Effect of Glutaredoxin and Protein Disulfide Isomerase on the Glutathione-Dependent Folding of Ribonuclease A†

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ABSTRACT: Protein folding, associated with oxidation and isomerization of disulfide bonds, was studied using reduced and denatured RNase A (rd-RNase A) and mixed disulfide between glutathione and reduced RNase A derivative (GS-RNase A) as starting materials. Folding was initiated by addition of free glutathione (GSH + GSSG) and was monitored by electrospray mass spectrometry (ESMS) time-course analysis and recovery of the native catalytic activity. The ESMS analysis permitted both the identification and quantitation of the population of intermediates present during the refolding process. Refolding of rd-RNase A and GS-RNase A was also performed in the presence of glutaredoxin (Grx) and/or protein disulfide isomerase (PDI). All the analyses indicate a pathway of sequential reactions in the formation of native RNase A. First, the reduced protein reacts with a single glutathione molecule to form a mixed disulfide which then evolves to an intramolecular S−S bond via thiol−disulfide exchange. Only at this stage, the intermediate containing one intramolecular S−S reacts with a further glutathione molecule, reiterating the process. An analogous mechanism occurs in the refolding of GS-RNase A. The structural analysis of the intermediates formed during the refolding of RNase A showed for the first time that Grx is actually able to catalyze both formation and reduction of mixed disulfides involving glutatione. In both refolding processes, starting from either rd-RNase A or GS-RNase A, Grx displays a significant catalysis at the early stages of the process. Addition of PDI led to a net catalysis of the entire process without appearing to alter the refolding pathway. In the presence of both Grx and PDI, the two enzymes showed a synergistic activity either starting from rd-RNase A, as previously reported [Lundström, J., and Holmgren, A. (1995) *J. Biol. Chem.* 270, 7822−7828], or starting from GS-RNase A. Present data suggest that the synergistic effect can be explained assuming that Grx actually facilitates PDI action by catalyzing formation or reduction of mixed disulfides. The mixed disulfides are then rapidly converted into intramolecular disulfides in the presence of PDI. These steps are repeated sequentially throughout the whole refolding, resulting in an immediate formation of fully oxidized species even at the very beginning of the reaction. Finally, a Grx mutant, C14S Grx, in which one of the active site cysteine residues (Cys14) had been replaced by serine, had a similar effect on the distribution of folding intermediates, compared to the wild-type protein, thus demonstrating that Grx acts by a monothiol mechanism either in the reduction or in the oxidation step.

RNase A, which contains four disulfide bonds, has been the archetypal model for understanding the folding problem of disulfide-bonded proteins for 3 decades since the fundamental studies of Anfinsen (*1*−*3*). Its refolding has been studied using several different approaches and a variety of different redox conditions. Several folding pathways were deduced depending essentially on the E_0' value of the redox buffer (*4*−*8*). These studies pointed out that the intermediates containing one and two disulfide bonds are largely conformationally disordered and that the formation of further disulfide bonds constitutes the rate-limiting steps in the regeneration of fully native RNase A (*9*). Recently, the relative abundance of the one-disulfide intermediates, formed during the reoxidation of RNase A in the presence of DTT^{ox},[1] has been determined, showing that the distribution of the molecular species within this population is not statistically

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[1] Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, reduced dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ER, endoplasmic reticulum; ESMS, electrospray mass spectrometry; Grx, *E. coli* glutaredoxin; Grx C14S, *E. coli* glutaredoxin with Cys-14 mutated to Ser; GSH, reduced glutathione; GSSG, oxidized glutathione; HPLC, high-performance liquid chromatography; GS-RNase A, ribonuclease A with the eight cysteine residues involved in mixed disulfides with glutathione; IAM, iodoacetamide; PDI, protein disulfide-isomerase; rd-RNase A, reduced and denatured ribonuclease A; TFA, trifluoroacetic acid.

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random (*10*). Likewise, during the refolding of rd-RNase A in the presence of GSH/GSSG, preferential coupling between some cysteine residues has been observed, showing that the formation of S−S bonds does not take place at random during the folding process (*11*).

In the endoplasmic reticulum of eucaryotic cells, protein disulfide bond formation is dependent on protein disulfide isomerase (PDI) which is present at high concentration and functions in the presence of millimolar concentrations of GSH and GSSG (*12, 13*). Previous studies showed that the addition of PDI catalyzes the refolding of reduced and denatured RNase A up to 23-fold at a molar ratio of 1:8 (*14, 15*).

Together with thioredoxin and PDI, glutaredoxin (Grx) is a member of a growing superfamily of well-characterized proteins that share a common fold and an exposed dithiol/disulfide active site. Glutaredoxin was first discovered as a GSH-dependent hydrogen donor for ribonucleotide reductase in *Escherichia coli* cells lacking thioredoxin (*16*). Glutaredoxin has been shown to readily reduce mixed disulfides between proteins and GSH (*17*). In addition, a mutant protein, C14S Grx, was used to generate a mixed disulfide complex between GSH and Grx (Grx-SSG), which has been structurally characterized, demonstrating the occurrence of a specific binding site for GSH within the protein structure (*18, 19*).

Lundström-Ljung and Holmgren (*20*) showed that Grx displayed synergistic activity together with PDI during the early stages of oxidative refolding of rd-RNase A, by monitoring the recovery of native catalytic activity. However, this study reports only on the formation of the final product, and it does not provide any data on the refolding intermediates. The lack of structural data on the intermediates did not allow an interpretation of the observed synergistic activity.

In previous papers (*21, 22*), the use of electrospray mass spectrometry (ESMS) time-course analysis has been described to obtain significant data for kinetic and structural studies on the refolding pathway of disulfide-containing proteins. The experimental approach is based upon the determination of the accurate molecular mass of the alkylated disulfide-bonded intermediates formed during the refolding process on a time-course basis. The alkyl group introduced with the quenching reaction increased the molecular mass of the intermediates by a fixed value, thus allowing the separation by mass of intermediates containing different numbers of disulfide bonds and the determination of their relative concentration during the refolding. This approach was employed to investigate the refolding of RNase T1 (*22*), providing evidence for a pathway of sequential reactions.

In this paper, we describe the application of the ESMS[1] time-course analysis illustrated above, in the structural characterization of the disulfide-bonded intermediates present in the refolding of rd-RNase A or GS-RNase A, a derivative in which the eight cysteine residues are all involved in mixed disulfides with glutathione. The refolding reactions were also performed in the presence of wild-type Grx and Grx mutant (C14S) and/or PDI in order to evaluate the effect of different starting materials on the catalysis and to provide an explanation for the synergistic activity shown by Grx and PDI.

Figure 1 represents all possible intermediates and equilibria in the refolding of a four-disulfide-containing protein in the presence of a glutathione redox system. Each population of intermediates is described in terms of the number of intramolecular disulfides (indicated as *n*S), mixed disulfides with glutathione (*n*G), and free cysteine residues (*n*H). The scheme shown in Figure 1 is derived from an analogous scheme previously reported by Konishi *et al.* (*23*).

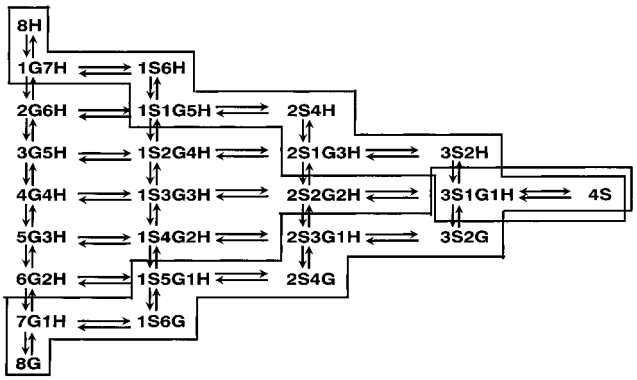

FIGURE 1: Theoretical intermediates and equilibria in the refolding of a four disulfide bond containing protein in the presence of a glutathione redox system. Intramolecular disulfide bonds are indicated as *n*S, mixed disulfides with glutathione as *n*G, and free cysteine residues as *n*H. Free glutathione (GSH/GSSG) is not shown. Zig-zag boxes indicate the pathway of refolding elucidated for rd-RNase and GS-RNase derivatives.

The present studies showed that refolding of RNase A proceeds through a pathway of sequential reactions similar to that proposed for the RNase T1 refolding (*22*). The structural characterization of intermediates present in the Grx-assisted refolding showed, for the first time, that Grx catalyzes both formation and reduction of mixed disulfides involving glutathione. Moreover, the synergistic effect displayed by Grx with PDI has been explained showing that Grx facilitates PDI action by catalyzing formation or reduction of mixed disulfides which are then rapidly converted into intramolecular disulfides in the presence of PDI. These catalytic steps repeat sequentially throughout the refolding, thus resulting in an immediate formation of 4S species even at the early stages of the process. The Grx mutant had similar effects on the refolding process, thus indicating that Grx acts through a monothiol mechanism.

MATERIALS AND METHODS

E. coli Grx and C14S Grx were kind gifts from Drs. O. Björnberg and J. Bushweller (Karolinska Institute, Stockholm, Sweeden). PDI was purified from bovine liver as previously described (*24*).

2′,3′-cCMP, DTNB, DTT, EDTA, GSH, GSSG, guanidinium chloride, bovine pancreatic RNase A, and horse heart myoglobin were obtained from Sigma Chemical Co.; iodoacetamide (IAM) and Tris were purchased from Fluka. Prepacked Sephadex G-25M PD10 columns were acquired from Pharmacia. All other reagents were HPLC grade from Carlo Erba.

The concentrations of solutions of native RNase A and of all the modified forms were determined using an absorption of 0.695 at 278 nm for a 1 mg/mL solution (*25*). Concentrations of PDI and glutaredoxins were determined using molar extinction coefficients at 280 nm of 47 300 M^{-1} cm^{-1} (*24*) and 12 500 M^{-1} cm^{-1} (*26*), respectively.

Preparation of Reduced and Denatured RNase A (rd-RNase A) and GS-RNase A. RNase A was reduced and

denatured as previously described (*21*). The preparation of GS-RNase A was carried out according to the previous procedure (*27*). The preparation of GS-RNase A was structurally characterized by ESMS analysis showing the presence of two molecular species, the GS-RNase A and a derivative with one disulfide bond and six glutathione moieties at almost equal percentage. The GS-RNase A containing eight glutathionyl groups was then purified by HPLC using a linear gradient of solvent B from 24% to 29% at flow rate of 1 mL/min using the chromatographic system described below. The first eluted peak was indeed the pure GS-RNase A product as confirmed by ESMS analysis (measured molecular mass = 16 132.6 ± 1.35 Da; expected molecular mass = 16 132.7 Da).

SH Titration. The free SH content of all the modified forms of RNase A was estimated by DTNB titration in 0.3 M Tris-HCl and 1 mM EDTA (pH 7.5) containing 6 M guanidinium chloride, and the formation of the 2-nitro-5-thiobenzoate anion was measured at 412 nm (extinction coefficient, 13 600 M^{-1} cm^{-1}) (*28*).

Refolding Reactions. Reduced and oxidized glutathione stock solutions were made fresh daily in 0.1 M Tris-HCl (pH 7.5) at a concentration of 50 mM; 1 mM EDTA was added to the buffers to prevent oxidation of SH groups catalyzed by traces of heavy metals.

Lyophilized rd-RNase A or GS-RNase A was dissolved to a concentration of approximately 3 mg/mL in 0.1% CH_3-COOH and then diluted into the refolding buffer (0.1 M Tris-HCl, 1 mM EDTA, pH 7.5) to a final concentration of 1 mg/mL. The desired amounts of GSH and GSSG stock solutions were added to initiate refolding; typically, final concentrations of the glutathione species were 1.5 mM GSH/0.3 mM GSSG. The pH of the solution was adjusted to 7.5 with Tris-base, and the reaction carried out at 25 °C under a nitrogen atmosphere.

When the refolding was carried out in the presence of Grx and/or PDI, the enzymes were dissolved in 0.1 M Tris-HCl, 1 mM EDTA, pH 7.5, and preincubated in the presence of 1.5 mM GSH/0.3 mM GSSG redox buffers for 10 min at 25 °C. This mixture was then added to rd-RNase A or GS-RNase A at different absolute concentrations and the refolding continued at 25 °C under a nitrogen atmosphere, as described. Typically the Grx concentration was fixed to 0.5 μM, and the PDI concentrations were either 1 μM or 10 μM. For the reactions in the presence of C14S Grx, the mutant was used at the same concentration as the wild-type protein. Grx concentration and the redox potential used were found to be optimal for PDI activity and allowed strong Grx enhancement of activity (*14*, *15*, *20*).

Each set of data was obtained as the mean of two independent folding experiments. The differences between refolding experiments performed completely independent of each other were about 2%.

Alkylation of the Refolding Aliquots. The refolding was monitored on a time-course basis by sampling aliquots of the refolding mixture at appropriate intervals. The protein samples were alkylated as described (*21*, *22*, *29*). IAM was freshly dissolved in 0.1 M Tris-HCl, containing 1 mM EDTA (pH 7.5), at 65 °C and cooled down to room temperature before use. During preparation of the reagents, the solutions were protected from light to minimize photolytic production of iodine which is a very potent oxidizing agents for thiols. The refolding aliquots (100 μL) were added to an equal volume of a 2.2 M IAM solution. Alkylation was performed for 30 s, in the dark, at room temperature, under a nitrogen atmosphere. After 30 s, 500 μL of 5% acetic acid was added, and the aliquots were quickly vortexed and desalted on a prepacked PD10 column equilibrated and eluted with 1% acetic acid. The protein fraction was then recovered and lyophilized. For the experiments in the presence of a higher concentration of PDI, which interferes with the subsequent ESMS analysis, the desalted samples were further purified by rapid HPLC separation.

In control experiments, some aliquots of the nonassisted rd-RNase A refolding were alkylated with IAM in the presence of a final [guanidinium chloride] = 1 M.

Purification by HPLC. The desalted samples containing PDI were further purified by HPLC using a Vydac TP 214 reversed-phase C4 column (0.46 cm × 25 cm). The elution system consisted of 0.1% TFA in water (solvent A) and 0.07% TFA in 95% acetonitrile/5% water (solvent B). Refolding intermediates were separated from PDI with a linear gradient of solvent B from 15% to 95% at a flow rate of 1 mL/min. Eluted proteins were monitored at 220 nm, and PDI was eluted in the washing of the column, thus not interfering with the subsequent analysis.

Electrospray Mass Analysis. ESMS analyses were carried out using a BIO-Q triple quadrupole mass spectrometer equipped with an electrospray ion source (Micromass). The protein samples were dissolved in a mixture of H_2O/CH_3-CN (50/50) containing 1% acetic acid, and 10 μL (10−20 pmol/μL) was introduced into the ion source via loop injection at a flow rate of 10 μL/min. Spectra were recorded by scanning the quadrupole at 10 s/scan. Data were acquired and elaborated by the MassLynx software. Mass-scale calibration was performed by means of multiply charged ions from a separate injection of horse heart myoglobin (average molecular mass 16 951.5 Da).

Activity Data. Desalted and alkylated aliquots from the refolding reaction were concentrated by lyophilization and dissolved in 300 μL of distilled water. Protein concentration was determined from the absorbance at 280 nm. In refolding aliquots containing high concentrations of PDI, RNase A concentration was determined by multiplying the absorbance at 280 nm by the RNase fraction of total protein as estimated by integrating HPLC peaks. The activity of RNase A was determined in triplicate by mixing 100 μL of sample diluted in distilled water with 450 μL of an assay mixture containing 50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, and 200 μL/mL of 2′,3′-cCMP. The specific activity of RNase A was determined from the rate of increase of the absorbance at 288 nm and related to a standard curve of native RNase A activity. Control experiments where native RNase A had been subject to the alkylation with iodoacetamide, desalted, and lyophilized showed that this procedure did not affect the enzymatic activity.

RESULTS

Oxidative Refolding of rd-RNase A. rd-RNase A was incubated in the presence of 1.5 mM GSH/0.3 mM GSSG. Assuming an E_0' standard = −0.25 V (*30*) for the glutathione redox system, the redox mixtures give an initial E_0' = −0.19 V. Aliquots of the refolding process were withdrawn at different intervals, trapped by alkylation of the free thiol groups as described, and analyzed by ESMS to identify the

Table 1: ESMS Analysis of the Intermediates Formed in the Refolding of rd-RNase A

component	measured molecular mass (Da)	identification[a]	expected molecular mass (Da)	possible isomers
A	14147.41 ± 0.73	8CAM (8H)	14146.26	1
B	14030.14 ± 0.77	1S (1S6H) 6CAM	14030.60	28
C	14394.87 ± 1.98	1G (1G7H) 7CAM	14394.57	8
D	13913.68 ± 1.01	2S (2S4H) 4CAM	13914.40	90
E	14278.35 ± 1.59	1S (1S1G5H) 1G 5CAM	14278.55	168
F	13798.33 ± 1.01	3S (3S2H) 2CAM	13798.30	235
G	14162.12 ± 0.88	2S (2S1G3H) 1G 3CAM	14162.51	360
J	14046.35 ± 1.29	3S (3S1G1H) 1G 1CAM	14046.51	470
I	13682.01 ± 0.79	4S (4S)	13682.20	105

[a] Abbreviations: CAM = carboxamidomethyl groups; G = mixed disulfide with glutathione; S = intramolecular disulfide; H = free cysteine.

disulfide-bonded intermediates formed. The carboxamidomethylation reaction used to trap the free SH groups increased the molecular mass of the intermediates by a fixed amount, 57 Da for each free SH group, thus allowing the separation by mass of intermediates containing different numbers of disulfide bonds and the determination of their relative concentration during the refolding.

Table 1 shows the measured molecular masses, the expected mass values, and the identification of the various intermediates. Each population of trapped intermediates is characterized by a different number of intramolecular disulfide bonds (indicated as *n*S in Table 1), mixed disulfides with the exogenous glutathione (*n*G), and carboxamidomethyl groups (*n*CAM). The number of CAM groups corresponds to the number of free thiols present in the refolding intermediates and is therefore indicated as *n*H. It is important to emphasize that each observed molecular mass corresponds to a population of isomers, in which the total number of disulfides, free thiols, and protein−SSG mixed disulfides is defined, whereas the positions of the effective cysteine residues involved are not identified. The number of possible isomers within each population is indicated in Table 1.

In the use of ESMS, a further bonus is that each population of intermediates can be accurately quantitated by measuring the total ion current produced by each species (*22, 31*). The relative intensity of the disulfide bond intermediates formed during the folding process is shown in black staples in Figure 2A,B. The fully reduced species (8H) disappeared after 20 min, and the 2S4H intermediate predominates from 20 min up to about 4 h when the relative concentration of the species 4S increases. At 24 h, only the fully oxidized protein, species 4S, is present. These analyses indicate that mixed disulfide containing species (Figure 2B) do not accumulate to a concentration higher than 10% throughout the entire process, thus confirming that they constitute transient species that rapidly evolve toward the formation of intramolecular disulfide bonds.

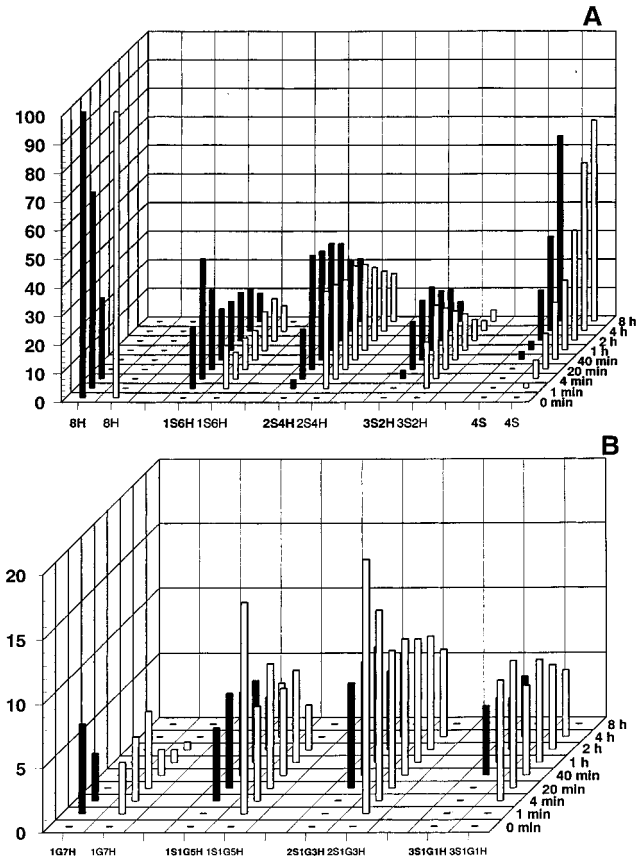

FIGURE 2: Time-course analysis of the refolding of rd-RNase A in the presence of 1.5 mM GSH/0.3 mM GSSG. Black staples represent the reaction in the absence of Grx and white staples the reaction in the presence of Grx. Grx was incubated with 1.5 mM GSH/0.3 mM GSSG for 10 min at 25 °C and then added to rd-RNase A (1 mg/mL) at a concentration = 0.5 μM. Intramolecular disulfide intermediates are reported in panel A; components containing mixed disulfides are reported in panel B.

As observed before in different redox conditions (*21*), the predominance of the species containing two intramolecular disulfide bonds along the pathway is quite surprising. On a purely statistical basis in fact, a larger number of three disulfide containing intermediates was expected. The accumulation of two disulfide containing species could be then ascribed to their intrinsic thermodynamic stability. The same results (data not shown) were obtained when the alkylation reaction was carried out in the presence of guanidinium chloride, thus indicating that the relatively low population of three-disulfide species should not be ascribed to the inaccessibility of some buried thiol groups to the alkylating reagent.

Figure 2 shows the time-course analysis of rd-RNase A refolding in the presence of 0.5 μM Grx (white staples) in comparison to the nonassisted reaction (black staples). Addition of Grx led to a net catalysis of the formation of intermediates containing mixed disulfides with glutathione (Figure 2B) at the early stages of the process where they are, in fact, present at higher concentration than in the spontaneous refolding. Moreover, in the presence of Grx, some components containing more than one mixed disulfide with glutathione, such as 2G6H, 1S2G4H, and 2S2G2H, could be identified at very low concentration (2%). Due to their low percentage, these intermediates are not reported in Figure 2. In addition, the analysis indicates that the presence of Grx suppresses the initial lag phase for the appearence of

Table 2: Appearance of 50% 4S Product in Various Conditions[a]

substrate	8H (min)	8G (min)
no addition of enzymes	400	140
0. 5 μM Grx	180	30
1 μM PDI	110	10
0. 5 μM Grx + 1 μM PDI	90	3
10 μM PDI	30	1
0. 5 μM Grx + 10 μM PDI	18	0.5

[a] Derived from data in Figures 2−7. See legends for detailed conditions.

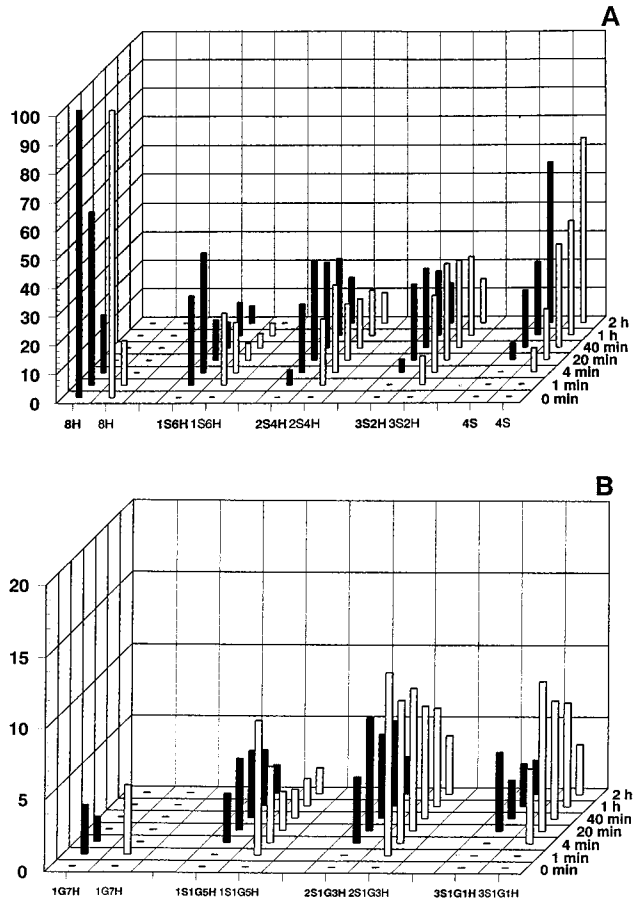

FIGURE 3: Time-course analysis of the refolding of rd-RNase A in the presence of PDI (black staples) or PDI + Grx (white staples). PDI and Grx were incubated with 1.5 mM GSH/0.3 mM GSSG for 10 min at 25 °C and then added to rd-RNase A (1 mg/mL) at a [Grx] = 0.5 μM and [PDI] = 1 μM. Intramolecular disulfide-containing species are reported in panel A; intermediates containing mixed disulfides are reported in panel B.

species 4S detected in the nonassisted refolding (see also Figure 4). The fully oxidized protein, in fact, accumulates at higher yield and rate (Table 2) at the early stage of the refolding, but it reaches almost the same value observed in the nonassisted process at later stages. Again at 24 h only the 4S species is present.

Figure 3 shows a comparison of the time-course analyses of rd-RNase A refolding in the presence of either 1 μM PDI (black staples) or 1 μM PDI + 0.5 μM Grx (white staples). Addition of PDI led to an increase in the rate of the whole refolding reaction (Table 2), but it appears not to change the distribution of intermediates containing only intramolecular disulfide bonds (compare Figure 2A, black staples). However, the intermediates containing mixed disulfides with glutathione (Figure 3B) show the tendency to accumulate at a slightly lesser extent than in the nonassisted process (Figure 2B, black staples). As shown in Figure 4, even in the presence of PDI the lag phase in the appearance of the 4S species exists altough it is less pronounced as compared to the nonassisted refolding. The PDI-catalyzed refolding was completed after 4 h when the 4S species reached about 95%.

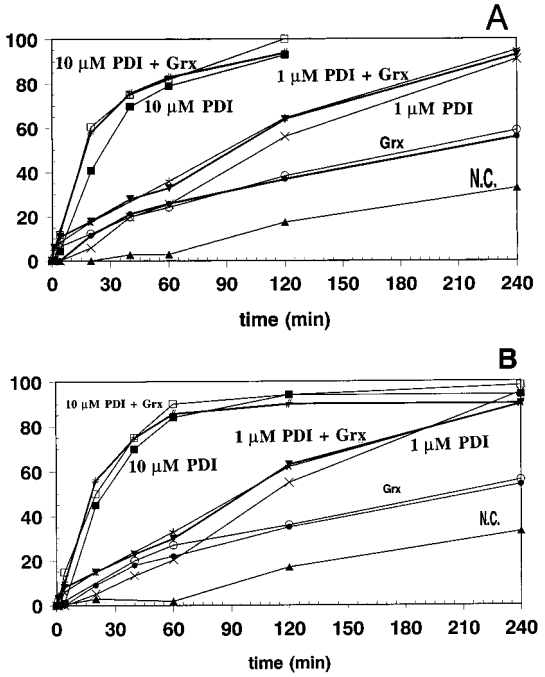

FIGURE 4: (A) Appearance of species 4S in the rd-RNase A refolding detected by ESMS analysis in the different conditions used. (B) Recovery of enzymatic activity from aliquots of the same refolding reactions. (▲) Noncatalyzed reaction; (●) 0.5 μM Grx; (○) 0.5 μM C14S Grx; (×) 1 μM PDI; (*) 1 μM PDI + 0.5 μM Grx; (▼) 1 μM PDI + 0.5 μM C14S Grx; (■) 10 μM PDI; (□) 10 μM PDI + 0.5 μM Grx; (#) 10 μM PDI + 0.5 μM C14S Grx. Reactions in the presence of the Grx mutant are represented with boldface lines.

The PDI-assisted refolding was also performed in the presence of a higher concentration of PDI (10 μM final concentration). As shown in Figure 4, addition of higher amounts of the enzyme led to a net catalysis of the refolding process, altough a short lag phase in the appearance of 4S species is still present, at the very early stages of the process. It is, however, clear that the rate of appearance of the fully oxidized protein is greatly increased using PDI alone, despite the enzyme concentration (Table 2).

The refolding of rd-RNase A was then carried out in the presence of both Grx and PDI (Figure 3, white staples). When both the enzymes are present, the appearance of species 4S is immediate, and no lag phase is observed (see also Figure 4 and Table 2). However, no synergistic effect was observed on the final yield of the fully oxidized species, 4S, which is produced to the same extent as that observed in the reoxidation assisted by PDI alone. In the presence of a higher concentration of PDI (10 μM final concentration), the addition of Grx at 0.5 μM again resulted in a synergism with PDI (Figure 4 and Table 2), as shown by the disappearance of the lag phase in the formation of 4S species at early stages of the reaction.

The refolding reactions were also performed in the presence of the mutant, C14S Grx. Addition of C14S Grx had the same effect as the wild-type protein (see Figure 4), thus strongly supporting a monothiol mechanism as the mode of action of Grx in this system (*20*).

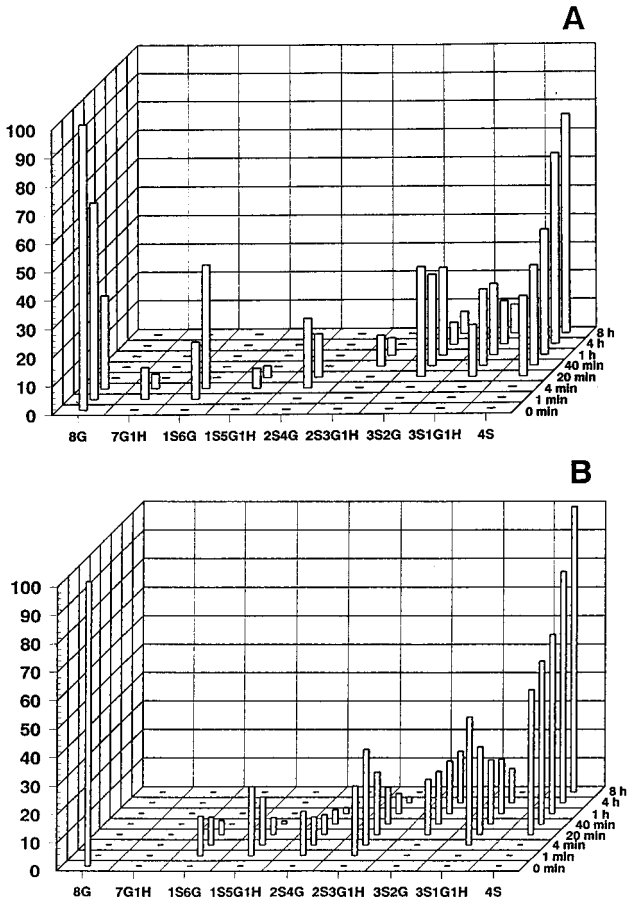

FIGURE 5: Time-course analysis of the refolding of GS-RNase A in the presence of 1.5 mM GSH/0.3 mM GSSG (A) and in the presence of Grx (B). Grx was incubated with 1.5 mM GSH/0.3 mM GSSG for 10 min at 25 °C and then added to GS-RNase A (1 mg/mL) at a concentration = 0.5 μM.

Altogether these results suggest that Grx catalyzes formation of mixed disulfides with glutathione which are then rapidly converted into intramolecular disulfides by PDI.

Figure 4 compares the rate of appearance of the 4S species under different refolding conditions (Figure 4A) with the recovery of RNase A activity (Figure 4B) assayed on aliquots of the refolding mixtures from the same experiments. A perfect correlation seems to exist between the percentage of the fully oxidized protein and the recovery of enzymatic activity, thus demonstrating that the refolding generates genuine native RNase A, rather than significant amounts of isomers containing non native conformations.

GS-RNase A Refolding. The mixed disulfide derivative GS-RNase A was incubated in the presence of the same redox buffer (1.5 mM GSH/0.3 mM GSSG) used for the refolding of the reduced and denatured protein. Aliquots were sampled from the refolding mixture at different intervals, alkylated with IAM, and analyzed by ESMS, as described above.

Figure 5A shows the time course of the GS-RNase A refolding in the absence of folding factors. The spectrum of the aliquot withdrawn at time zero shows the presence of a single component exhibiting a molecular mass of 16 131.7 $\pm$ 1.9 Da, corresponding to the protein carrying 8 exogenous glutathione groups (8G). The GS-RNase A disappeared at the same rate as the reduced protein (Figure 2A), whereas the whole reaction seems to proceed faster than the reoxidation of rd-RNase A (see Table 2). The time-course analysis clearly shows that at the early stages of refolding the populations of different intermediates tend to appear and disappear very quickly, indicating an initial fast phase. At late stage, a steady state is established between the intermediates 3S2G and 3S1G1H. At 24 h, only species 4S is present. As emphasized for rd-RNase A, each observed intermediate characterized by a single molecular mass might correspond to a population of isomeric species. The 9 components observed during the GS-RNase A refolding experiments and represented in Figure 5 could therefore give rise to almost 1400 theoretically possible isomers.

The effect of folding catalysts on the refolding of GS-RNase A was also investigated. Grx and/or PDI were incubated in the presence of the redox buffer, 1.5 mM GSH/0.3 mM GSSG, and then added to GS-RNase A as previously described. Figure 5B shows the time-course analysis of Grx-assisted GS-RNase A refolding. Addition of Grx led to a net catalysis of the refolding reaction, without substantially altering the distribution of the intermediates along the refolding pathway. GS-RNase A disappears completely after only 1 min, and the species 7G1H detected in the nonassisted reaction could not be observed; it is possible that the reaction is too fast at the early stages in the presence of Grx to allow detection of species 7G1H even when the reaction mixture is sampled after only 1 min. The steady state between species 3S2G and 3S1G1H is established earlier than in the nonassisted refolding; finally, species 4S is predominant after only 20 min (the appearance of 50% 4S product in these conditions is reported in Table 2).

Figure 6A shows the time-course analysis of the GS-RNase A refolding in the presence of 1 μM PDI. PDI seems to catalyze the whole process more efficiently than Grx (Table 2); PDI thus has almost the same effect in the refolding either starting from the reduced protein or starting from the GS derivative. Addition of a higher concentration of PDI, 10 μM, resulted in a further increase in the rate of the process which is completed within 40 min (see Figure 7 and Table 2).

When Grx and PDI are used together (Figure 6B), Grx displayed a synergistic activity together with PDI, which is again maximal at the early stages of refolding. The time-course analysis shows, in fact, the absence of the early appearing intermediates 7G1H, 1S6G, and 1S5G1H, detected in the other reactions; in the presence of both enzymes, it is possible that the reaction is too fast at the early stages to allow detection of these species even when the reaction mixture is sampled after only 1 min.

Figure 7A shows the appearance of the 4S species in the refolding reactions of GS-RNase A performed under different experimental conditions. The corresponding recovery of enzymatic activity detected in aliquots of the refolding mixtures from the same experiments is displayed in Figure 7B. At the early stages of the refolding, in the noncatalyzed process or, to a higher extent, in the presence of Grx, a fully oxidized 4S species was detected at significant amounts long before the appearance of a corresponding recovery of enzymatic activity. As an example, at 20 min the percentage of 4S is about 28% and 50% in the noncatalyzed and in the Grx-mediated process, respectively, while the corresponding recovery of RNase A activity is about 15% and 22%, respectively. Therefore, catalysis by Grx results in a rapid formation of 4S species from GS-RNase A, via fast reduction of mixed disulfide with glutathione, but the 4S species is

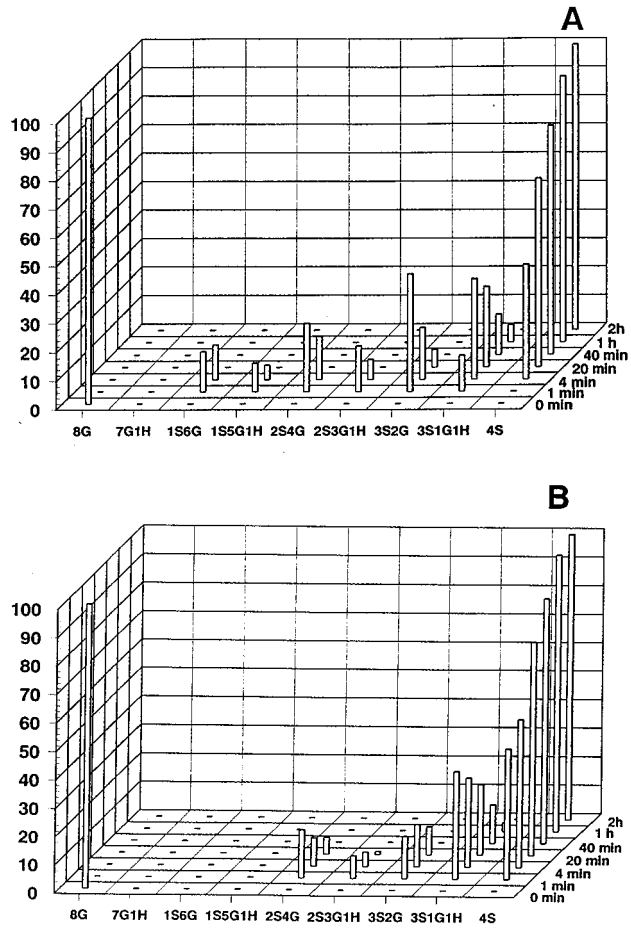

FIGURE 6: Time-course analysis of the refolding of GS-RNase A in the presence of PDI (A) and in the presence of Grx and PDI (B). PDI alone or PDI and Grx were incubated with 1.5 mM GSH/0.3 mM GSSG for 10 min at 25 °C and then added to GS-RNase A (1 mg/mL) at [Grx] = 0.5 μM and [PDI] = 1 μM.

very likely constituted by a mixture of native and non-native isomers, thus displaying a lower specific activity.

In the presence of PDI, a good correlation between the appearance of the 4S species and the recovery of enzymatic activity exists, thus suggesting that the PDI-assisted GS-derivative refolding produces genuine native RNase A.

DISCUSSION

Formation of native disulfide bonds is a rate-limiting process in the folding of disulfide-containing proteins during their residence in the endoplasmic reticulum (*32, 33*) where the major low molecular mass thiol redox system is provided by glutathione (*34*). Folding studies *in vitro* have been performed using reduced and oxidized glutathione with different ratios and different concentrations, and several folding pathways have been deduced depending on the physicochemical conditions and the protein under study (*35*). Recently, mixed disulfides between reduced and denatured proteins and glutathione (GS derivative) have been shown to be a good model of the denatured state and to constitute an effective alternative to reduced and denatured protein as starting material in protein folding studies (*27, 36*). It seems conceivable that mixed disulfides between proteins and glutathione may form *in vivo* and they may be important folding intermediates. Since the nascent chain emerges into the ER lumen into conditions of high oxidized glutathione concentration, it may well be that unpaired cysteine residues

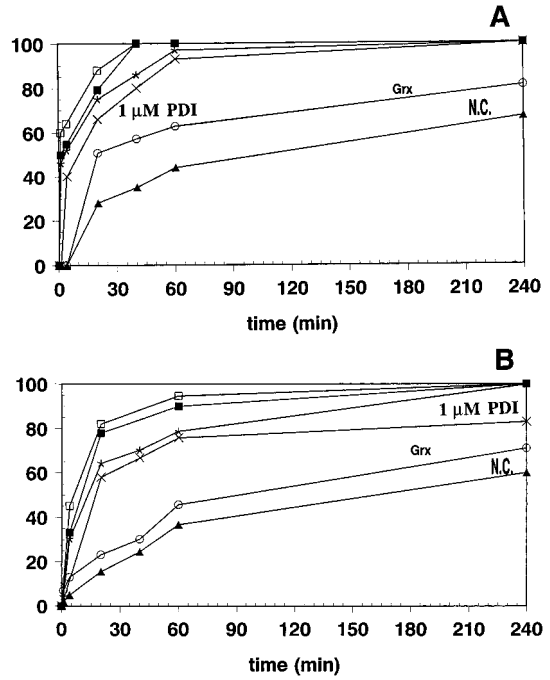

FIGURE 7: (A) Appearance of species 4S in the GS-RNase A refolding detected by ESMS analysis in the different conditions used. (B) Recovery of enzymatic activity from aliquots of the same refolding reactions. (▲) Noncatalyzed reaction; (○) 0.5 μM Grx; (×) 1 μM PDI; (*) 1 μM PDI + 0.5 μM Grx; (■) 10 μM PDI; (□) 10 μM PDI + 0.5 μM Grx.

are converted to mixed disulfides even before the translocation is completed.

In a previous paper (*22*), an ESMS time-course procedure was employed, providing crucial data for kinetic and structural studies on the refolding pathway of RNase T1. The definition of the precise molecular mass of the refolding intermediates allowed us to identify the isomeric species formed during the process and to establish a pathway of sequential reactions for the refolding.

In this work, we have reinvestigated the refolding of RNase A starting from both the reduced protein and the GS derivative. The ESMS time-course experiments allowed us to determine a pathway of sequential reactions for both refolding processes similar to that already observed for the refolding of RNase T1 (see zig-zag boxes in Figure 1). The present analysis demonstrates that only a limited number of intermediates are formed in the processes as compared to all those theoretically possible, thus significantly decreasing the number of possible refolding pathways for both rd-RNase A and GS-RNase A.

As stated in the proposed pathway of sequential reactions for the RNase T1 refolding (*22*), the first obligate step in the refolding of rd-RNase is the formation of a mixed disulfide with glutathione, producing species 1G7H, while the first step in the GS-RNase A refolding is the reduction of a single mixed disulfide, producing species 7G1H. Then, both species undergo an internal attack by a free SH group to form an intramolecular disulfide bond, leading to the species 1S6H and 6G1S, respectively. It is worth emphasizing that the formation of an intramolecular disulfide is in both cases favored over a further reaction with exogenous GSH or GSSG and this significantly restricts the pathway. Species 2G6H or 6G2H were in fact never found in the nonassisted or in the PDI-assisted refolding whereas they

have been detected, only at very low level, when the rd-RNase refolding was carried out in the presence of Grx (see below). This indicates that only when the first S−S bond has been formed, the one S−S intermediate will react with a second glutathione molecule to reiterate the process.

It should be noted that the intermediates identified by the ESMS analysis are populations of molecular species characterized by the same number of disulfide bonds and GS mixed disulfides. These intermediates may include nearly all possible disulfide-bonded isomeric species. However, earlier study on rd-RNase A refolding (*11*) showed that only a limited number of disulfide bonds are formed, thus suggesting that the formation of disulfide bonds along the refolding pathway is not statistically random and that the coupling of some cysteine residues is preferred to others. Therefore, it might be possible that only a limited number of isomers is effectively formed within an intermediate population due to kinetic or thermodynamic constraints, thus further restricting the possible folding pathways.

Two different mechanisms had been proposed for the oxidative refolding of RNase A, one in which a single pathway exists and other in which multiple pathways are adopted (*37*, *38*). The data presented in this paper do otherwise fit both the "single pathway" and the "multiple pathway model" since it is clearly evident that the protein refolds through only a limited number of pathways compared to all those theoretically possible.

The refolding experiments were also performed in the presence of Grx and/or PDI to investigate the role of enzyme catalysis in the formation of disulfide bonds.

Grx seems to act as a catalyst of both refolding processes either starting from rd-RNase A or starting from GS-RNase A (Table 2). The addition of the enzyme results in a net catalysis of the early stages of both processes where its presence suppresses the initial lag phase in the appearance of species 4S observed in the nonassisted refolding (see Figure 4). In the presence of Grx in fact, a more rapid formation of the fully oxidized 4S species at the early stages of the reaction is observed. Conversely, at late stages of the refolding where isomerizations and conformational transitions become rate-limiting, Grx does not seem to have any catalytic effect, and the process proceeds as the nonassisted refolding. However, it cannot be ruled out that Grx is not able to exert its catalysis at the late stages because the protein has already acquired a substantial degree of compact structure which might prevent the accessibility of thiols or mixed disulfides to the enzyme.

Here we show for the first time that the addition of Grx resulted in an effective catalysis of steps involving both formation and reduction of mixed disulfides. Glutaredoxin from *E. coli* has a low redox potential, −240 mV, and is believed to maintain cytosolic protein disulfides in a reduced state together with glutathione, NADPH, and glutathione reductase (*39*). It is therefore not surprising that Grx catalyzes GS-RNase refolding which proceeds throughout the reduction of mixed disulfides and the subsequent formation of intramolecular disulfide bonds. The finding that Grx is also able to catalyze rd-RNase A refolding by enhancing the rate of formation of mixed disulfide with glutathione is in some way unexpected. It is conceivable that the formation of mixed disulfides intermediates is a rate limiting step in the rd-RNase refolding process and that Grx catalysis actually makes this reaction competitive with the formation of intramolecular disulfide bonds. This hypothesis is confirmed by the observation of species containing two mixed disulfides with glutathione such as 2G6H, 1S2G4H, and 2S2G2H, even if at a very low extent (2%), only in the presence of Grx (see scheme in Figure 1). These results suggest that Grx can catalyze either reduction or formation of mixed disulfides, depending on the redox status of the protein substrate, by recognizing the glutathione moiety.

Activity data show that although Grx catalyzes the formation of a fully oxidized species starting from GS-RNase A, it is not able to guide the protein into a productive folding pathway. The appearance of a fully oxidized species in fact precedes the recovery of enzymatic activity during most of the process, although the final yield of 4S species corresponds to the percentage of recovered activity.

The addition of PDI resulted in a net catalysis of the entire refolding process (Table 2), but it did not seem to alter the pathway other than small changes in the relative distribution of the various populations of intermediates. In a previous paper (*22*), it was suggested that PDI catalyzes formation and reduction of mixed disulfides as well as formation of intramolecular disulfides, confirming the broad range of activities of the enzyme (*12*, *40*). The present study confirms that PDI can catalyze both the oxidation/reduction and the isomerization steps during refolding. However, it was not possible to develop any quantitative analysis of PDI catalysis since the complexity of the process did not allow the calculation of any rate constants as we did in studying the PDI-assisted RNase T1 refolding (*22*).

PDI could form directly a mixed disulfide with the substrate protein, and such species are potential intermediates in the PDI-catalyzed process observed here. With the analytical method and PDI concentration employed here, it was not possible to detect or quantitate such transient intermediate species. It is also important to underline that the present study did not allow us to detect any effect of PDI on the possible isomerization of disulfide bonds within a single population of intermediates. However, in the presence of PDI, the recovery of enzymatic activity is in good agreement with the appearance of 4S species monitored by ESMS analysis. It is therefore reasonable to assume that the 4S species contain only the native isomer, suggesting that PDI does catalyze the formation of native disulfide bonds, thus further restricting the folding pathways.

The addition of Grx together with PDI resulted in a synergistic activity of the two enzymes (Table 2) which abolishes the lag phase in the appearance of the 4S species at the early stages of the refolding process.

In a previous study, it was suggested that Grx assists PDI in the oxidative refolding of rd-RNase A by catalyzing early steps in the refolding reaction (*20*). However, the lack of structural characterization of intermediates present in the process did not allow a detailed interpretation of the observed synergistic activity. The data presented in this paper seem to suggest that the synergistic effect of Grx and PDI could be due to the Grx catalysis of steps involving glutathione as reactant such as the oxidation/reduction steps shown as vertical arrows in the scheme of Figure 1. Conversely, PDI might catalyze both reactions represented by vertical and horizontal arrows, but it could be much more efficient in the catalysis of intramolecular disulfide bond formation. Therefore, Grx actually facilitates PDI action by catalyzing the formation or reduction of mixed disulfides which are then

rapidly converted into intramolecular disulfides by PDI. These elementary catalytic steps when sequentially repeated throughout the whole process result in an immediate formation of 4S species even at the very beginning of the reaction. At later stages, it could be possible that only PDI action predominates when isomerizations constitute the final rate-limiting steps. Finally, in the presence of higher concentrations of PDI, the synergistic effect of the two enzymes is much less detectable, possibly because in these conditions PDI catalysis of the oxidation/reduction of mixed disulfides (vertical arrows in Figure 1) is equally efficient as the Grx activity.

It should be noted that *in vivo* PDI is present at very high concentration in the ER lumen. No ER-resident Grx has been identified, and all known Grx proteins are localized in the cell cytosol. Therefore, Grx and PDI may not function within the same cellular compartment *in vivo*. However, it is possible that although PDI lacks a proper glutathione binding site, it is still able to exert a Grx-like activity promoting formation and reduction of mixed disulfide within the ER. It is worth considering that PDI is a multidomain enzyme, and one might speculate that each single domain has different catalytic efficiency with respect to the oxidation/reduction and isomerization steps, respectively.

Data presented in this paper may lead to a more general conclusion on the refolding mechanism. In quasi-physiological conditions, it appears that the refolding of single-domain disulfide-containing proteins occurs via reiteration of two sequential steps: (i) formation of a mixed disulfide with glutathione; (ii) internal attack of a free SH group to form an intramolecular disulfide bond. The proposed mechanism predicts that only a limited number of intermediates actually accumulates and that isomerization between species with the same number of disulfides could be extensive only at late stages of the process where slow conformational transitions become significant. Moreover, on purely theoretical bases, the number of intermediates during the refolding process should increase exponentially with the increase in the number of cysteine residues. However, according to the above mechanism, the number of populations of intermediates, which do form, is only linearly increased with the increase of disulfide bonds [compare RNase T1 (*22*) and RNase A, present study]. The proposed sequential mechanism which limits the population of isomers occurring during the folding process and the presence of a limited number of S−S bonds within each population due to conformational and/or kinetic constraints severely restrict the possible folding pathways. If the number of isomers actually formed *in vivo* is further restricted by the activity of folding factors, this picture suggests an explanation of the extremely high rate and efficiency of the folding process in the cell.

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