Reduction-Reoxidation Cycles Contribute to Catalysis of disulfide Isomerization by Protein-disulfide Isomerase*

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Protein-disulfide isomerase (PDI) catalyzes the formation and isomerization of disulfides during oxidative protein folding. This process can be error-prone in its early stages, and any incorrect disulfides that form must be rearranged to their native configuration. When the second cysteine (CGHC) in the PDI active site is mutated to Ser, the isomerase activity drops by 7–8-fold, and a covalent intermediate with the substrate accumulates. This led to the proposal that the second active site cysteine provides an escape mechanism, preventing PDI from becoming trapped with substrates that isomerize slowly (Walker, K. W., and Gilbert, H. F. (1997) J. Biol. Chem. 272, 8845–8848). Escape also reduces the substrate, and if it is invoked frequently, disulfide isomerization will involve cycles of reduction and reoxidation in preference to intramolecular isomerization of the PDI-bound substrate. Using a gel-shift assay that adds a polyethylene glycol-conjugated maleimide of 5 kDa for each sulfhydryl group, we find that PDI reduction and oxidation are kinetically competent and essential for isomerization. Oxidants inhibit isomerization and oxidize PDI when a redox buffer is not present to maintain the PDI redox state. Reductants also inhibit isomerization as they deplete oxidized PDI. These rapid cycles of PDI oxidation and reduction suggest that PDI catalyzes isomerization by trial and error, reducing disulfides and oxidizing them in a different configuration. Disulfide reduction-reoxidation may set up critical folding intermediates for intramolecular isomerization, or it may serve as the only isomerization mechanism. In the absence of a redox buffer, these steady-state reduction-reoxidation cycles can balance the redox state of PDI and support effective catalysis of disulfide isomerization.

The folding of proteins destined for the secretory pathway occurs in the endoplasmic reticulum where a quality control system ensures that secreted proteins are correctly folded (1), including the correct formation of disulfide bonds. Disulfides that form early in the folding process are often incorrect; cysteines can be mispaired (2), or disulfides can be formed in the wrong temporal order, making it difficult to oxidize buried cysteines (3). To rectify these errors, the incorrect disulfides must be broken and new ones formed in a different configuration.

The endoplasmic reticulum contains folding assistants that help proteins achieve their correct disulfide arrangement. Protein-disulfide isomerase (PDI) is a 55-kDa protein of the endoplasmic reticulum which catalyzes disulfide formation (oxidase activity) as well as the rearrangement of incorrect disulfide pairings (isomerase activity) (4), accelerating both processes without drastically altering the refolding pathway (1). PDI catalyzes the chemical changes but does not appear to guide the process or unfold misfolded substrates actively (5, 6). PDI has two active sites, one near the amino terminus and the other near the carboxyl terminus (7). Each active site contains two cysteines in the sequence WCGHCK which mediate the catalytic activities. The oxidase activity of the enzyme clearly requires the reaction of an oxidized PDI active site with a reduced substrate to introduce a substrate disulfide. Isomerization results in no net redox state change, but a substrate disulfide must be broken to initiate rearrangement. The first step of isomerization involves a reduced PDI active site attacking a substrate disulfide (8). After the initial reaction, two different mechanisms could result in substrate isomerization (Fig. 1). In the first mechanism, the sulfhydryl of the substrate cysteine that was released from a disulfide reacts intramolecularly with a different substrate disulfide, resulting in a different disulfide configuration. The intramolecular rearrangement concludes when a substrate cysteine displaces PDI from the covalent complex, forming another disulfide in the substrate and regenerating reduced PDI for another round of catalysis. In the second model, reduced PDI could simply engage in cycles of reducing substrate disulfides and reoxidizing them in a different orientation, much in the same way that the ATP-dependent chaperonin, GroEL/ES provides a folding substrate with numerous attempts at reaching the native state through cycles of substrate binding and release (9).

To account for the observation that PDI active sites lacking the carboxyl cysteine (CGHSI) are deficient in catalyzing disulfide isomerization unless supported by a glutathione redox buffer, Walker and Gilbert (10) proposed the scanning and escape model for PDI-assisted disulfide isomerization of scrambled ribonuclease. In this model, the first (more amino-terminal) active site cysteine of PDI initiates a disulfide rearrangement by attacking a reactive disulfide within the substrate and forming a covalent complex (scanning). The resolution of this complex depends on the relative rates of an intramolecular isomerization pathway versus the escape of PDI from the covalent complex using the second active site cysteine (Fig. 1). By

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1 The abbreviations used are: PDI, protein-disulfide isomerase; DTT, dithiothreitol; ImPDI, internal mutant PDI; Mal-PEG, a conjugate of polymeric polyethylene glycol and maleimide; RNase A, bovine pancreatic ribonuclease; sRNase, scrambled RNase.
**Reduction-Reoxidation Cycles**

**Fig. 1. Mechanism of PDI-catalyzed disulfide isomerization.** Two alternative pathways can accomplish disulfide isomerization. The top (blue) pathway represents an intramolecular rearrangement of the substrate while bound to PDI as a disulfide. The bottom (tan) pathway represents cycles of disulfide reduction and reoxidation in a different orientation. GSH (red) inhibits the reduction-reoxidation pathway by depleting oxidized PDI, which is required to reoxidize the reduced substrate.

The scanning and escape model predicts that both oxidized and reduced PDI are necessary for the efficient catalysis of isomerization, even when there is no net disulfide reduction or oxidation. We have monitored the redox state of PDI during steady-state disulfide isomerization and found that both reduced and oxidized PDI are essential to observe net isomerization. Rapid reduction-reoxidation cycles accompany the isomerization of sRNase. They are kinetically competitive to account for substrate isomerization, and intramolecular substrate isomerization is a relatively rare event during the PDI-assisted isomerization of sRNase A. These reduction-reoxidation cycles can also balance the redox state of PDI in a nonequilibrium steady state because of the relatively slow reaction between PDI and a glutathione redox buffer.

**EXPERIMENTAL PROCEDURES**

**Materials**—Ribonuclease A, glutathione disulfide (GSSG), glutathione (GSH), cCMP, DTT, 5,5'-dithiobis(2-nitrobenzoic acid), glutathione reductase, and NADPH were obtained from Sigma. Mal-PEG was obtained from Shearwater Corporation (Huntsville, AL). PDI was purified as described (11). The concentration was determined by absorbance at 290 nm using an E of 0.15 of 0.94 (mg/ml)-1 cm-1 (12). Mutation of the two noncatalytic cysteines of PDI was performed using the QuikChange® Multi Site-directed Mutagenesis kit purchased from Stratagene. The mutations were verified by dyeoxy sequencing. The production and isolation of other mutants have been described previously (13). Scrambled ribonuclease was prepared by the method of Hillson et al. (14) with minor changes.

**Assay of RNase Refolding**—PDI activity was measured by observing the activity caused by the formation of native RNase using cCMP as a substrate (12, 15). The cCMP at an initial concentration of 4.5 mM was monitored continuously at 296 nm using a Δε of 0.19 mM−1 cm−1. The active RNase concentration at any time was calculated from the first derivative of the absorbance versus the time curve. The assay was performed at pH 8.0, 230 °C in 0.1 M Tris-HCl containing various components of a glutathione redox buffer and PDI as described in the figure legends. The reaction was initiated by the addition of sRNase.

In experiments where it was necessary to exclude GSSG, the rate of refolding of sRNase was measured in the presence of 0.75 unit/ml glutathione reductase and 0.27 mM NADPH. Assays were performed in 100 mM Tris-HCl, 2 mM EDTA, pH 8.0.

**Partitioning between Reduction and Isomerization**—At higher concentrations of GSH, the background oxidation of NADPH caused absorbance changes at 296 nm which interfered with continuously monitoring the cCMP concentration over the time course of native RNase formation. Consequently, we monitored the partitioning between sRNase reduction and isomerization to native RNase. The rate of reduction of 27 μM sRNase was determined at various concentrations of GSH ranging from 0 to 7 mM. All assays were done in the presence of 0.75 unit/ml glutathione reductase and 0.27 mM NADPH to reduce any GSSG present. The PDI concentration was as indicated in the figure legends. Assays were conducted in a buffer of 90 mM Tris-HCl, 1.8 mM EDTA, pH 8.0, at 25 °C. The rates of reduction were determined from the slope of absorbance at 340 nm versus time using an extinction coefficient of 6.23 mM−1 cm−1.

To measure the partitioning between sRNase reduction and refolding, the amount of native RNase was determined by measuring its enzymatic activity after an overnight incubation of sRNase with PDI, various concentrations of GSH, and 0.75 unit/ml of glutathione reductase and 0.27 mM NADPH. After overnight incubation at room temperature, cCMP was added to a concentration of 4.5 mM, and the initial rate of hydrolysis was measured. A small background rate of NADPH oxidation caused by GSH oxidation by air was subtracted from the observed rate.

**Preparation of Reduced and Oxidized PDI**—Reduced PDI was prepared by incubating PDI with 0.5 mM DTT overnight at room temperature. The DTT was removed by centrifugal gel filtration over Sephadex G-50 coarse from Amersham Biosciences. Oxidized PDI was prepared by incubating PDI with 10 mM GSSG overnight at room temperature. The GSSG was removed by centrifugal gel filtration over Sephadex G-50 coarse from Amersham Biosciences. Controls showed no significant carryover of DTT or GSSG during gel filtration.

**Gel-shift Assays of the PDI Redox State**—To capture the redox state of PDI during steady-state turnover or in equilibrium with a glutathione-redox buffer, the reaction mixture was quenched at the appropriate time by adding an equal volume of ice-cold 40% trichloroacetic acid to precipitate the proteins and stop further thiol/disulfide exchange. After a 1-h incubation on ice, the precipitated proteins were isolated by centrifugation, and the pellets were washed twice with acetone to remove the trichloroacetic acid. The pellet was dissolved in 30 μl of nonreducing 2× SDS sample buffer and split into two equal portions. One portion immediately received 5 mM Mal-PEG (final concentration), and the other received an equivalent amount of buffer only. This provides an internal recovery control for each sample. Controls in which the trichloroacetic acid pellet was dissolved directly into Mal-PEG showed that the short time before the addition of Mal-PEG to the split samples did not result in any redox changes. The unbound Mal-PEG in the sample buffer resulted in distortion of the protein migration so it was removed by dialysis (1 h at room temperature) of the split samples against sample buffer using 10,000 MWCO microdialysis devices from Pierce. SDS-PAGE was performed on a nonreducing 4–20% Tris-HCl polyacrylamide gel. Protein bands were visualized by staining with Coomassie Blue, or the proteins were transferred to nitrocellulose and detected using a monoclonal anti-PDI antibody (Stress-Gen) or a polyclonal anti-PDI antibody.

**RESULTS**

**Substrate Inhibition**—One distinction between an intramolecular mechanism of disulfide rearrangement and a mechanism involving cycles of substrate reduction and reoxidation lies in the consequences of releasing a reduced substrate and forming a molecule of oxidized PDI. If reductive escape happens frequently, increasing the concentration of an oxidized
substrate such as sRNase would tend to oxidize more and more of the PDI. As the concentration of sRNase rises, it should also become more difficult for this oxidized PDI to locate an appropriately reduced substrate to complete the catalytic cycle. A large excess of fully oxidized substrate should compete with the much lower concentration of partially reduced substrate for binding to oxidized PDI. Consequently, substrate inhibition at high concentrations of sRNase would be expected if reductive escape is a frequent phenomenon, but simple saturation behavior would be characteristic of an exclusively intramolecular mechanism.

When the isomerization of sRNase (four disulfides) is initiated by the addition of reduced PDI, isomerization proceeds rapidly when the concentration of sRNase is low (8 μM). Under these conditions, a glutathione redox buffer has little effect on the isomerization rate (Fig. 2), suggesting that there is no large imbalance in the PDI redox state. However, when the substrate concentration is increased to 32 μM, the initial velocity of native RNase formation actually falls 7-fold (Fig. 2), even though the $K_n$ for this substrate is high (40 μM), and the rate would have been expected to increase by almost 4-fold. If the excess substrate is preventing the oxidized PDI that is formed from locating a reduced substrate, adding more oxidized PDI should increase the rate of RNase oxidation and help restore the isomerization activity by increasing the rate of substrate oxidation and by generating more reduced PDI in the process. Adding oxidized PDI to the assay, along with reduced PDI, does increase the isomerase activity, but the increase is still significantly less than that produced by adding a redox buffer (Fig. 2).

**PDI Redox State**—The depletion of reduced PDI during the early stages of the reaction could also compromise initiating new rounds of substrate reduction. To visualize the redox state of PDI during steady-state turnover, a gel-shift assay was developed to detect the number of available sulfhydryl groups present on PDI under turnover conditions. The basis of the assay is the modification of the available PDI sulfhydryl groups by a PEG-conjugated maleimide (Mal-PEG) with an average molecular weight of 5,000 (16). Because disulfides are not maleimide-reactive, only reduced PDI molecules show a shift. PDI normally has two cysteines outside the active site (in the b' domain). They do not contribute to catalysis (17), but their presence creates ambiguity in the site(s) of modification. Consequently, the initial experiments were performed with a mutant PDI (internal mutant, ImPDI) in which the two internal, nonactive site cysteines are mutated to serines. This restricts the location of the modification by Mal-PEG to the active site cysteines. The mutation of the internal cysteines to serine has no effect on the catalytic activity compared with wild-type PDI (data not shown).

The ability of Mal-PEG to trap the cysteines of reduced PDI and capture the PDI redox state accurately was examined by incubating ImPDI with glutathione redox buffers designed to set the active site redox state at various extents of reduction. It is essential to quench redox changes rapidly and to remove small molecule thiols before trapping the free cysteines with Mal-PEG. Consequently, after equilibration, the PDI was precipitated with cold trichloroacetic acid, washed extensively with acetone to remove traces of trichloroacetic acid, and the precipitates were dissolved under denaturing conditions in the presence of Mal-PEG. If Mal-PEG is added directly to the isomerization reaction mixture without trichloroacetic acid precipitation, the competing reactions of PDI disulfide reduction by the GSH and the modification of the PDI thiols with Mal-PEG shift the PDI redox state toward more reduced PDI (data not shown). As the redox buffer is varied, individual bands corresponding to zero, one, two, three, and four Mal-PEG shifts are observed (Fig. 3). The most predominant bands are at zero, two, and four SH groups. Bands visible at one and three bands to disappear but at the risk of reaction at residues other than cysteine. A molecular mass shift of ~15 kDa/Mal-PEG addition is observed (Fig. 3), consistent with the addition of a bulky PEG molecule that is highly solvated and interacts weakly with SDS. The presence of two active sites means that a shift of one or two SH groups identifies a molecule where one of the two active sites is oxidized, whereas a shift of three or four SH groups indicates a completely reduced PDI.

**Fig. 2.** Substrate inhibition at high concentrations of sRNase. Assays for the formation of native RNase were performed in the absence and presence of a glutathione redox buffer (1 mM GSH, 0.2 mM GSSG) at pH 8.0, 0.1 M Tris-HCl, 25 °C. The sRNase concentration was either 8 or 32 μM, as indicated. Reactions were initiated by the addition of 3.5 μM reduced, gel-filtered PDI. In experiments with PDIox, oxidized PDI was added after gel filtration to a final concentration of 3.5 μM. The values are the averages of three or more replicates under each condition, and error bars represent the S.D.

**Fig. 3.** Gel-shift assay of PDI redox state. ImPDI was equilibrated with various glutathione redox buffers at pH 8.0 (0.1 M Tris-HCl, 25 °C), and the free sulfhydryl groups were captured by modification with Mal-PEG alkylation as described under “Experimental Procedures.” Lane 1, 100 μM 5,5'-dithiobis(2-nitrobenzoic acid); lane 2, 0.5 mM GSH and 1 mM GSSG; lane 3, 1 mM GSH and 1 mM GSSG; lane 4, 1.5 mM GSH and 1 mM GSSG; lane 5, 100 μM DTT. The average redox potential for the PDI active sites was 0.7 mM ± 0.2 mM (n = 9).
A glutathione redox buffer (1 mM GSH, 0.2 mM GSSG) also initiates by adding reduced, gel-filtered PDI, 3.5 μM/H11006 increases the amount of PDI in its reduced form (74% formation catalyzed by wild-type PDI (Fig. 4). Confirm that the indicated bands contain PDI.

4°/H11006 contributes to the inhibition by high concentrations of the substrate reduction. At even higher GSH concentrations (Fig. 5), without a requirement for GSSG. Only small amounts of GSSG, the GSSG would inhibit isomerization. To eliminate even small amounts of GSSG, the GSSG was rapidly recycled to GSH using NADPH and glutathione reductase. Under these conditions, GSH by itself can relieve the substrate inhibition (Fig. 5), without a requirement for GSSG.

The two independent active sites have similar redox potentials and are expected to titrate together. When the active sites are half-oxidized, a statistical distribution of redox states would generate 25% unshifted PDI (both sites oxidized), 25% fully shifted PDI (both sites reduced), and 50% shifted by two modifications (one site oxidized, one site reduced). From integrating the band intensities and calculating the fraction of the active sites that were reduced, the average redox potential

\[ K_n = \frac{[PDI_{red}][GSH]}{[PDI_{ox}][GSSG]} \quad (Eq. 1) \]

of the two sites at pH 8.0 is 0.7 ± 0.2 mM (n = 9), comparable with the values of 1.3 mM (at pH 7.4) reported by Darby and Creighton (18) and 3 mM (at pH 7) found by Lundstrom and Holmgren (19). Allowing for variation of the equilibrium constant with pH, the trapping procedure accurately captures the PDI redox state.

Using this gel-shift assay, the redox state of PDI was examined during sRNase isomerization. Reduced ImPDI was incubated for a short time (1 min) with high or low concentrations of sRNase in the presence or absence of a glutathione redox buffer (Fig. 4A). As predicted, reduced PDI was oxidized more extensively by 40 μM sRNase in the absence of a redox buffer, but the presence of a redox buffer helps maintain a higher concentration of reduced PDI without eliminating the oxidized PDI. In the absence of a glutathione redox buffer, changing the substrate concentration from 10 to 40 μM shifts the PDI redox state from 48 ± 4% reduced (n = 4) to 28 ± 4% reduced (n = 4). A glutathione redox buffer (1 mM GSH, 0.2 mM GSSG) also increases the amount of PDI in its reduced form (74 ± 4%, n = 4), suggesting that depletion of the amount of reduced PDI contributes to the inhibition by high concentrations of the sRNase substrate. Control experiments without PDI verify that sRNase does not shift significantly under these conditions so that sRNase multimers are distinguishable from PDI (Fig. 4A). Western blots to visualize only the PDI (data not shown) confirm that the indicated bands contain PDI.

The Mal-PEG assay was also performed during isomerization catalyzed by wild-type PDI (Fig. 4B) with comparable results. Oxidized PDI shifts ~30 kDa upon the addition of Mal-PEG-MAL, suggesting that the two internal, nonactive site cysteines are still reduced (Fig. 4B). PDI in the completely reduced control shifts the equivalent of six Mal-PEG additions, as expected. As with ImPDI, the active site cysteines become more oxidized as the sRNase concentration increases.

Role of the Redox Buffer—For a reduction-reoxidation mechanism of isomerization, both oxidized and reduced PDI would have to participate in the reaction. By contrast, an intramolecular isomerization would only require reduced PDI. If oxidized PDI is necessary to complete a redox cycle during isomerization, one might expect that eliminating the oxidant, GSSG, from the redox buffer would inhibit isomerization. To eliminate even small amounts of GSSG, the GSSG was rapidly recycled to GSH using NADPH and glutathione reductase. Under these conditions, GSH by itself can relieve the substrate inhibition (Fig. 5), without a requirement for GSSG.

Although GSH with an NADPH trap will completely reduce PDI in the absence of substrate, low concentrations of GSH (<0.5 mM) are not sufficient to deplete the steady-state concentration of oxidized PDI in the presence of high concentrations of sRNase (Fig. 6). Thus, the reduction of PDI by GSH must be slower than the formation of oxidized PDI through substrate reduction. At even higher GSH concentrations (>0.5 mM) a substantial fraction of the PDI becomes reduced.

A technical limitation of the isomerization assay precludes continuously monitoring the RNase-catalyzed hydrolysis of cCMP at GSH concentrations >0.5 mM. The background oxidation of NADPH as the reduction of the sRNase substrate produces absorbance changes at 296 nm which are too large to permit an accurate assessment of the isomerization rate during a continuous assay. Although sRNase is reduced by GSH and NADPH, native RNase is sufficiently stable that once formed, it is resistant to reduction by the highest GSH used (data not shown). The partitioning between reduction and isomerization will depend on the ratio of the velocities of the two alternative pathways. By measuring the velocity of sRNase reduction from...
0.27 mM NADPH and 0.75 unit/ml glutathione reductase were included to remove traces of GSSG that might be formed during the reaction.

To determine the effect of increasing GSH concentration (Fig. 6A), the isomerization reaction will be given by

\[ v_{\text{iso}} = \frac{v_{\text{red}}F_{\text{iso}}}{1 - F_{\text{iso}}} \]  

(Eq. 2)

where \( v_{\text{iso}} \) is the velocity of isomerization, \( v_{\text{red}} \) is the velocity of reduction, and \( F_{\text{iso}} \) is the fraction of the total RNase that partitions to native RNase through the isomerization pathway. To account for the observed partitioning between reduction and isomerization, the isomerization rate must actually decrease with increasing GSH concentration (Fig. 6C). The PDI redox state is also changing over this range of GSH concentrations from almost fully oxidized to almost fully reduced (Fig. 6D), showing that as the amount of oxidized PDI is depleted further and further, the isomerization rate slows down. Both oxidized and reduced PDI are required to effect the isomerization of sRNase.

**DISCUSSION**

Two mechanisms can be envisioned for the PDI-catalyzed disulfide isomerization of sRNase (Fig. 1). In the intramolecular rearrangement mechanism, a free thiol is generated in the misoxidized substrate by attack of the more amino-terminal thiolate of the PDI active site. The free thiol of the substrate would then be required to attack another disulfide of the substrate, leading eventually to rearrangement and release of the substrate by reforming a different disulfide and expelling PDI. There is precedence for such a rearrangement in the uncatalyzed, intramolecular rearrangement of folding intermediates of bovine pancreatic trypsin inhibitor (20).

The alternative is that disulfide isomerization results from cycles of reduction and oxidation, simply trying again and again until isomerization leads to a product that is resistant to further rearrangement (10). Walker and Gilbert (10) suggested that the loss of activity resulting from mutation of the second (more carboxyl-terminal) cysteine in either active site could be most easily accomplished by a reduction-reoxidation mechanism because this cysteine does not participate in the intramolecular isomerization pathway. The timing of the intramolecular clock provided by the second active site cysteine will govern the partitioning between intramolecular isomerization and reduction-reoxidation pathways. If intramolecular substrate isomerization takes too long, the second active site cysteine will initiate escape and reduce the substrate. Using equilibrium and rate constants for the equilibration of the PDI active site with glutathione, Darby and Creighton (18) estimated the half-time for the intramolecular expulsion of glutathione to be ~50 ms, although the rate constant could be different for protein substrates. Whether or not reductive escape is an important feature of the mechanism depends on how frequently it occurs during substrate turnover and whether or not reoxidation of the substrate and catalyst are essential during catalysis of isomerization.

Substrate reduction and oxidation by PDI are both kinetically competent to participate in a reduction-reoxidation mechanism of isomerization. When reduced PDI and sRNase (40 μM) are mixed, the redox state changes are finished within 1 min (\( k_{\text{obs}} \), for reducing sRNase is >8 μm substrate reduced/min/μM PDI) (Fig. 6A), and at high substrate, most of the PDI is quickly converted to its catalytic form (Fig. 6A). The oxidation of reduced substrate by PDI is also fast. Lyles and Gilbert (15) found that a catalytic amount of PDI will convert a reduced RNase substrate to an oxidized RNase within 1 min after 0.1 mM GSSG is added. This places a lower limit of >26 μM substrate formed/min/μM PDI on the rate constant for oxidation of a reduced substrate by PDI. Because the turnover number for sRNase isomerization is 1 μM native RNase formed/min/μM PDI, both substrate oxidation and substrate reduction are sufficiently fast that they would be catalytically competent to participate in an isomerization mechanism composed of reduction-reoxidation cycles.

A reduction-reoxidation mechanism also predicts that high concentrations of an oxidized substrate should inhibit the isomerization reaction by competing with the low concentration of reduced substrate for binding to oxidized PDI. The accumulation of oxidized PDI resulting from a rapid reaction of reduced PDI with the oxidized substrate is verified by a gel-shift assay designed to monitor the redox state of PDI during sub-
strate turnover under inhibiting conditions (Fig. 4). The substrate inhibition is decreased by either dropping the concentration of the substrate (Fig. 4), adding oxidized PDI (Fig. 2), or by making more reduced PDI available by adding GSH (Figs. 5 and 6). These diverse effects on the rate of isomerization are more easily accounted for by a reduction-reoxidation mechanism than by intramolecular isomerization (Fig. 1).

Increasing concentrations of GSH (in the presence of an NADPH/glutathione trap to remove GSSG), initially stimulate isomerization as they convert some of the PDI active site back into the reduced state (Fig. 6), but at higher concentrations significant inhibition is observed concomitant with the loss of oxidized PDI during steady-state turnover. A simple intramolecular isomerization mechanism would predict that there should be no requirement for oxidized PDI and that increasing the extent of PDI reduction should only stimulate isomerization, not inhibit it.

A reduction-reoxidation cycle also requires that the PDI active site disulfide be able to survive long enough to reoxidize the reduced substrate. In the presence of GSH and NADPH, redox equilibrium would predict complete reduction of the PDI active site. However, under steady-state conditions, the rapid reduction of the substrate by PDI and the relatively slow reaction of the PDI active site with GSH result in a significant steady-state accumulation of oxidized PDI, sufficient to support the reoxidation of the reduced substrate. The rate constant for the reduction of the PDI active site by 1 mM GSH is 12 min⁻¹ (22), suggesting that the survival of the oxidized PDI active site during steady-state isomerization is caused by the relatively slow reduction of PDI by GSH and the rapid oxidation of protein substrates. At higher GSH, the rate of active site reduction by GSH becomes competitive with protein substrate oxidation, and the rate of isomerization falls.

The observation of rapid cycles of reduction and reoxidation does not exclude the occurrence of intramolecular isomerization reactions during the formation of native RNase. In fact, the reduction-reoxidation cycles may serve only to set up the formation of a critical intermediate during sRNase refolding, and the rate of isomerization falls.

The occurrence of cycles of reduction and oxidation may also have implications toward the mechanism of disulfide formation in vivo. Previously we reported that the PDI redox state could be maintained in a steady state that was effective for catalysis of disulfide isomerization by using a sulphydryl oxidase to catalyze oxidation of a reduced RNase substrate and GSH to maintain PDI in a reduced state (24). The GSH was required to offset the oxidation of the PDI active site by either the sulphydryl oxidase or through oxidation of the reduced substrate. Thus, the PDI redox state can be maintained by a nonequilibrium, steady-state balance between the kinetics of transfer of oxidizing and reducing equivalents to the substrate. Frand and Kaiser (25) along with Pollard et al. (26) have found that PDI is oxidized in the yeast endoplasmic reticulum by an oxidase, Ero1p. Interestingly, a mutant allele of this gene is suppressed by a mutation in the GSH1 gene (21), responsible for the biosynthesis of glutathione, suggesting that GSH may be used to balance the oxidative activity of Ero1p.

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REFERENCES
1. Helenius, A. (2001) Phil. Trans. R. Soc. Lond. B Biol. Sci. 356, 147–150
2. Rothwarf, D. M., Li, Y. J., and Scheraga, H. A. (1998) Biochemistry 37, 3760–3766
3. Creighton, T. E., and Goldenberg, D. P. (1984) J. Mol. Biol. 179, 497–526
4. Gilbert, H. F. (1998) Methods Enzymol. 290, 26–50
5. Huth, J. R., Perini, F., Leckridge, O., Bedows, E., and Ruddon, R. W. (1993) J. Biol. Chem. 268, 16472–16482
6. Weissman, J. S., and Kim, P. S. (1993) Nature 365, 185–188
7. Edman, J. C., Ellis, L., Blacher, R. W., Roth, R. A., and Rutter, W. J. (1985) Nature 317, 267–270
8. Lamb, N., and Freedman, R. B. (1988) Biochem. J. 213, 235–243
9. Todd, M. J., Lorimer, G. H., and Thirumalai, D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4035–4035
10. Walker, K. W., and Gilbert, H. F. (1997) J. Biol. Chem. 272, 8845–8848
11. Gilbert, H. F., Kruzel, M. L., Lyles, M. M., and Harper, J. W. (1991) Protein Expr. Purif. 2, 194–198
12. Lyles, M. M., and Gilbert, H. F. (1991) Biochemistry 30, 619–619
13. Walker, K. W., Lyles, M. M., and Gilbert, H. F. (1996) Biochemistry 35, 1572–1580
14. Hilleman, D. A., Lamb, N., and Freedman, R. B. (1984) Methods Enzymol. 107, 281–294
15. Lyles, M. M., and Gilbert, H. F. (1991) Biochemistry 30, 619–625
16. Wu, H. H., Thomas, J. A., and Memand, J. (2000) Biochem. J. 351, 87–93
17. Lyles, M. M., and Gilbert, H. F. (1994) J. Biol. Chem. 269, 30946–30952
18. Darby, N. J., and Creighton, T. E. (1995) Biochemistry 34, 16770–16780
19. Lundstrom, J., and Holmgren, A. (1993) Biochemistry 32, 6649–6655
20. Creighton, T. E., Darby, N. J., and Kemmink, J. (1996) PASEF J. 16, 110–118
21. Cuozzo, J. W., and Kaiser, C. A. (1999) Nat. Cell Biol. 1, 130–135
22. Gilbert, H. F. (1989) Biochemistry 28, 7363–7365
23. Shin, H. C., Song, M. C., and Scheraga, H. A. (2002) FEBS Lett. 521, 77–80
24. Hooper, K. L., Sheasly, S. L., Gilbert, H. F., and Thorpe, C. (1999) J. Biol. Chem. 274, 22147–22150
25. Frand, A. R., and Kaiser, C. A. (1998) Mol. Cell 1, 161–170
26. Pollard, M. G., Travers, K. J., and Weissman, J. S. (1998) Mol. Cell 1, 171–182