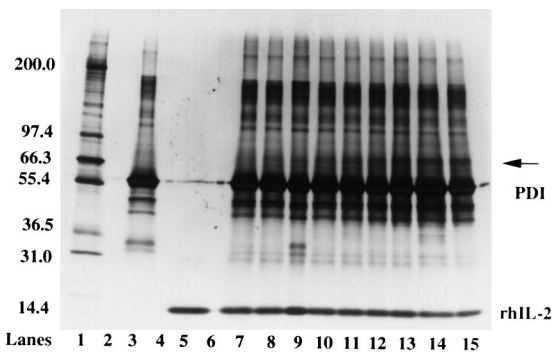


FIG. 1. SDS-PAGE of the reaction mixtures of PDI and rhIL-2 from Reaction 1. Lane 1 is the molecular weight marker. Lanes 2 and 4 are blank. Lane 3 and 5 are PDI and rhIL-2 controls, respectively. Lanes 7–15 are reaction mixtures withdrawn at 0, 5, 15, 30, 45, 60, 75, 90, and 105 min, respectively.

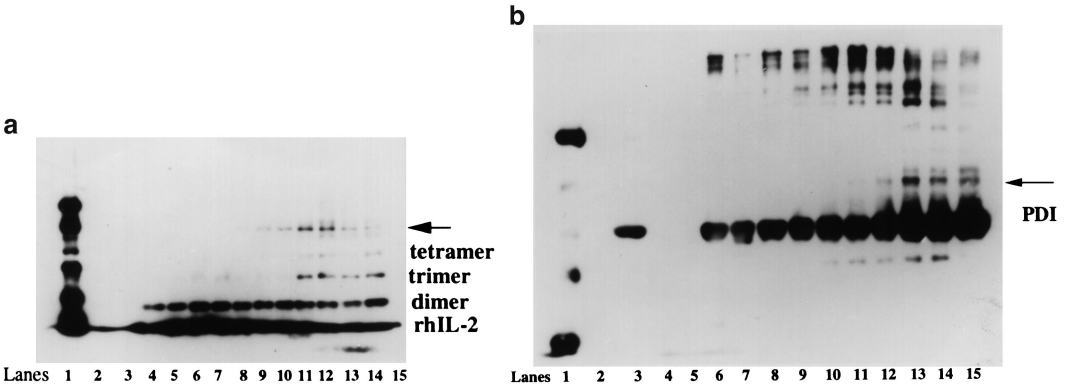


FIG. 2. Western blot results of the reaction mixtures from Reaction 1. (a) Detection of rhIL-2 using anti-rhIL-2 antibody; Lane 1 is the ECL molecular weight markers, Lane 2 is blank, Lanes 3 and 4 are the PDI and rhIL-2 controls, Lanes 5–14 are the reaction mixtures withdrawn at 0, 5, 15, 30, 45, 60, 75, 90, 105, and 120 min, respectively. (b) Detection of PDI using anti-PDI antibody; Lane 1 is ECL molecular weight markers, Lanes 2 and 4 are blank, Lanes 3 and 5 are PDI and rhIL-2 controls, respectively, Lanes 6–15 are the reaction mixtures withdrawn at 0, 5, 15, 30, 45, 60, 75, 90, 105, and 120 min, respectively.

control experiment (data not shown). This band appears after 45 min of the reaction and the intensity changes as the reaction progresses. Therefore, the protein at this band results from the reaction of PDI with rhIL-2 and contains rhIL-2. Figure 2b is the western blot of the reaction of reduced-PDI with reduced rhIL-2, as detected by the reaction with anti-PDI antibody. This figure shows bands at molecular weights of 57 and 71–72 kDa, respectively, which bind to anti-PDI antibody. The 57 kDa band is PDI, as demonstrated by the PDI control sample (Lane 3). The 71–72 kDa band appears after 45 min of the reaction and also has the same molecular weight as the band detected on the SDS-PAGE gel in Figure 1 and it is absent in the PDI control. This band, therefore, also results from the reaction of PDI with rhIL-2 and contains PDI. The results from western blot analysis demonstrate the formation of PDI-rhIL-2 associate. The lighter bands that appeared below PDI and above the PDI-rhIL-2 heterodimer appear to result from degradation or aggregation of PDI since they became more visible when the reaction mixtures were boiled for longer periods to quench the reaction.

Figure 3 demonstrates that the PDI-rhIL-2 association is through a covalent disulfide bond. Duplicate aliquots were withdrawn periodically, one of which was treated with 2-mercaptoethanol and the other was treated with SDS-sample buffer and acetic acid. Lanes 1 and 3 are PDI and rhIL-2 controls, respectively. Lanes 4–8 are the reaction mixtures quenched with SDS-sample buffer and acetic acid only. Lane 10 is the ECL molecular weight marker. Lanes 11–15 are the reaction mixtures that were treated with 2-mercaptoethanol. The PDI-rhIL-2 associate appears at the positions as expected on Lanes 4–8. This band disappears on Lanes 11–15 because the disulfide bond between PDI and rhIL-2 is cleaved by 2-mercaptoethanol. The results clearly demonstrate that PDI-rhIL-2 is covalently associated. Similar results were obtained by using anti-PDI antibody in the detection procedure (data not shown).

DISCUSSION

PDI is a pleiotropic protein associated with several different enzymes in the lumen of ER in eukaryotic cells. It has two active sites, each of which contains a dithiol/disulfide thioredoxin-like center (-CGHC-), and a peptide/protein binding site which binds peptides and proteins with little specificity (3,23,24). It catalyzes the in vitro and in vivo formation and isomerization of intramolecular disulfide bridges involving thiol/disulfide interchanges (1,2,25). It exhibits broad specificity, and has been shown to catalyze the folding of a variety of proteins. It has also demonstrated chaperone activity by promoting the folding of chains in addition to the formation of disulfide

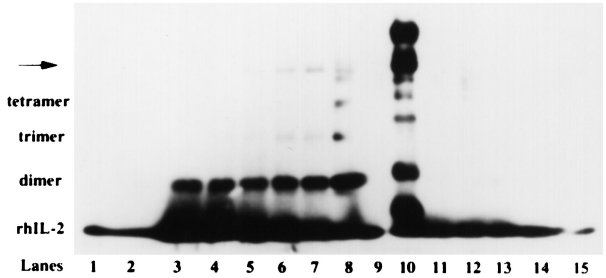
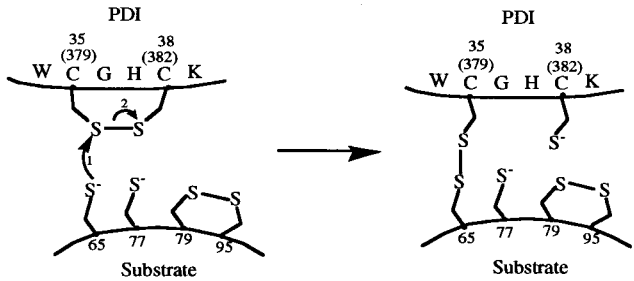


FIG. 3. Western blot results showing PDI-rhIL-2 disulfide bridge. PDI (30 μM, 60 μL) and rhIL-2 (20 μM, 60 μL) were each individually preincubated with 0.5 mM DTT in the degassed folding buffer (100 mM HEPES, 20 mM EDTA, and 5 mM $MgCl_2$, pH 7.0) at 37°C for 30 min, respectively. The reaction was initiated by adding the reduced rhIL-2 to the reduced PDI and warming at 37°C. Duplicate aliquots of the reaction mixture (10 μL each) were withdrawn at 10, 30, 45, 60, and 90 min, respectively. One aliquot from each time point was quenched with SDS sample buffer (10 μL) and 2 mM DTNB containing 0.5 M acetic acid and immediately heated in boiling water for 30 seconds to stop the reaction, and the other one was treated with 4% 2-mercaptoethanol in SDS-sample buffer. The samples were loaded onto a 4–12% gel, after which SDS-PAGE was performed. The bands were identified using Western blot analysis with anti-rhIL-2 for the detection. Lanes 1 and 3 are PDI and rhIL-2 controls. Lane 2 is blank. Lanes 4–8 are the reaction mixtures withdrawn at 10, 30, 45, 60, and 90 min, respectively, which were not reduced by 2-mercaptoethanol. Lane 9 is blank. Lane 10 is ECL molecular weight markers. Lanes 11–15 are the same reaction mixtures as those in Lanes 4–8 but reduced with 4% 2-mercaptoethanol.

bonds (11,12). PDI is a multifunctional protein: it is identical to the β-subunit of prolyl hydroxylase, the glycosylation site binding protein, and the thyronine binding protein (2). Despite its multifunctionality, the essential function of PDI is still not known (26).

As an enzyme, the two thioredoxin-like active sites of PDI (-CGHC-) provide thiol/disulfide groups that are essential for the enzymatic function and each site operates independently (13,27,28). Mutagenesis of either site causes a loss of 50% of the PDI activity (27). The thiol groups have a pKa of 6.7 (29) and are ionized under neutral conditions; therefore they act as nucleophiles in the formation and isomerization of disulfide bridges. It is also known that PDI has a high redox potential (−110 mV) (30) and its activity is maximized when the redox environment is more oxidizing, as is found in the lumen of ER (31). It has been demonstrated that the redox environment is important for the activity of PDI (19–21). In PDI-catalyzed folding of ribonuclease A, the folding rate increases as the ratio of GSSG to GSH increases and reaches maximum at a ratio of 0.2: 1.0 (20). This suggests that when the active site cysteines of PDI are in the reduced form, PDI has the highest activity. On the other hand, the oxidized cysteine groups in the active sites of PDI are less stable than the reduced forms, and therefore have a tendency to be reduced by free thiol groups in the substrate. This reaction subsequently drives the formation of disulfide bridges in the substrate. Therefore, it is most likely that the oxidation or rearrangement of an incorrect disulfide bond is through the formation of a mixed disulfide of PDI and the substrate, as supported by the previous findings of PDI-ribonuclease A covalent associate (13).

In vitro and in vivo studies (31,32) have suggested that the mechanism of the catalysis of disulfide bond formation by PDI involves the intermolecular disulfide exchange between PDI and the substrate (31). Kanaya, et al. proposed mechanisms in which the free thiol group in mutant human lysozyme (the substrate) attacks the disulfide bond at the active center of PDI, and leads to the formation of a PDI-substrate covalent intermediate (31). This covalent intermediate plays a key function in the rearrangements of the disulfide bonds of the substrate. It is known that mutation of both active sites in PDI destroys its isomerase activity (27). However, mutation or even deletion of residues other than the residues in the active sites, including those conserved residues, have little effect on PDI activity (26). Therefore, the covalent interaction of PDI with the substrate during the catalytic process must be through the cysteine groups at the active sites of PDI, as proposed by Kanaya, et al. and shown in Scheme 1.



SCHEME 1.

In the present study, PDI was placed in the folding buffer containing DTT to obtain the reduced form of PDI at the active sites. rhIL-2 was then added to the PDI solution. PDI-rhIL-2 associate formed during this reaction, as shown by the arrows in Figures 1 and 2. When rhIL-2 is reduced by DTT in the folding buffer prior to its addition to the reduced PDI to initiate the reaction, dimers, trimers, and even tetramers of rhIL-2 are formed as seen in Figures 2a and 3 and result from the intermolecular disulfide bond formation. The aggregates form as a function of time, appearing immediately, and are clearly of a covalent nature because they are stable in SDS, but are broken when subject to 2-mercaptoethanol treatment (Figure 3). This observation suggests that thiol groups of rhIL-2 prefer to exist in the oxidized form. We have also observed that whether rhIL-2 is reduced or not reduced by DTT prior to the addition to PDI solution, there is little effect on the formation of PDI-rhIL-2 associate. These results imply that the formation of PDI-rhIL-2 associate is through the nucleophilic attack of the ionized thiol group of PDI at the active center on the disulfide bridge of rhIL-2, which is consistent with other literature results (20,21,29,30). However, it is likely that the more oxidizing environment like that used by Kanaya et al. (31) causes the disulfide bridge between PDI and the substrate to form by nucleophilic attack of the substrate's sulfur anion on the cystine at the active sites of PDI (31).

It is concluded that when the cysteine residues in the active centers of PDI are oxidized, PDI functions as an oxidoreductase and facilitates disulfide formation in nascent polypeptide chains (28,31). This function is involved in the co-translational modifications of secretory proteins (14,32). Alternatively, reduced cysteines at the active centers in PDI perform the isomerase function and rearrange the incorrect disulfide bridges to form native disulfide bonds, which is involved in the post-translational modifications of scrambled proteins (33,34). Therefore, a clear understanding of the reaction mechanisms involving these translational modifications are important, and further studies on the nature of reaction of PDI with rhIL-2 are in progress to elucidate the reaction mechanism of catalysis of intermediate formation.

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