Improved Folding Yields of a Model Protein Using Protein Disulfide Isomerase

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Purpose. To study the effects of recombinant human protein disulfide isomerase (rhPDI) concentration, reduced glutathione:oxidized glutathione ratio (GSH:GSSG) and temperature on the efficiency of oxidative folding of a model protein, recombinant human interleukin 2 (C125A mutation) (C125A rhIL-2).

Methods. C125A rhIL-2 inclusion bodies were reduced and denatured by guanidium hydrochloride (Gdm.Cl) and 100 mM GSH. The solution was diluted 10 times into folding buffer, allowing C125A rhIL-2 to fold either in the absence or presence of rhPDI. The renatured and unfolded C125A rhIL-2 species were quantitated by reversed phase-HPLC.

Results. The initial folding rate of C125A rhIL-2 linearly increased with rhPDI:C125A rhIL-2 molar ratio in the first 2.5 minutes, and reached the highest rate when the rhPDI:C125A rhIL-2 ratio was 1:1. The oxidative folding of C125A rhIL-2 linearly increased as the GSH:GSSG molar ratio decreased from 10:0 to 10:3. The folding of C125A rhIL-2 was also dependent on temperature, and optimum folding was realized at 23°C.

Conclusions. These results demonstrate that under optimal redox potential and temperature, rhPDI enhances the oxidative folding of C125A rhIL-2. In the oxidative folding of C125A rhIL-2, rhPDI exerts its effect on folding by the acceleration of thiol/disulfide interchange.

KEY WORDS: protein folding; protein disulfide isomerase; interleukin 2; protein renaturation; disulfide bonds; chaperone.

INTRODUCTION

Expression of recombinant protein in transformed cells provides large quantities of protein that would be hard to obtain from natural sources. However, high level expression of eukaryotic protein in *Escherichia coli* can result in the formation of inclusion bodies in the cell cytoplasm or periplasm (1). Inclusion bodies are insoluble aggregates of misfolded recombinant protein having no biological activities. Thus the inclusion body protein must be folded to its native structure in order to attain its biological activities. The folding of recombinant protein from inclusion bodies is typically accomplished by solubilizing inclusion bodies in a strong chaotrope such as guanidium hydrochloride (Gdm.Cl) or urea. Additionally, a reducing environment is necessary to break disulfide bonds. The denatured, reduced protein is then added to a folding buffer to allow the polypeptide to fold into its native structure. The folding buffer should be at an optimal pH and redox potential to enable correct disulfide bond formation (2).

Conventional protein-folding protocols are often not efficient for the renaturation of hydrophobic, disulfide-bond containing proteins because of the slow chemical conversion of thiols to disulfides and the tendency to aggregate (3). Utilization of a folding system that closely mimics production of native proteins in their natural host organism could prove to be a promising alternative for the efficient renaturation of recombinant proteins. Folding pathways *in vivo* are facilitated by molecular chaperones and folding enzymes such as protein disulfide isomerase (PDI). Chaperones are responsible for correct folding and assembly of the nascent peptides into a mature protein, and the folding process does not involve the formation of covalent bonds. It is believed that chaperones assist in the correct folding of proteins by preventing misfolding and aggregation (3,4).

PDI is a multifunctional enzyme (5) comprised of approximately 500 amino acid residues. There are copious amounts of PDI located in the lumen of the endoplasmic reticulum, the site of thiol-disulfide exchange reactions that are essential for the post-translational formation of disulfide bonds in newly synthesized proteins. PDI concentrations of 0.2–0.5 mM have been estimated (6), levels that are comparable to or in excess of the levels of newly synthesized proteins (7). PDI also exhibits chaperone activity by increasing folding yields and decreasing aggregation (8). For example, PDI displays chaperone activity in the renaturation of denatured D-glyceraldehyde-3-phosphate dehydrogenase (9), and rhodanese (10), two proteins that contain no disulfide bonds and that tend to aggregate in the denatured state.

There are many examples in which PDI catalyzes disulfide bond formation and isomerization (11,12). Most of these studies have been performed on water soluble proteins, such as bovine pancreatic trypsin inhibitor and ribonuclease A. It was found that the disulfide bond formation and exchange rates are increased approximately one hundred to six thousand fold in the presence of PDI. However, there is a lack of systematic studies performed on PDI-catalyzed folding of disulfide-containing hydrophobic proteins. Such an investigation would be useful to determine whether PDI can be exploited to fold therapeutic proteins more efficiently.

Interleukin 2 (IL-2) is a key cytokine involved in the activation of T lymphocytes. It plays a crucial role early in the immune response, and is used for cancer therapy and the treatment of both immunodeficient and autoimmune diseases (13). It also contains structural attributes that make it a good

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ABBREVIATIONS: C125A rhIL-2, Recombinant Human Interleukin 2 (C125A mutation); DTNB, 5,5′-Dithiobis(2-Nitrobenzoic Acid); Gdm.Cl, Guanidium Hydrochloride; GSH, Reduced Glutathione; GSSG, Oxidized Glutathione; GST, Glutathione S-transferase; IPTG, isopropyl-β-D-thiogalacto-pyranoside; MALDI-TOF, Matrix Assisting Laser Desorption Ionization-Time Of Flight; PDI, Protein Disulfide Isomerase; rhIL-2, Recombinant Human Interleukin 2; rhPDI, Recombinant Human Protein Disulfide Isomerase; PBS, Phosphate Buffered Saline; RNase, Ribonuclease; RP-HPLC, Reversed Phased-High Pressure Liquid Chromatography; SDS-PAGE, Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis; TEA, Triethanolamine; TFA, Trifluoroacetic Acid.

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model to study PDI-assisted folding. Native human IL-2 contains a free thiol group at cysteine 125 and a disulfide bond between cysteine 58 and cysteine 105. Recombinant human IL-2 (rhIL-2) has been modified via substitution of alanine for cysteine 125 (C125A rhIL-2), thus eliminating the possibility of incorrect disulfide isomer formation. Methods have been published in which very good yields of correctly folded rhIL-2 can be obtained by the polypeptide in the presence of copper salt (14–16). Addition of 0.1% sodium dodecyl sulfate to the mixture retards aggregation of the protein. However, the protein folding problem for many proteins is not as easily overcome, and incorrect disulfide bond formation and aggregation seriously impede sufficient yields of the target protein. Therefore, we probed an alternate means of folding our hydrophobic, disulfide-bonded model protein, C125A rhIL-2, using PDI.

The aims of this study were to assess if recombinant human PDI (rhPDI) can facilitate C125A rhIL-2 folding from inclusion bodies. Since it is known that the folding conditions affect the folding of substrate protein as well as rhPDI's function as an enzyme, we also investigated the effect of temperature, rhPDI:C125A rhIL-2 molar ratio and reduced glutathione (GSH):oxidized glutathione (GSSG) molar ratio on rhPDI-assisted C125A rhIL-2 folding.

MATERIALS AND METHODS

Human PDI cDNA was cloned in the expression vector pGEX4T-1 (Pharmacia) and the recombinant vector was used to transform JM109 *E. coli* (17) (provided by Robert Freedman, University of Kent at Canterbury). rhPDI was expressed as a glutathione S-transferase (GST) fusion protein in media (10 g tryptone, 5 g yeast extract, 5 g NaCl, 100 mg ampicillin and 2.0 ml 1N NaOH per liter H_2O) at 37°C overnight. The rhPDI-GST fusion proteins were induced by addition of isopropyl-β-D-thiogalacto-pyranoside (IPTG, Sigma Chemical Co.) to a final concentration of 2 mM. Following a 6 hr induction period, the *E. coli* cells were harvested by centrifugation. The cell pellets were suspended and incubated in 50 ml of ice-cold lysis buffer (1X PBS, containing 100 mg lysozyme) for 30 min, followed by 2 min sonication at power level 5 and 70% duty cycle pulse with a VC 600 sonicator and a CV17 probe from Sonics & Materials INC (Danbury, CT). The lysates were centrifuged at 15,000 rpm in a Beckman JA20 rotor for 15 min at 4°C and the pellets were discarded. The supernatant containing rhPDI-fusion proteins were then purified by "glutathione sepharose 4B," a commercial GST fusion protein purification kit from Pharmacia Biotech Inc. (Piscataway, NJ). 1.5 bed volumes of supernatant containing rhPDI-GST were incubated with the glutathione sephrose 4B beads at room temperature for 30 min and then 4°C overnight. The glutathione sepharose beads with bound rhPDI-GST were collected by filtration and washed with 10 bed volumes of PBS containing 1% Triton X-100 three times to remove non-specifically bound proteins. The rhPDI was then cleaved from GST by incubation of the beads with 1 ml of thrombin (50 units of thrombin in 1 ml PBS) at room temperature for 16 hours. The rhPDI was then eluted from the matrix by PBS. Residual thrombin was removed by incubation of 5 ml of the eluent with 0.5 ml *p*-aminobenzamidine agarose beads. rhPDI was stored at −20°C until use.

rhPDI purity was judged by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with silver stain detection. The molecular weight of rhPDI was determined by Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry. For mass spectrometry, rhPDI was first desalted and adjusted to approximately 1 mg/ml using Kwikspin macro ultrafiltration units (30,000 molecular weight cut off) (Pierce, Rockford, IL). rhPDI was then mixed with the matrix (10 mg/ml sinapinic acid in 50:40:10 acetonitrile/water/1.5% trifluoroacetic acid (TFA)) with a 1:1 ratio. 1.3 μl of the mixture was then applied to the target and allowed to air dry. A Voyager Biospectrometry Workstation with Delayed Extraction Technology MALDI-TOF mass spectrometer (Framingham, MA) was used for analysis. The specific activity of rhPDI was assayed by measuring the re-activation of reduced bovine ribonuclease (RNase) according to the protocol from Calbiochem (San Diego, CA).

C125A rhIL-2 inclusion bodies were harvested from *E. coli.* harboring plasmid cDNA containing the C125A rhIL-2 gene (provided by Tom Ciardelli, Boston University) according to the literature (18). Briefly, *E. coli* harboring the plasmid C125A rhIL-2 gene were grown in M9 media containing 1% ampicillin at 37°C to an OD_{590} of 0.275–0.3. C125A rhIL-2 expression was induced overnight by the addition of IPTG to a final concentration of 2 mM. The cells were harvested by centrifugation at 5000 rpm for 10 min. The cell pellets from 1 liter culture were resuspended and incubated in 50 ml sonication buffer (10 mM sodium phosphate, pH = 7.4, 150 mM NaCl, 1 mM EDTA, 1 mg/ml lysozyme) for 30 min followed by 1 min sonication at power level 5 and 70% duty cycle pulse with a VC 600 sonicator and a CV17 probe from Sonics & Materials INC (Danbury, CT). The pellets containing C125A rhIL-2 inclusion bodies were washed with 5% triethanolamine-HCl (TEA), (pH = 8.5) six times, followed by two 4 M urea washes in 5% TEA solution. The inclusion bodies were then lyophilized and kept at 4°C until use.

The purity of C125A rhIL-2 was determined by SDS-PAGE with silver stain detection and by reversed phase HPLC (RP-HPLC). The molecular weight of reduced C125A rhIL-2 was determined by MALDI-TOF mass spectrometry. For mass spectrometry measurements, C125A rhIL-2 inclusion bodies were dissolved into 6 M Gdm.Cl, 100 mM GSH, 350 mM Tris-HCl, (pH = 8.0) to give a concentration of 1.4 mM. C125A rhIL-2 (1.4 mM) was then diluted 100 fold into the matrix (10 mg/ml sinapinic acid solution in 30:60:10 acetonitrile/water/3% TFA). The mass spectrometry experiments for C125A rhIL-2 were the same as for rhPDI.

All other reagents were analytical grade and used as purchased without further purification.

RP-HPLC Method

RP-HPLC was used to resolve denatured, reduced C125A rhIL-2 from native C125A rhIL-2. The HPLC system consisted of two Shimadzu LC-10AD pumps, a SCL-10A system controller, a CR501 Chromatopac integrator, a SPD-10AV UV-VIS detector, a SIL-10A autoinjector, a FRC-10A autofraction collector, and a sample cooler. A Vydac reversed-phase C-8 column (4.6 × 250 mm, 5-μm resin) with a guard column was used for all analytical measurements. The analysis of C125A rhIL-2 was carried out using a linear gradient elution method

consisting of 5% acetonitrile in water and 0.2% TFA as mobile phase A, and 5% water in acetonitrile and 0.2% TFA as mobile phase B. The total flow rate was 0.7 ml/min. The detection wavelength was 280 nm. The injection volume was 50 μL. The gradient started at 68% B, increased to 80% B in 11 min, and then decreased to 68% B in 1 min. The column was allowed to equilibrate for 8 min before the next injection.

SDS-PAGE

SDS-PAGE (19) was used to monitor C125A rhIL-2 aggregation during oxidative folding. Mini polyacrylamide gels were purchased from Novex (San Diego, CA). Protein bands were detected on the 14% polyacrylamide gel with silver stain.

Denaturation of C125A rhIL-2

C125A rhIL-2 inclusion bodies were dissolved in 6 M Gdm.Cl and 100 mM GSH, 350 mM Tris-HCl (pH = 8.0), 20 mM EDTA to give a concentration of 140 μM. The C125A rhIL-2 concentrations were determined by the method of Bradford (20) with BSA as a standard. C125A rhIL-2 was denatured and reduced at room temperature for one hour. The number of free thiol groups per reduced C125A rhIL-2 were determined by using the method of Ellman (21) following purification by a desalting column (Bio-Gel P-6 DG desalting gel, Bio-Rad, CA). The column was equilibrated with 6 M Gdm.Cl (pH = 4.0). The free thiol content of reduced C125A rhIL-2 was estimated by DTNB titration in 0.1 Tris/HCl and 2 mM EDTA (pH = 8.0) containing 6 M Gdm.Cl and the formation of the 2-nitro-5-thiobenzoate dianion was measured at 412 nm.

The Folding of C125A rhIL-2

Folding of C125A rhIL-2 was initiated by addition of 9 volumes of folding buffer to 1 volume of denatured and reduced C125A rhIL-2 at various temperatures. The final concentrations of the components of the folding buffer were: 14 μM C125A rhIL-2, 10 mM GSH, 100 mM Tris-HCl (pH = 7.75), 2 mM EDTA, various concentrations of GSSG, and BSA or rhPDI. At various times, an aliquot of the folding reaction was quenched with 0.05 volumes of 2 M HCl and kept at 4°C until HPLC analysis. Each reaction was repeated three times to generate statistical data. Quantitation of the product was based on the peak areas of the oxidized or reduced species as a percentage of the reduced peak area at the start of the reaction.

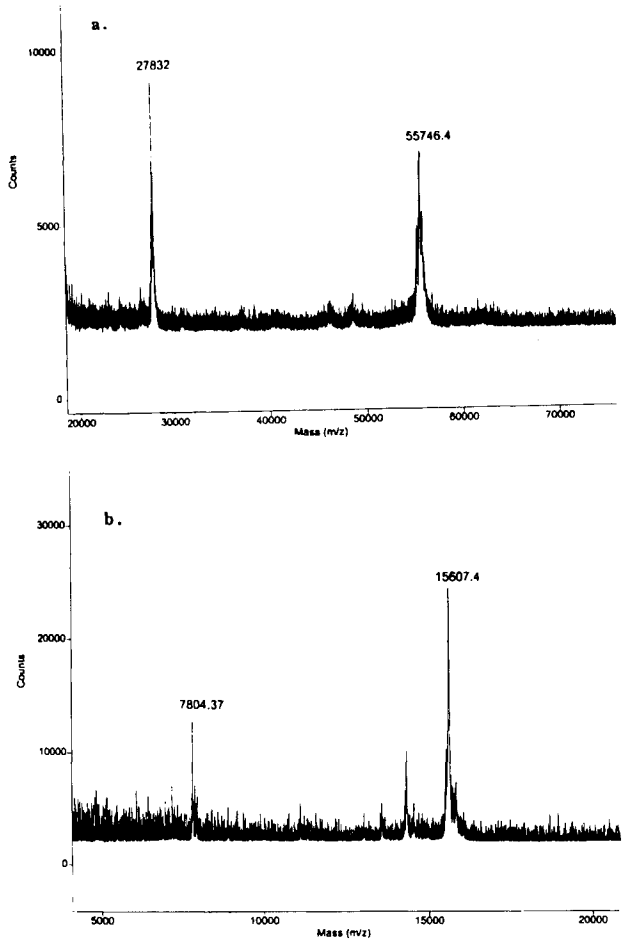


Fig. 1. Mass spectra of rhPDI and C125A rhIL-2. a. Mass spectrum of rhPDI. Mass per cationic charge peaks (m/z^+) corresponding to rhPDI appears at 55,746.4. Double-charged rhPDI shows a peak at 27,832. b. Mass spectrum of C125A rhIL-2. Mass per cationic charge peaks (m/z^+) of reduced, denatured C125A rhIL-2 appears at 15,607.4. Double charged C125A rhIL-2 shows a peak at 7,804.4.

RESULTS AND DISCUSSION

Protein Expression and Characterization

Production of rhPDI yielded 15 mg of protein per liter of broth with a specific activity of 550 unit/mg. rhPDI appeared to be ≥95% pure as judged by SDS-PAGE with silver stain detection. Figure 1a shows the mass spectrum of rhPDI. The molecular weight of rhPDI is 55,746.4. The double-charged ion species of rhPDI is 27,832. Production of C125A rhIL-2 yielded 30 mg of inclusion body protein per liter of broth. The C125A rhIL-2 in inclusion bodies appeared to have ≥95% purity as judged by SDS-PAGE with silver stain detection and by RP-HPLC. Incubation of C125A rhIL-2 inclusion bodies with 6 M Gdm.Cl and 100 mM GSH containing 350 mM Tris-HCl (pH = 8.0), 20 mM EDTA at room temperature for 1 hr completely reduced C125A rhIL-2. Ellman's assay showed that the average free cysteine groups in one mole of reduced, denatured C125A IL-2 are 1.97 ± 0.093 (n = 5). Figure 1b shows the mass spectrum of C125A rhIL-2. The molecular weight of reduced C125A rhIL-2 was 15,607.4. The double-charged ion species of C125A rhIL-2 is 7,804.4.

RP-HPLC Chromatograms of C125A rhIL-2

Figure 2 shows the RP-HPLC chromatograms of C125A rhIL-2 (14 μM) folding in the presence of rhPDI (7 μM). Reduced and oxidized rhIL-2 were resolved on an RP-HPLC system employing a C8 column with an aqueous-acetonitrile gradient. In Figure 2a, the single peak that elutes at 13.9 minutes is reduced C125A rhIL-2. Native C125A rhIL-2 has less hydrophobicity exposed than reduced C125A rhIL-2, so it elutes earlier (9.1 minutes). Figures 2b and 2c show the folding reaction at 2.5 min and 30 min respectively. The peak area of native C125A rhIL-2 increases as the folding reaction proceeds. In addition to the native and reduced C125A rhIL-2 peaks, there

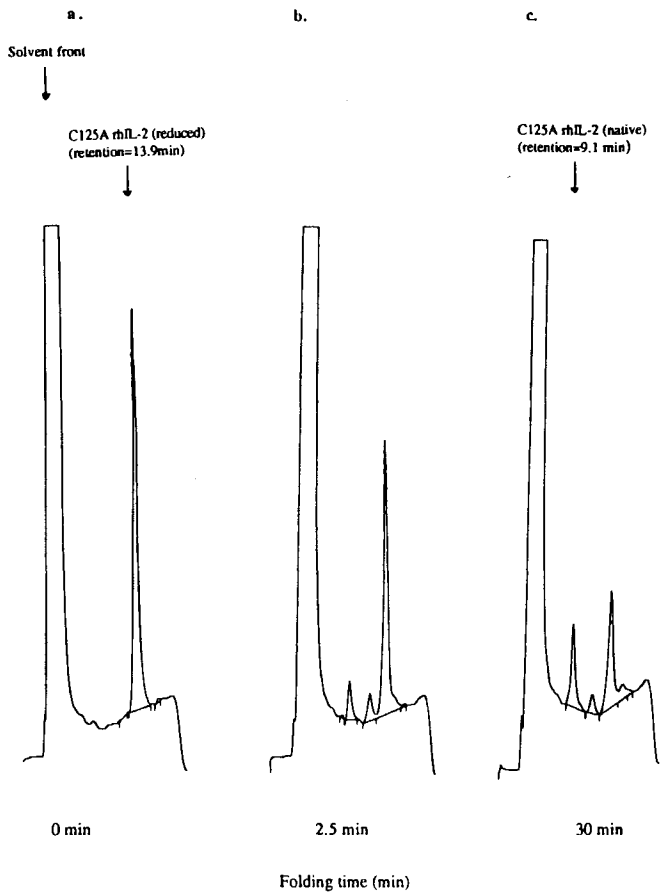


Fig. 2. Reverse phase-HPLC chromatograms for the formation of native C125A rhIL-2 from unfolded C125A rhIL-2. Reverse phase-HPLC chromatograms show folding of C125A rhIL-2 in the presence of 7 μM rhPDI. Chromatogram **a** shows reduced C125A rhIL-2 before the folding reaction, and reduced C125A rhIL-2 elutes as a single peak with a retention time of 13.9 minutes. The native C125A rhIL-2 elutes at 9.1 minutes. Chromatograms **b** and **c** show the reaction mixture after 2.5 min and 30 min reaction times, respectively. The amount of reduced C125A rhIL-2 decreases as the amount of native C125A rhIL-2 increases.

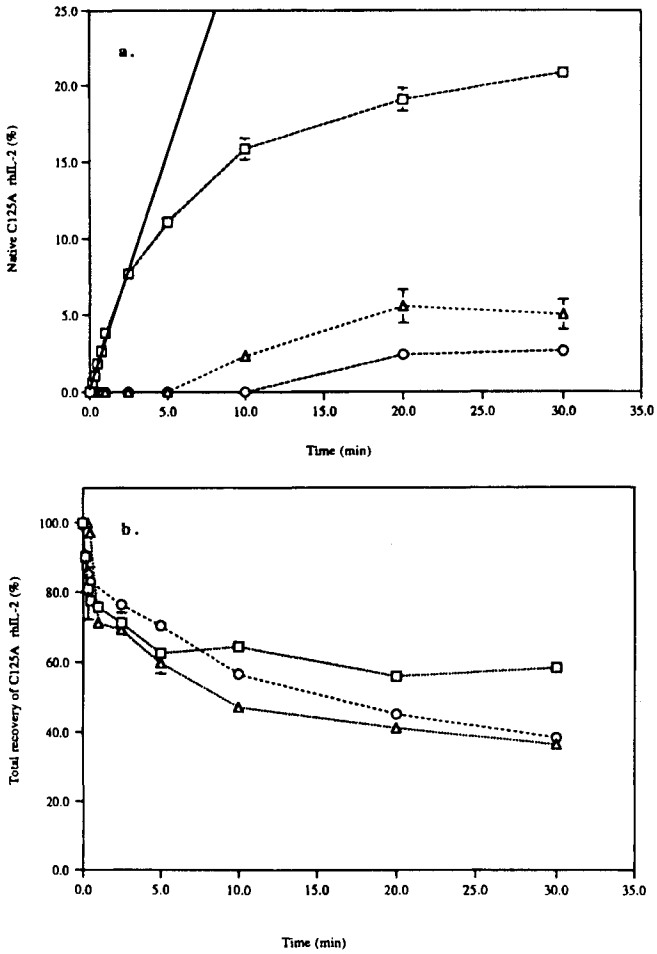


Fig. 3. Time course of folding of C125A rhIL-2. a. The appearance of native C125A rhIL-2 *vs* time. The peak areas for the native species are shown as a percentage of the initial peak areas of reduced C125A rhIL-2. Folding reactions were performed at 23°C. The final concentrations of the components of the folding buffer were: 14 μM C125A rhIL-2, 10 mM GSH, 1.5 mM GSSG, 100 mM Tris-HCl pH = 7.75, 2 mM EDTA, 7 μM PDI (□) or 7 μM BSA (△) or lacked added protein (○). The solid line demonstrates that the formation of native C125A rhIL-2 is linear within 2.5 minutes reaction time (r^2 = 0.989) when 7 μM rhPDI is present. The dashed lines are drawn to guide the eye. b. The total recovery of C125A rhIL-2 *vs* time. The total recovery of C125A rhIL-2 is defined as the peak areas of both native and reduced, unfolded C125A rhIL-2, and are shown as a percentage of the initial peak areas. The control reaction (○) lacked added proteins. The experimental reactions were performed with either 7 μM PDI (□) or 7 μM BSA (△). The lines were drawn to guide the eye. All reactions were performed in triplicate, and no error bar appears where the error is smaller than the size of data point symbol.

are two small peaks in the chromatograms which may be reaction intermediates or side products.

Time Course of Folding of C125A rhIL-2

Figure 3a shows the time course of the C125A rhIL-2 (14 μM) folding reaction in the presence of either rhPDI (7 μM), BSA (7 μM), or no added protein. It shows that under the optimum conditions the formation of native C125A rhIL-2 was boosted by the addition of rhPDI. More than 17% of C125A rhIL-2 folded in the presence of 7 μM rhPDI in 30 minutes, contrasted with 5% C125A rhIL-2 folded in the presence of BSA and 3% yields in the absence of added protein. The folding yield of C125A rhIL-2 in the presence and absence of PDI slowly increased after 30 minutes reaction and reached plateau levels of 24% and 11% at 3 hr respectively. However, considering that the autooxidative reaction of C125A rhIL-2 by air may be involved at later time, we focus our discussion on the first 30 minutes of the folding reaction in this paper. The formation of native C125A rhIL-2 in the presence of rhPDI linearly increased in the first 2.5 minutes of the folding reaction and then slowed down. The formation of native C125A rhIL-2 in the absence of PDI appeared to have a 5- to 10-minute lag time. We attributed this to the detection limit of our RP-HPLC system. The decreased yield of native C125A rhIL-2 with time may be due to several factors. First, rhPDI is inactivated in the folding buffer. rhPDI is very sensitive to inactivation by denaturants, e.g., Gdm.Cl or urea. It was reported that 50% inactivation of PDI was observed in the presence of 0.2 M

Gdm.Cl with complete inactivation at 1 M Gdm.Cl (22), as measured by both conformational change via the intrinsic fluorescence of its five tryptophan residues, and the specific activity in the reactivation of scrambled RNase. It is thought that PDI is most active in its dimeric form and dissociates completely to the protomer in the presence of more than 0.4 M Gdm.Cl (23). In our studies, rhPDI-assisted C125A rhIL-2 folding involves 10 fold dilution of 6 M Gdm.Cl denaturant into folding buffer. The final folding solution therefore contains 0.6 M Gdm.Cl. The rhPDI may be denatured and inactivated while it is exposed to the residual Gdm.Cl in folding buffer, which will result in the gradual decrease of catalytic potency. We found that preincubation of rhPDI in 0.6 M Gdm.Cl for 30 min resulted in significantly lower folding yields (data not shown), which agrees with data from other laboratories (22). In order to minimize the complication of residual Gdm.Cl on the activity of rhPDI, we decreased the Gdm.Cl concentration in the final folding buffer by a greater dilution. However, such an approach results in lower yields of folded C125A rhIL-2, apparently because of increased aggregation of C125A rhIL-2. It was reported that complete removal of denaturant such as Gdm.Cl resulted in the precipitation of reduced IL-2 and the optimum concentration to solubilize reduced IL-2 and to bring about correct folding was in the range of 1.5–3.0 M Gdm.Cl (15).

It is interesting to compare the time course of rhPDI-assisted C125A rhIL-2 folding with the PDI-assisted RNase A folding. RNase A has 4 disulfides; therefore there are 764 possible intramolecular isomer species. In the renaturation of RNase A with a mixture of oxidized and reduced glutathiones, the formation of disulfide bonds is crucial in directing the formation of subsequent native structure (24). It was shown that under optimum conditions, the PDI-assisted oxidative folding of reduced RNase exhibits two phase kinetic behavior: a rapid phase of RNase activation, followed by a slower, steady-state velocity (11). The rate limiting step appeared to be the conversion of scrambled disulfide species to active RNase.

In contrast, C125A rhIL-2 only has one disulfide bond, so reduction or isomerization of other disulfides in the molecule is unlikely to be involved in the rate determining steps. An alternative explanation for the decreased rhPDI-assisted C125A rhIL-2 folding rate is the depletion of C125A rhIL-2 having a suitable conformation for disulfide formation. After the initially reduced, denatured C125A rhIL-2 is diluted into folding buffer, it may spontaneously fold to a metastable conformation with buried cysteine groups, or one in which the cysteines are in unfavorable proximity for the disulfide bond formation. Our experiments showed that rhPDI had no catalytic activity toward the regeneration of native C125A rhIL-2 if rhPDI was added to the folding buffer more than five minutes after reduced C125A rhIL-2 was incubated with GSH/GSSG buffer. The result suggests that the formation of a metastable conformation of reduced C125A rhIL-2 in the folding buffer may inhibit the access of rhPDI. If this conformation forms rapidly and rearranges slowly, it will slow down the rhPDI-assisted folding reaction of C125A rhIL-2. In this respect, C125A rhIL-2 folding is similar to the oxidative folding of β-lactamase. This protein has two cysteines. The native β-lactamase has one disulfide bond which is buried. It was found that the buried cysteines have 500-fold less reactivity than exposed cysteines (25). PDI improves the oxidative folding of β-lactamase by 1.7–3 fold, which is less than the rate of spontaneous β-lactamase unfolding (25).

Figure 3b shows that the total recovery of C125A rhIL-2 (both native C125A rhIL-2 and reduced, denatured C125A rhIL-2 peaks) decrease as the folding reaction proceeded and rhPDI appeared to improve the total recovery of C125A rhIL-2 during the 30 minute reaction. These results suggest that the reduced C125A rhIL-2 forms unstable intermediates first. These intermediates are faced with the alternatives of either folding and assembly to native C125A rhIL-2 by forming intra-molecular disulfide bonds or forming non-native C125A rhIL-2 species. In fact, we found that the loss of reduced, denatured C125A rhIL-2 in the absence of rhPDI obeyed second-order kinetics in the folding reaction (data not shown). In order to determine if the soluble aggregates of C125A rhIL-2 account for the decreased recovery of C125A rhIL-2 during oxidative folding, the solvent front of the RP-HPLC analysis was collected and concentrated. The concentrated fractions were then analyzed by SEC-HPLC, SDS-PAGE and MALDI-TOF mass spectrometry. Our results indicate that the multimeric C125A rhIL-2 eluted at the solvent front in our RP-HPLC system. Multimeric C125A rhIL-2 formation resulted in decreased recovery of native C125A rhIL-2 during oxidative folding. It appeared that non-native C125A rhIL-2 exposed hydrophobic surfaces in aqueous solution and intermolecular hydrophobic interactions drive the formation of C125A rhIL-2 aggregates. The aggregates exposed hydrophilic surfaces, resulting in decreased retention time in the RP-HPLC. These results were consistent with our previous work that showed the formation of dimeric and tetrameric C125A rhIL-2 in the folding reaction both in the presence of rhPDI and absence of rhPDI (21). Based on these results, we thought that the folding of C125A rhIL-2 could be improved in two ways: catalyzing the intramolecular disulfide bond formation so that this pathway kinetically competes with aggregation pathways, or inhibiting the aggregation pathway to favor the formation of native C125A rhIL-2.

In the absence of molecular chaperones and folding catalysts, the folding of disulfide-containing proteins is often slow. PDI catalyzes the intra-molecular disulfide bond formation of substrate proteins so that this pathway kinetically competes with aggregation pathways. In addition to its catalytic capability, PDI also shows chaperone activity (3,8) by nonspecific peptide binding, which causes major conformation changes in polypeptides and prevents incorrect association leading to aggregate formation. It is known that during oxidative protein folding, stabilized intermediate structures form that can bury cysteine thiols, thus inhibiting access of oxidizing agents. If the intermediate structures form rapidly, they will kinetically trap the folding protein in a metastable, incompletely oxidized state (25). In order to complete folding, these kinetically trapped intermediates have to unfold sufficiently to expose sterically hindered cysteine residues to oxidant or they must rearrange to species with accessible cysteines. Weissman and Kim (12) reported that PDI accelerates the rearrangement of kinetically trapped BPTI folding intermediates with buried sulfhydryls by 4000- to 6000-fold compared to the approximately 140-fold rate enhancement observed in PDI-catalyzed folding of RNase (11). Walker and Gilbert (25) provided evidence that PDI unfolds the kinetically trapped intermediate via a noncovalent interaction with substrate proteins. They reasoned that the mechanisms by which PDI catalyzes the oxidative folding of β-lactamase

are the high kinetic reactivity of rhPDI as an oxidant and its ability to unfold kinetically trapped protein by noncovalent interactions (25). Since there is relatively weak binding observed between PDI and peptides or unfolded protein substrates (6,26), a high concentration of PDI is needed to drive the equilibrium of binding between PDI and substrate proteins to unfold kinetically trapped intermediates. In the case of rhPDI-assisted C125A rhIL-2 folding, we used a high concentration of rhPDI in our study to utilize both its catalytic and chaperone functionalities. When reduced, denatured C125A rhIL-2 is diluted into folding buffer, it may rapidly form an intermediate structure in which its cysteine thiols are buried. Such intermediate structures tend to form aggregates, therefore decreasing the folding rate and folding recovery. rhPDI can react rapidly with exposed cysteine groups of C125A rhIL-2 and partially avoid the kinetic traps by increasing the oxidation rate and productive folding. Once kinetically trapped intermediates form, rhPDI then interacts noncovalently with metastable intermediates of C125A rhIL-2 by its chaperone functionalities. The chaperone function stabilizes unfolded intermediate structures and makes the cysteine thiols of C125A rhIL-2 accessible. Therefore, the folding recovery of C125A rhIL-2 is improved. This effect, although small at the PDI concentration employed in an *in vitro* experiment, would be more significant at the ER lumen where PDI concentrations reach 0.5 mM.

PDI is a multifunctional protein. The structural and functional properties of the isolated domains confirm that the intact molecule has a modular structure. It was found that the two thioredoxin-like domains of PDI had virtually all the activity of complete PDI in introducing disulfides into the reduced peptides. However, the thioredoxin-like domains have an apparently lower activity in catalyzing disulfide formation in scrambled peptides needing intramolecular disulfide rearrangement (27). Whether disulfide rearrangement requires unfolding kinetically trapped intermediates before the disulfide exchange occurs is not clear now. However, it is clear that the complete PDI structure is necessary for all of its activity to be demonstrated.

Effect of rhPDI and GSSG Concentration on Folding Yield

As shown in Figure 4, the initial folding rate of C125A rhIL-2 linearly increased when rhPDI concentration increased from 0 to 14 μM. Further increases of rhPDI concentration had only a slight effect on the folding rate of C125A rhIL-2. BSA, which was used as a control, had no effect on the folding rate of C125A rhIL-2 under the same conditions (Figure 4). The rhPDI-assisted C125A rhIL-2 folding rate was also strongly dependent on the redox potential of the folding buffer. The rhPDI-assisted C125A rhIL-2 folding rate increased linearly with increased GSSG concentration up to 3 mM (Figure 5) and then reached a plateau. Further increasing GSSG concentration up 5 mM had no effect on the folding rate of C125A rhIL-2. The effect of GSSG concentration on the folding reaction of C125A rhIL-2 in the absence of rhPDI was also studied as a control. There was no detectable native C125A rhIL-2 formed in one minute. These results indicate that both rhPDI and GSSG participate in rhPDI-assisted folding reactions. It appears that rhPDI assisted-C125A rhIL-2 folding is a multi-step process (Equation 1). Denatured, reduced C125A rhIL-2 reacts with

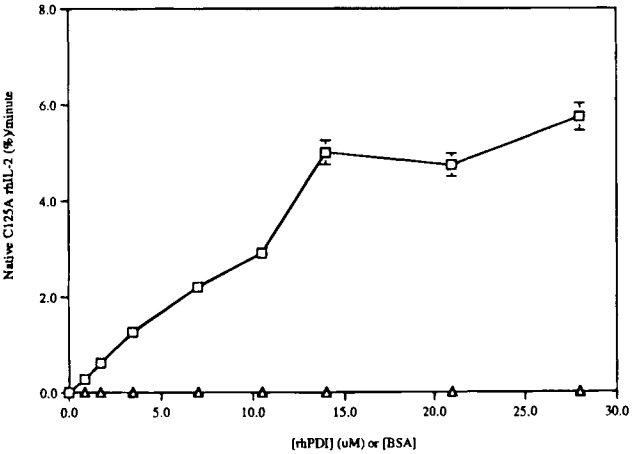


Fig. 4. The effect of rhPDI:C125A rhIL-2 ratio on the initial rate C125A rhIL-2 folding. The initial rate of C125A rhIL-2 folding as a function of rhPDI:C125A rhIL-2 ratio. Folding reactions were performed at 23°C for 1.0 minute. The final concentrations of the components of the folding buffer were: 14 μM C125A rhIL-2, 10 mM GSH, 1.5 mM GSSG, 100 mM Tris-HCl pH = 7.75, 2 mM EDTA, various concentrations of rhPDI (□) or BSA (△). All reactions were performed in triplicate, and no error bar appears where there the error is smaller than the size of data point symbol.

oxidized rhPDI to form a covalent rhPDI-C125A rhIL-2 complex with an intermolecular disulfide bond. The rhPDI-C125A rhIL-2 complex then dissociates to reduced rhPDI and native C125A rhIL-2. The reduced rhPDI is then oxidized by GSSG to generate oxidized rhPDI (Equation 2). Since the oxidative folding of C125A rhIL-2 is dependent on the concentration of rhPDI, it is likely that the second step is rate-limiting. The oxidative folding of C125A rhIL-2 increases with increased

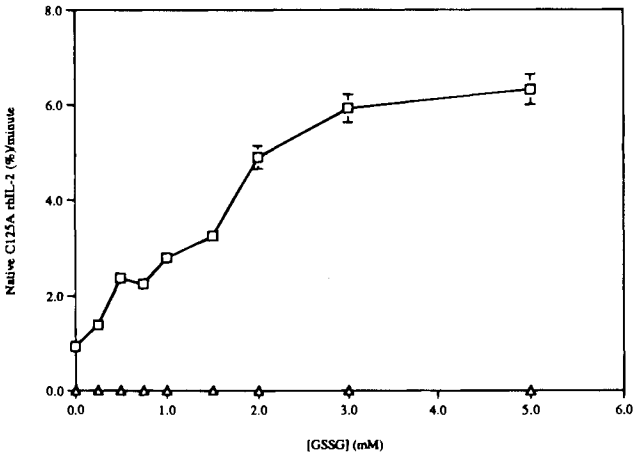
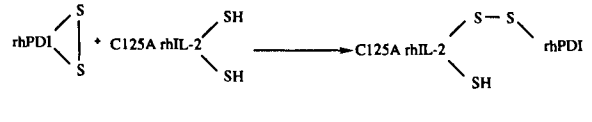
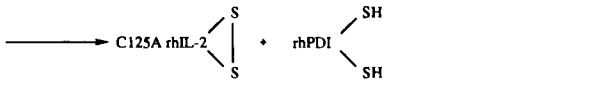


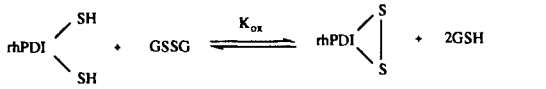
Fig. 5. The effect of GSH:GSSG ratio on the initial rate of C125A rhIL-2 folding in the presence of rhPDI. The data shows the initial rate C125A rhIL-2 folding *vs* GSH:GSSG ratio. Folding reactions were performed at 23°C. The final concentrations of the components of the folding buffer were: 14 μM C125A rhIL-2, 10 mM GSH, various concentrations of GSSG, 100 mM Tris-HCl pH = 7.75, 2 mM EDTA, 7 μM rhPDI (□) or 7 μM BSA (△). The folding reactions were quenched at 1 minute. All reactions were performed in triplicate, and no error bar appears where the error is smaller than the size of data point symbol.

rhPDI concentration until saturation is reached. At the saturated rhPDI concentration, the rate limiting intramolecular disulfide formation of C125A rhIL-2 is a rhPDI-independent step, therefore continuous increase of rhPDI concentration in the folding buffer has no effect on the folding rate. Our laboratory previously provided physical evidence to support this model: we found the rhPDI-C125A rhIL-2 complex accumulates during the folding reaction (28). By SDS-PAGE and Western blot analysis we characterized a covalent associate formed between PDI and C125A rhIL-2 via an intermolecular disulfide bond when folding buffer contained a high ratio of PDI *vs* C125A rhIL-2 concentration (1.5:1). The covalent intermediate between PDI and substrate protein was also demonstrated in the folding reaction of scrambled ribonuclease A *in vitro* and lysozyme *in vivo* (29,30). Further studies are in progress to test this model.



(1)





(2)

$$f_{rhPDIox} = \frac{[rhPDI_{ox}]}{[rhPDI_{total}]} = \frac{K_{ox}}{K_{ox} + [GSH]^2/[GSSG]} \quad (3)$$

In the absence of GSSG, the initial rate of rhPDI-assisted C125A rhIL-2 folding was higher than the rate of C125A rhIL-2 folding without any added rhPDI or GSSG. This result reveals that rhPDI not only catalyzes the oxidative folding of C125A rhIL-2 but also directly provides oxidizing equivalents by utilizing its disulfide bonds as oxidizing agents. In fact, the oxidizing equivalents of PDI form disulfide bonds in RNase in a stoichiometric reaction (11). Since the C125A rhIL-2 folding rate increased linearly with increasing GSSG concentration, the folding rate therefore appears to be dependent on the fraction of oxidized PDI ($f_{rhPDIox}$), as shown in Equation 3. However, the catalytically active rhPDI has to be regenerated from the reduced form, at the expense of oxidizing equivalents from GSSG (Equation 2). K_{ox} is an equilibrium constant of thiol/disulfide exchange between PDI and glutathione, and was previously determined to be 0.06 mM (6). In our studies, the ratio of $[GSH]^2/[GSSG]$ was $\geq$33 mM. Since K_{ox} is much smaller than $[GSH]^2/[GSSG]$, the fraction of oxidized rhPDI which is catalytically active will be a function of $[GSSG]/[GSH]^2$ (Equation 3). The [GSH] was maintained at 10 mM, therefore, the fraction of oxidized rhPDI should be a linear function of [GSSG] concentration until the rhPDI active sites are all oxidized. However, the optimum [GSSG] was 3 mM. Based on the calculation from Equation 3, the fractions of rhPDI active site disulfides were 0.18%. This result indicates that PDI active site disulfides are far from saturation when the folding rate reaches a plateau. It appeared that reduced rhPDI was necessary for the optimal oxidative folding of C125A rhIL-2. One explanation is that the rate-limiting step involves intermolecular disulfide isomerization which needs an optimal $[GSH]^2/[GSSG]$ ratio. Intra-molecular disulfide isomerization does not occur in C125A rhIL-2 folding since C125A rhIL-2 has only two cysteine groups. However, the intermolecular disulfide bond formation is competing with the intramolecular disulfide bond formation. We previously demonstrated covalent aggregates of C125A rhIL-2 (28). It is possible that rhPDI catalyzes the isomerization of intermolecular bonds to native intramolecular disulfide bonds under optimal redox conditions, but we have no evidence for this reaction presently.

In the absence of rhPDI, the dependence of the folding rate of C125A rhIL-2 on the GSSG concentrations of the folding redox buffer was also studied as a control. However, the production of native C125A rhIL-2 in the uncatalyzed reaction was undetectable under our experimental conditions. Therefore, we were unable to determine the effect of GSSG concentration on the uncatalyzed folding reaction. Generally, the establishment of a proper redox environment of the glutathione redox buffer is required to support optimal oxidative folding for both the PDI-catalyzed reaction and the uncatalyzed reaction. For the uncatalyzed folding reaction of RNase, too high a GSH concentration has been suggested to inhibit disulfide bond formation in the intermediates leading to native RNase, and too high a GSSG concentration has been suggested to promote the formation of nonproductive mixed disulfides between glutathione and RNase (6).

Effect of Temperature on the Folding Yield

The initial rate of rhPDI assisted C125A rhIL-2 folding is also dependent on temperature (Figure 6). The folding rate increased with increasing temperature and was maximized at 23°C. The decreased folding yield at high temperatures may be due to increased aggregation of C125A rhIL-2. This result is consistent with the temperature effect of the regeneration of native RNase from its reduced form. In that case, the regenera-

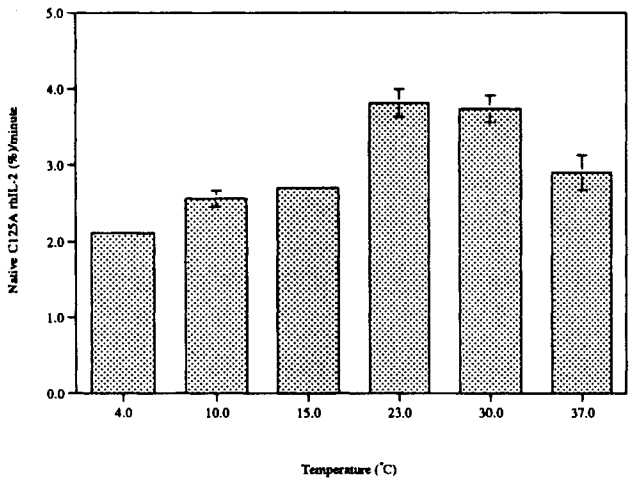


Fig. 6. The effect of temperature on the initial rate of C125A rhIL-2 folding. The initial rate of C125A rhIL-2 folding as a function of temperature. Folding reactions were performed at different temperatures. The final concentrations of the components of the folding buffer were: 14 μM C125A rhIL-2, 10 mM GSH, 1.5 mM GSSG, 100 mM Tris-HCl pH = 7.75, 2 mM EDTA, and 7 μM PDI. The data showed the initial rate of C125A rhIL-2 folding in 1 minute. All reactions were performed in triplicate, and no error bar appears where the error is smaller than the size of data point symbol.

tion of native RNase increased from 15–25°C, then decreased by a factor of 10 when the temperature was increased from 25 to 37°C (24).

Folding of inclusion body proteins *in vitro* is considered to be an extremely difficult task. However it is generally believed that the production of recombinant protein via inclusion bodies is likely to remain an important manufacturing route for some time to come. Using a system that more closely mimics production of a protein in its natural host organisms could prove extremely useful to the large-scale manufacture of recombinant products. Our studies indicate that rhPDI is capable of assisting C125A rhIL-2 (0.2 mg/ml) folding, achieving a folding yield of 65–70% when rhPDI and GSSG concentration were 28 μM and 3 mM respectively, compared with a folding yield of 20% in the absence of rhPDI (data not shown). This is most likely due to the catalysis of thiol/disulfide exchange and the chaperone functionality of rhPDI, which enhance formation of the native C125A rhIL-2 and decrease self-aggregation during folding.

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