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Refolding by Disulfide Isomerization: The Mixed Disulfide between Ribonuclease T_1 and Glutathione as a Model Refolding Substrate†

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ABSTRACT: Protein folding, associated with isomerization of disulfide bonds, was studied using the mixed disulfide between glutathione and reduced ribonuclease T_1 (GS-RNase T_1) as a stable soluble and homogenous starting material; conditions were selected to model those within the lumen of the endoplasmic reticulum where native disulfide bonds are formed in protein biosynthesis. Folding was initiated by addition of free glutathione (GSH ± GSSG) to promote thiol−disulfide interchange and was monitored by intrinsic protein fluorescence, appearance of native ribonuclease activity, HPLC, and nonreducing SDS−PAGE. All the analyses indicated that native RNase T_1 was recovered in high yield in a variety of redox conditions. Appearance of native activity followed first-order kinetics; kinetic analysis of the intrinsic fluorescence changes indicated an additional rapid process in some conditions, interpreted as the formation of a nonnative intermediate state. Analysis by HPLC and SDS−PAGE also indicated the formation of transient intermediates. In 1.5 M NaCl, GS-RNase T_1 adopts a compact native-like conformation; refolding by thiol−disulfide interchange in these conditions was accelerated approximately 2-fold. Refolding of GS-RNase T_1 was catalyzed by protein disulfide isomerase (PDI); substoichiometric quantities of PDI accelerated refolding several-fold. GS-RNase T_1 refolding was inhibited by BiP; refolding was completely blocked in presence of a 5-fold molar excess of BiP, and the yield of refolding was substantially reduced by equimolar concentrations of BiP; the refolding was then restored by the addition of ATP. GS-RNase T_1 is a convenient model substrate for studying protein folding linked to native disulfide formation in conditions comparable to those within the lumen of the endoplasmic reticulum.

Our understanding of the mechanism that governs the folding of a protein into the native structure is still far from satisfactory: this process is therefore the subject of many theoretical and experimental studies. In secretory and extracellular proteins, disulfide bonds have a dramatic role in the maintenance of biological activity and conformational stability. Folding of these proteins is therefore intimately linked to disulfide bond formation; as a consequence, many studies have focused on the roles of disulfides as constraints in the native protein structure and on the identities of disulfides in transient intermediates in protein folding (Creighton, 1992). Some of these studies have been directed at understanding the process of initial biosynthetic formation of native disulfides which *in vivo* takes place in the endoplasmic reticulum (ER)[1] (Creighton et al., 1993; Freedman, 1995).

Fully reduced derivatives are the natural starting materials for studying the folding of secretory proteins at biosynthesis (Rowling & Freedman, 1993), but these derivatives are liable to ill-controlled oxidation. Preparations containing nonnative disulfides—"scrambled proteins", generated by reoxidizing proteins in denaturing conditions—have also been used, but these materials are heterogeneous, ill-defined, and irreproducible. Here we have explored the properties of mixed disulfides between reduced unfolded proteins and the physiological thiol, glutathione; in appropriate conditions such substrates can refold and form native disulfides by disulfide isomerization, rather than net oxidation.

For this purpose we have used as a model, RNase T_1 a small protein of 104 residues which contains two disulfide bonds (Cys2−Cys10 and Cys6−Cys103). The two disulfide bonds are spatially close to each other in the folded protein (Figure 1). Remarkable features of RNase T_1 are its very low isoelectric point and its strong stabilization in the presence of high concentrations of NaCl. RNase T_1 can be unfolded, leaving its native disulfides intact, and on refolding under strongly native conditions it rapidly re-forms ordered, native-like secondary structure and some tertiary contacts; in this case, the kinetics of refolding of RNase T_1 are greatly determined by the slow *trans−cis* isomerization of the two prolyl residues Pro39 and Pro55 (Kiefhaber et al., 1990a,b, 1992). When the disulfide bonds are broken, RNase T_1 is essentially unfolded but can adopt compact native-like structure even in the absence of disulfide bonds under favourable solvent conditions such as high concentrations of NaCl (Oobatake et al., 1979; Pace et al., 1988a). The refolding and reoxidation of reduced RNase T_1 have been studied previously by Pace and Creighton (1986) and Schönbrunner and Schmid (1992). Pace and Creighton (1986) reported that disulfide bond formation in reduced and denatured RNase T_1 produced primarily intermediates having

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; BiP, immunoglobulin heavy chain binding protein; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid, disodium salt; ER, endoplasmic reticulum; GSH, reduced glutathione; GSSG, oxidized glutathione; HPLC, high-performance liquid chromatography; λ_{em}, wavelength of maximum fluorescence emission; PDI, protein disulfide isomerase; RNase, ribonuclease; TFA, trifluoroacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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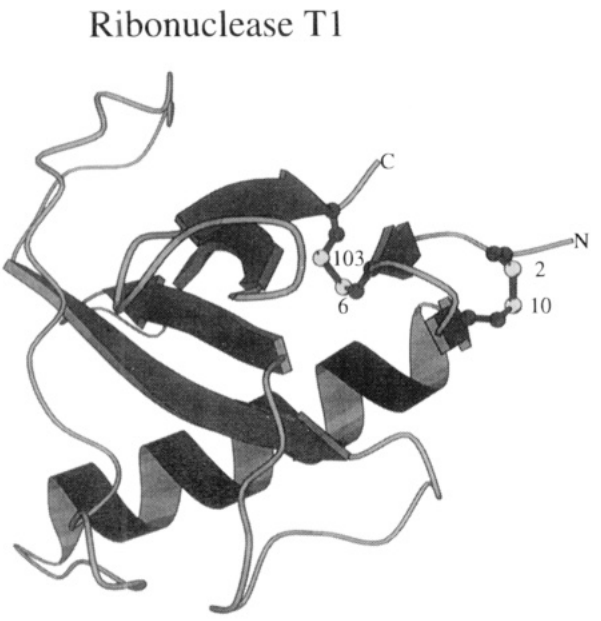

FIGURE 1: Structure of ribonuclease T_1. A Molscript representation indicating the side chains atoms forming the native disulfide bonds, from structural coordinates in the Brookhaven database (accession code 9RNT), based on Martinez-Oyanedel *et al.* (1991).

a single disulfide bond between any pair of the residues Cys2, Cys6, and Cys10. No initial disulfides were observed involving Cys103, probably due at least in part to its distance in the primary structure from the other three Cys residues. In the work of Schönbrunner and Schmid (1992), the kinetic analysis of refolding was mainly based on changes in intrinsic fluorescence. The kinetics of refolding indicate an initial fast-phase implying the formation of transient nonnative intermediates whose concentrations are low throughout the refolding process. To further investigate the role of disulfides for the folding mechanism, Mayr et al. (1994) compared the folding kinetics of the wild-type RNase T_1 with the folding of a variant that lacks the Cys2−Cys10 bond, by substituting Cys2 and Cys10 by Ser and Asn. Breaking of the Cys2−Cys10 bond does not change the folding mechanism of RNase T_1, rather the disruption of the disulfide leads to a general destabilization of folding intermediates in a similar manner as it destabilizes the native protein.

In the endoplasmic reticulum of eucaryotic cells, disulfide bond formation is dependent on protein disulfide isomerase (PDI); data from cross-linking studies in intact mammalian cells, from reconstitution studies on cotranslational folding *in vitro* using dog pancreas microsomes, and from genetic studies in *Saccharomyces cerevisiae* all illustrate the direct involvement of PDI in formation of native disulfide bonds [reviewed in Freedman et al. (1994)]. This confirms earlier data which pointed to correlations between the cellular and subcellular distributions of PDI and the activities of those cells and compartments in the biosynthesis of disulfide-bonded proteins [reviewed in Freedman (1984)]. It now appears that the major potential source of oxidizing equivalents for protein disulfide formation within the ER is oxidized glutathione (GSSG) (Hwang et al., 1992); this is present with reduced glutathione (GSH) at a total glutathione concentration probably close to that in the cytosol (5−10 mM) and at between a 1:1 and a 1:3 ratio of GSSG/GSH. Also within the ER, in addition to PDI, the hsp70 homolog known as BiP is believed to act as a molecular chaperone, interacting with nascent unfolded proteins and promoting protein folding and the assembly of multimeric proteins (Gething et al., 1986; Copeland et al., 1986; Flynn et al., 1989). The molecular role of this protein needs to be elucidated by examining its effect on a well-characterized folding reaction *in vitro*.

In our previous paper (Ruoppolo & Freedman, 1994) we prepared and characterized GS-RNase T_1, the product of converting each free Cys residue of reduced RNase T_1 to a mixed disulfide with glutathione. We showed that GS-RNase T_1 is unfolded in native conditions but can be refolded to a native-like state at NaCl concentration ≥1.5 M. We now describe the folding of this protein mixed disulfide (GS-RNase T_1), which is initiated by introducing thiol compounds to permit thiol−disulfide interchange and is linked to the isomerization of disulfide bonds (mixed disulfide to protein disulfide). In addition, we have investigated the effect of NaCl on this refolding and isomerization. In order to define further the action of PDI, we studied the effect of the enzyme as a catalyst of disulfide isomerization in the refolding of GS-RNase T_1, both in the absence and presence of NaCl. The addition of PDI resulted in catalysis of the refolding process, increasing the rate and the yield of the reaction. In our previous paper (Ruoppolo & Freedman, 1994), GS-RNase T_1 was shown to interact with BiP and to stimulate BiP ATPase activity. Here we have shown that, in the absence of ATP, BiP produced a block on the refolding of GS-RNase T_1; the refolding is then restored by the addition of Mg-ATP.

MATERIALS AND METHODS

RNase T_1 was provided as a generous gift by N. Pace (Texas A&M University Health Science Center). GS-RNase T_1 was prepared as previously described (Ruoppolo & Freedman, 1994); PDI was purified from bovine liver as described (Freedman et al., 1995); BiP was purified from bovine liver microsomes by a procedure (Rowling et al., 1994) modified from Flynn et al. (1989).

BSA (98−99% pure), Coomassie Blue R250, GSH, GSSG, hen egg-white lysozyme, iodoacetamide, and yeast RNA were obtained from Sigma Chemical Co. EDTA and Tris were purchased from Boehringer Mannheim GmbH. All the other reagents were HPLC grade from Fisons.

Reduced and oxidized glutathione stock solutions were made fresh daily in 0.1 M Tris-HCl and 0.2 M KCl (pH 7.5) at a concentration of 50 mM; 1 mM EDTA was added to the buffers to prevent oxidation catalyzed by traces of heavy metals. ATP and $MgCl_2$ solutions were made fresh daily in 0.1 M Tris-HCl, 0.2 M KCl (pH 7.5) at a concentration of 50 mM. For the experiments in the presence of high concentration of NaCl, the buffers contained 1.5 M NaCl.

The concentrations of solutions of native RNase T_1 and of all the modified forms were determined using an absorption of 1.67 at 278 nm for 1 mg/mL solution (Pace & Grimsley, 1988b). All other protein concentrations were determined by the method of Bradford (1976) as modified by Stoscheck (1990) with BSA as a standard.

Refolding Reaction. Lyophilized GS-RNase T_1 was dissolved to a concentration of approximately 100 μM in 0.01 M HCl and then diluted into the refolding buffer (0.1 M Tris-HCl, 0.2 M KCl, 1 mM EDTA pH 7.5). The final concentration of GS-RNase T_1 was 2 μM in experiments followed by fluorescence, 7.7 μM for experiments followed by RNase activity, 4 μM for HPLC analysis, and 30 μM for SDS−PAGE analysis. The refolding reactions were carried

out with different ratios of GSH and GSSG while maintaining a constant total glutathione concentration ([GSH] + 2[GSSG] = 5 mM). The desired amounts of GSH and GSSG stock solutions were added to initiate refolding; typically, final concentrations of the glutathione species were 5 mM GSH, 4 mM GSH/0.5 mM GSSG, and 2 mM GSH/1.5 mM GSSG. The pH of the solution was adjusted to 7.5 with Tris-base and the reaction carried out at 25 °C.

For the experiments in the presence of high concentration of NaCl, the samples were incubated in 0.1 M Tris, 0.2 M KCl, and 1 mM EDTA (pH 7.5) in the presence of 1.5 M NaCl at 25 °C for 6 or more hours to ensure that conformational equilibrium was reached, and then the disulfide interchange was started by adding GSH/GSSG.

When the refolding was carried out in the presence of PDI, the lyophilized enzyme was dissolved in 0.1 M Tris-HCl, 0.2 M KCl, and 1 mM EDTA, pH 7.5 and preincubated in the presence of the GSH/GSSG redox buffers for 10 min at 25 °C. This mixture was then added to the GS-RNase T_1 mixed disulfide, and the refolding continued at 25 °C as described.

For the refolding in the presence of BiP or lysozyme, the chaperone (or control protein) and GS-RNase T_1 were preincubated at 25 °C for 30 min, and then the reaction was started with the addition of GSH/GSSG. For the refolding in the presence of ATP, the chaperone and GS-RNase T_1 were incubated at equimolar concentrations and the reaction initiated by adding the glutathione species together with $MgCl_2$ and ATP; $MgCl_2$ and ATP final concentrations were 10 mM. Buffer conditions were as for nonenzymic refolding except that EDTA was excluded from the refolding buffer.

Fluorescence Analysis. The refolding reaction was monitored by the increase in protein fluorescence at 320 nm (2.5–5-nm bandwith) after excitation at 278 nm (10-nm bandwith) using 1-cm-square cuvettes in a Perkin-Elmer LS-5B spectrofluorimeter at 25 °C. Reactions in the presence of high concentration of PDI or in the presence of BiP, in which the absorbance of the sample at 280 nm was high, were carried out using an excitation wavelength of 295 nm. The excitation shutter was closed between the individual measurements to minimize radiation damage during the experiment. The kinetic data were analyzed by the programs Enzfitter (Elsevier-Biosoft, Cambridge, U.K.) and IGOR (P.O. Box 2088, Lake Oswego, OR 97035 USA). The results of the kinetic analysis were independent of the excitation wavelength.

Activity Assay. RNase T_1 activity was measured as previously described (Ruoppolo & Freedman, 1994) by following the decrease in A_{260} of a 82 μM RNA solution at 30 °C. The RNA solution was preincubated in the spectrophotometer for 2 min and checked for the absence of autohydrolysis. Then the protein solutions were added at a concentration ranging from 50 to 5 nM. The decrease in absorbance was measured for 3 min, and the initial slope $[\Delta A_{260}/\text{min}]/\mu g$ was used as a measure of the enzymatic activity.

The recovery of enzymatic activity at various time of refolding was measured as percent of residual activity relative to a solution of native RNase T_1 at the same concentration and maintained under the same experimental conditions.

Each set of data was obtained as the mean of three parallel, but independent folding experiments. The differences in activity recovery between refolding experiments performed in parallel were less than 3%; experiments performed completely independent of each other did not show larger variations.

HPLC Analysis. The refolding was monitored on a time-course basis by sampling aliquots (100 μL) of the refolding mixture at appropriate intervals. The aliquots were quenched by lowering the pH to 2 with the addition of an equal volume of 5% TFA and quickly vortexing. The acid-quenched samples were stored on ice prior to loading on the HPLC. At least duplicate experiments were performed, loading the samples in reverse order to check that the length of time prior to loading on HPLC did not influence the amounts of the various species observed.

The quenched refolding samples and standards were analyzed by HPLC using a Vydac 218TP54 reversed-phase C18 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 and standard GS-RNase T_1 and native RNase T_1 samples were eluted with a linear gradient of solvent B from 29% to 33% at flow rate of 1 mL/min. Protein monitoring was carried out at 220 nm.

SDS–PAGE Analysis. Samples (10 μL) of the refolding reaction were withdrawn at different times, and the free cysteines were alkylated by adding the same volume of 1 M iodoacetamide solution in the same refolding buffer. After 30 min at room temperature the aliquots were stored on ice. All stock solutions and incubations were kept in the dark. The alkylated samples were mixed with nonreducing SDS sample buffer just before the SDS–PAGE analysis which was carried out with the use of minigels containing 20% (w/v) acrylamide. After electrophoresis, the gels were stained for protein with Coomassie Blue R250. The band for the protein with native disulfides was well resolved from that of GS-RNase T_1.

PDI Assay. PDI was assayed by its reactivation of "scrambled" RNase A (s-RNase A) (Freedman et al., 1995), which contains incorrectly formed disulfide bonds. When incubated with s-RNase A, PDI catalyzes the reactivation of the substrate by isomerization of the incorrect disulfide bonds to the native ones. The rate at which RNase A activity increases is a measure of PDI activity.

RESULTS

Refolding by Disulfide Isomerization. The refolding of the unfolded mixed disulfide species GS-RNase T_1 arising from thiol–disulfide interchange in presence of GSH/GSSG was monitored by four independent methods. Intrinsic fluorescence monitors the major conformational change between folded and unfolded species and enzyme assay monitors the recovery of the stable native structure, while reverse-phase HPLC and SDS–PAGE in nonreducing conditions provide alternative characterizations of the surface properties and effective size of the protein.

Figure 2 shows the fluorescence changes accompanying GS-RNase T_1 refolding initiated by the addition of GSH/GSSG to permit thiol–disulfide interchange. There is an immediate blue-shift in the fluorescence emission maximum, from 349 nm (unfolded state) toward the native λ_{em} within the first minutes of reaction, followed by a slow increase in fluorescence intensity to a value just below that of a standard sample of native RNase T_1 maintained in the same conditions. Figure 3A shows the kinetics of the fluorescence

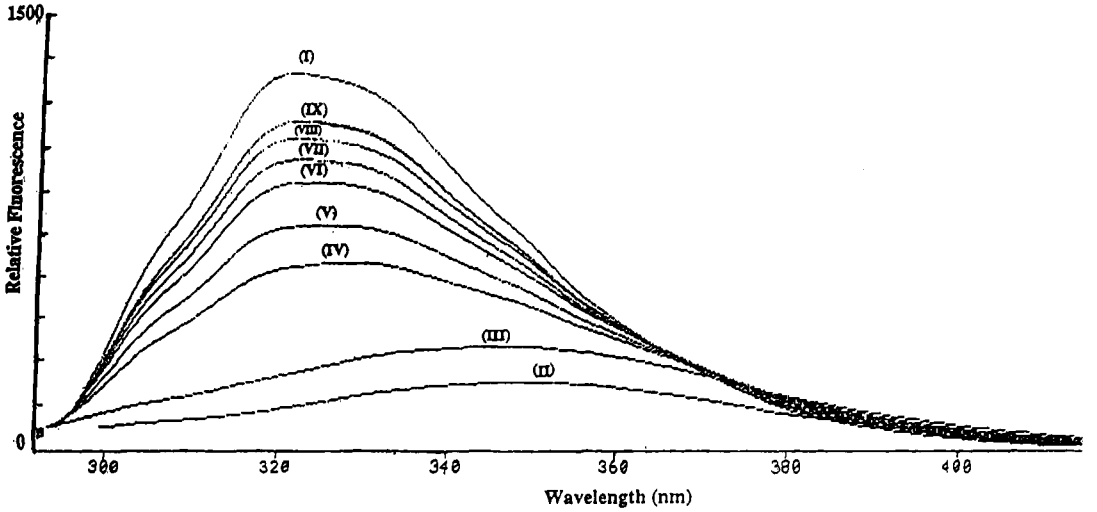

FIGURE 2: Fluorescence emission spectra of native RNase T_1 (I) and GS-RNase T_1 (II) in 0.1 M Tris, 0.2 M KCl, and 1 mM EDTA, pH 7.5. Refolding of 2 μM GS-RNase T_1 at 25 °C in the presence of 2 mM GSH/1.5 mM GSSG at time 30 s (III), 15 min (IV), 30 min (V), 1 h (VI), 2 h (VII), 3 h (VIII), and 4 and 5 h (IX).

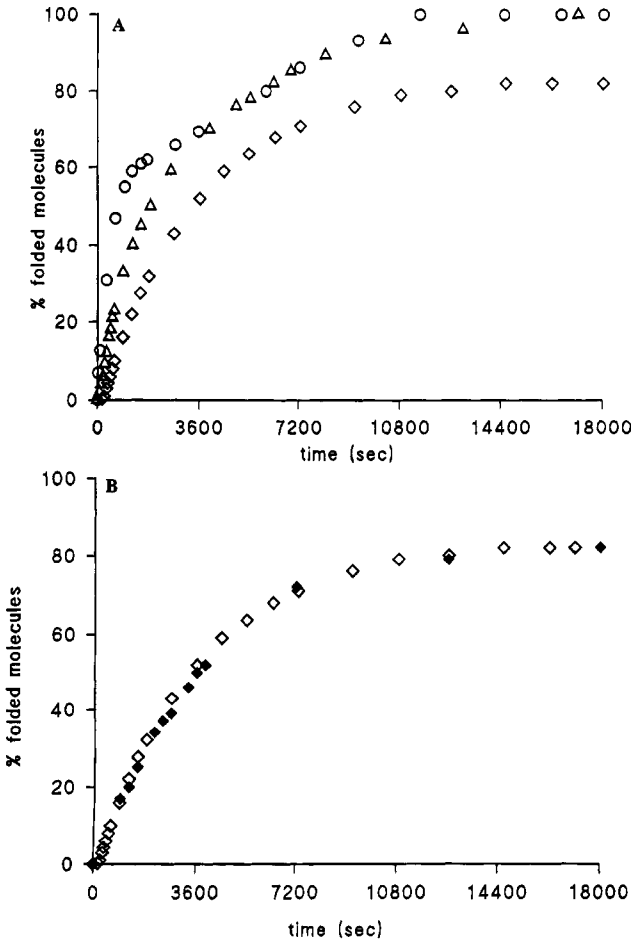

FIGURE 3: (A) Refolding of GS-RNase T_1 followed by the increase in fluorescence intensity in the presence of 5 mM GSH (○); 4 mM GSH/0.5 mM GSSG (△); 2 mM GSH/1.5 mM GSSG (◇). (B) Refolding of GS-RNase T_1 followed by fluorescence analysis (◇) and regain of the native enzymatic activity (◆) in the presence of 2 mM GSH/1.5 mM GSSG. The final protein concentrations were 2 μM for the fluorescence analysis and 7.7 μM for the activity analysis.

changes accompanying disulfide-linked refolding in the various redox conditions used. GS-RNase T_1 could be refolded in the presence of all the redox conditions used. The refolded product exhibited the fine structure of the fluorescence spectrum of the native protein; this together with the data from other techniques (below) supports the conclusion that the refolding in conditions of thiol−disulfide interchange generates primarily native RNase T_1. The yield, as determined by fluorescence, was high in every case, but the lowest yield (82%) was obtained in the presence of the most oxidative conditions (2 mM GSH/1.5 mM GSSG). Kinetic analysis of the data of Figure 3A shows that refolding in the presence of 2 mM GSH/1.5 mM GSSG showed an excellent fit with first-order kinetics, while refolding in the presence of 5 mM GSH or of 4 mM GSH/0.5 mM GSSG were well approximated by two sequential reactions of approximately equal amplitude. Data from the kinetic analyses are presented in Table 1.

Table 1: Kinetics of Refolding of GS-RNase T_1 by Disulfide Interchange

	fluorescence		activity	
GSH/ GSSG (mM)	rate constant (10^{-3} s^{-1})	yield (%)	rate constant (10^{-3} s^{-1})	yield (%)
5/0	2.77 ± 0.40 (k_1) 0.14 ± 0.02 (k_2) $r^2 = 0.996$	100	0.36 ± 0.01	95
4/0.5	1.10 ± 0.10 (k_1) 0.20 ± 0.13 (k_2) $r^2 = 0.999$	100	0.27 ± 0.01	95
2/1.5	0.28 ± 0.01	82	0.25 ± 0.01	82

The refolding of GS-RNase T_1 arising from disulfide isomerization was also monitored by following the regain of enzymatic activity, assaying aliquots of the refolding mixture at different times. Within the first minutes of refolding, a significant fraction of the native catalytic activity was regained. As shown in Figure 3B, for refolding in the presence of 2 mM GSH/1.5 mM GSSG, activity and fluorescence data show similar kinetics. In the presence of the other redox conditions (5 mM GSH or 4 mM GSH/0.5 mM GSSG), the kinetics of changes in fluorescence and activity accompanying refolding were not very similar, as shown in Table 1. Activity data in these conditions showed a good fit with first-order kinetics, while the fluorescence kinetics were more complex (see above). Possibly, in these conditions, some intermediates have an ordered structure around the single tryptophan residue contributing to the fluorescence-detected kinetics, but they do not have native catalytic activity.

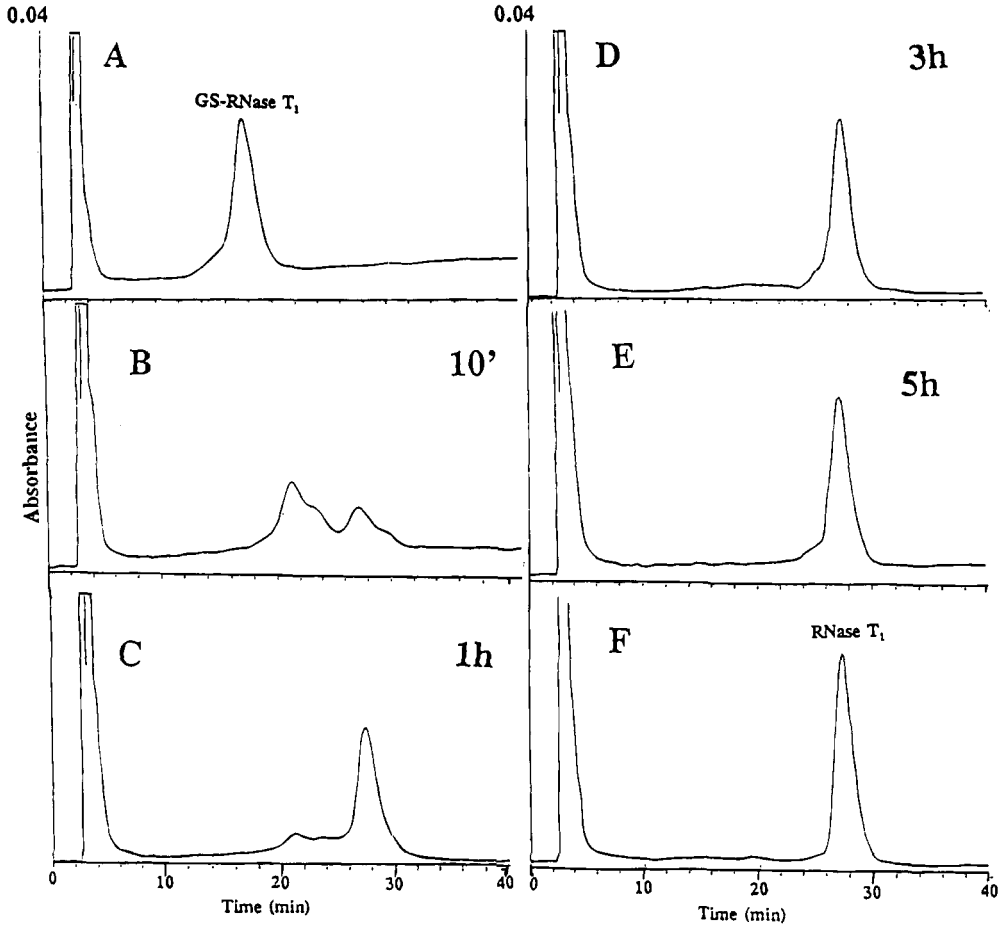

FIGURE 4: HPLC analysis of GS-RNase T_1 refolding reaction carried out in the presence of 2 mM GSH/1.5 mM GSSG. Aliquots were withdrawn from the refolding mixture at time 10 min (panel B), 1 h (panel C), 3 h (panel D), and 5 h (panel E). Refolding and analysis conditions are described in the text. Control aliquots of GS-RNase T_1 (panel A) and of native RNase T_1 (panel F) were also analyzed.

In a control experiment, GS-RNase T_1 was incubated in the refolding buffer without GSH/GSSG to check spontaneous isomerization. As expected, in the absence of thiol compounds, GS-RNase T_1 remained unfolded, by fluorescence criteria, and did not show any recovery of enzymatic activity, thus confirming that refolding is strictly linked to disulfide isomerization under the conditions described. Conversely, when incubated in the presence of redox buffer under the same experimental conditions described above, native RNase T_1 showed no changes in the intensity or λ_{em} of intrinsic fluorescence or in its catalytic activity.

Figure 4 shows the HPLC analysis of the mixture of species sampled at different times from the refolding reaction in the presence of 2 mM GSH/1.5 mM GSSG. At the different times, different acid-trapped disulfide intermediates were present. Under the HPLC conditions described in Materials and Methods the various species were not well-resolved, and no better separation of the intermediates was obtained in a very wide variety of modified gradient conditions. However, GS-RNase T_1 (Figure 4, panel A) and native RNase T_1 (Figure 4, panel F) were widely separated from each other and readily distinguished from the other species present. The HPLC analysis showed clearly that the initial mixed disulfide disappeared rapidly, yielding (at least) two distinct intermediates, while the final product of the refolding reaction (Figure 4, panel E) is eluted at the same retention time as the native protein.

Under the other redox conditions used above, the same intermediates accumulated according to HPLC analysis (data not shown), but at different rates and to different levels. The rate of appearance of the native RNase T_1 was much faster in the more reducing conditions than that observed in the presence of 2 mM GSH/1.5 mM GSSG. No detailed kinetic analysis of the appearance and disappearance of intermediates was performed because quantitation of individual species was not possible under the HPLC conditions used.

For SDS–PAGE analysis of refolding linked to disulfide isomerization, samples of the refolding reaction were withdrawn at different times, alkylated with iodoacetamide, and electrophoretically resolved under nonreducing conditions. The analysis (data not shown) showed that the band corresponding to the native protein increased with time, at the expense of the band corresponding to GS-RNase T_1. On the other hand, the concentrations of intermediate bands remained very small throughout the time course of the isomerization. The data essentially confirm those from the HPLC analysis.

Refolding in the Presence of High Salt Concentration. In the presence of 1.5 M NaCl, GS-RNase T_1 acquired a native-like structure, as monitored by fluorescence analysis, but exhibited only 2.5% of native enzyme activity showing that the salt-induced refolding is accompanied by a limited regain of the enzymatic activity (Ruoppolo & Freedman, 1994). When GS-RNase T_1 disulfide isomerization was studied in the presence of a high concentration of NaCl, the thiol/disulfide compounds were added to the mixture after GS-RNase T_1 was incubated in the presence of 1.5 M NaCl. The disulfide isomerization reaction was then monitored by the recovery of the native enzymatic activity. As shown in Table 2, under all the redox conditions used, the refolding

Table 2: Kinetics of Refolding of GS-RNase T_1 by Disulfide Interchange in the Presence of 1.5 M NaCl[a]

GSH/GSSG (mM)	1.5 M NaCl rate constant (10^{-3} s^{-1})	yield (%)	acceleration[b]
5/0	0.64 ± 0.01	93	1.8
4/0.5	0.65 ± 0.04	95	2.4
2/1.5	0.66 ± 0.06	83	2.6

[a] The reaction was monitored by RNase activity. [b] Acceleration is the ratio of the rate constant quoted in this table and the corresponding rate constant in the same redox conditions quoted in Table 1.

is faster than at lower ionic strength, ranging from 1.8 times faster in the presence of 5 mM GSH to 2.6 times faster in the presence of 2 mM GSH/1.5 mM GSSG. No significant changes were detected in the yield of the reaction in the presence of NaCl. The above results confirm that the presence of salt stabilizes a compact state of GS-RNase T_1 (Ruoppolo & Freedman, 1994); this appears to bring the cysteine residues together so that the rate of formation of intramolecular disulfide bonds is enhanced.

Refolding Assisted by PDI. In experiments studying the effect of protein disulfide isomerase (PDI), the enzyme was incubated in the presence of the redox buffer and then added to the GS-RNase T_1 mixed disulfide. The total concentration of glutathione species ([GSH] + 2[GSSG] = 5 mM) was much greater than that of substrate mixed disulfide groups and redox-active dithiol/disulfide couples of PDI, so that the GSH/GSSG redox pair determined the redox potential of the mixture. The PDI-assisted refolding reactions were followed by fluorescence analysis and recovery of ribonuclease activity.

Fluorescence analysis (Figure 5A and Table 3) showed that, in the presence of 2 mM GSH/1.5 mM GSSG, addition of PDI at a ratio of 0.025 ([PDI]/[GS-RNase T_1]) catalyzed the refolding reaction leading to a 2-fold increase in the rate of GS-RNase T_1 refolding, together with a small increase in the yield of reaction. An increase in PDI concentrations (to 0.1 and to 0.25 = [PDI]/[GS-RNase T_1]) led to further increases in the measured rate constants as well as in the yield of the reaction. The dependence of the rate constant for folding on PDI concentration shows a downward curvature (Figure 5B) that is reminiscent of a binding curve as previously observed by Schönbrunner and Schmid (1992) in the PDI catalysis of refolding of reduced and denatured RNase T_1. No change was observed when PDI was preincubated in the refolding buffer in the absence of thiol compounds and then added to GS-RNase T_1 (0.25 = [PDI]/[GS-RNase T_1]); the mixed disulfide remained unfolded by fluorescence criteria.

Ribonuclease activity data on the enzyme-catalyzed refolding (Table 3) yield kinetics similar to those from fluorescence at the lowest concentration of PDI. When PDI concentration was increased, the recovery of enzymatic activity was too fast to be monitored by the method described, where no attempt was made to prevent further refolding during the ribonuclease assay, so that the level of active ribonuclease was increasing during the 3 min of the assay. The experiments monitoring recovery of enzyme activity in the presence of the higher concentrations of PDI were used only to check the final yields of the reactions, which were in good agreement with those determined by fluorescence data.

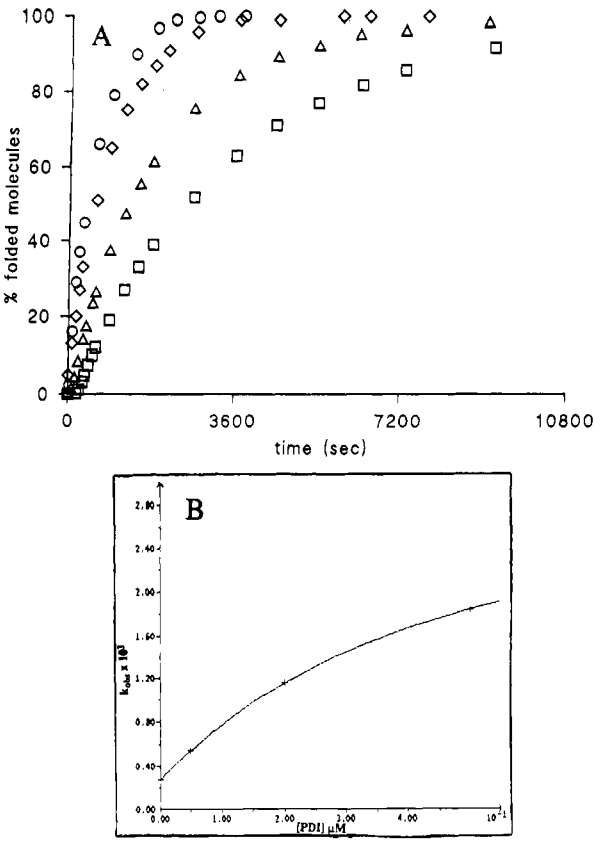

FIGURE 5: Refolding of GS-RNase T_1 in the presence of PDI followed by fluorescence analysis. (A) PDI was incubated with 2 mM GSH/1.5 mM GSSG for 10 min at 25 °C and then added to the mixed disulfide (2 μM) at a ratio of 0.025 (△), 0.1 (◇), and 0.25 (○) ([PDI]/[GS-RNase T_1]). The refolding reaction in the absence of PDI is shown as well (□). (B) The dependence of the rate constant for folding on PDI concentration (constants derived from the data shown in panel A).

Table 3: Kinetics of Refolding of GS-RNase T_1 in the Presence of PDI[a]

[PDI]/[GS-RNase T_1]	fluorescence rate constant (10^{-3} s^{-1})	fluorescence yield (%)	activity rate constant (10^{-3} s^{-1})	activity yield (%)
0	0.28 ± 0.01	82	0.25 ± 0.01	82
0.025	0.54 ± 0.01	86	0.51 ± 0.02	85
0.1	1.15 ± 0.01	100	N.D.	97
0.25	1.84 ± 0.07	100	N.D.	100

[a] The reaction was performed in the presence of 2 mM GSH/1.5 mM GSSG. [b] ND = not determined.

PDI-assisted refolding in more reducing conditions was monitored only by fluorescence. The kinetic analysis again indicated sequential kinetics (Table 4), as for the spontaneous refolding in these redox conditions, but both rate processes were clearly catalyzed by very low levels of PDI (Table 4, cf. Table 1). The catalysis of the first reaction of the sequential processes was approximately 2-fold at an enzyme/substrate ratio of 0.025 ([PDI]/[GS-RNase T_1]), similar to the overall catalysis by PDI observed at this stoichiometry in more oxidizing conditions (Table 4, cf. Table 3). Catalysis of the second of the sequential reactions was less marked. No further experiments were carried out on PDI catalysis of refolding in these redox conditions, since the kinetic analysis revealed the existence of an inactive folding intermediate, requiring more extensive characterization which was outside the scope of this study.

Table 4: Kinetics of Refolding of GS-RNase T_1 in the Presence of PDI in the Most Reducing Conditions

[PDI]/ [GS-RNase T_1]	GSH/ GSSG (mM)	fluorescence: rate constant (10^{-3} s^{-1})	fluorescence: yield (%)
0	5/0	2.77 ± 0.40 (k_1) 0.14 ± 0.02 (k_2) $r^2 = 0.996$	100
0.025	5/0	4.88 ± 0.34 (k_1) 0.19 ± 0.01 (k_2) $r^2 = 0.999$	100
0	4/0.5	1.10 ± 0.10 (k_1) 0.20 ± 0.13 (k_2) $r^2 = 0.999$	100
0.025	4/0.5	2.89 ± 0.22 (k_1) 0.37 ± 0.01 (k_2) $r^2 = 0.999$	100

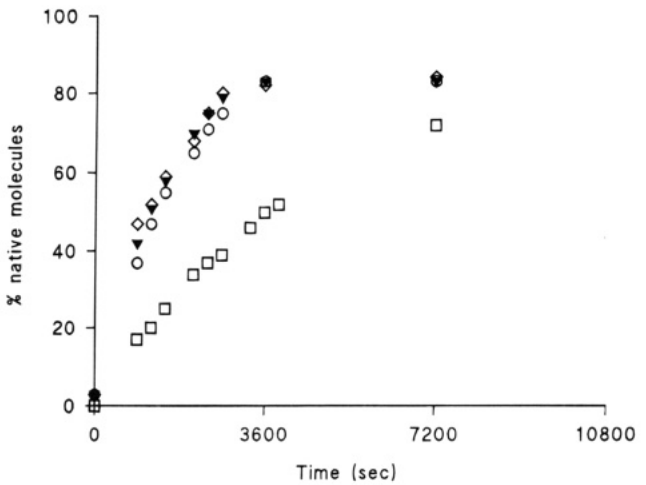

FIGURE 6: PDI-assisted refolding reaction of GS-RNase T_1 in the presence of 1.5 M NaCl followed by recovery of the native ribonuclease activity. Refolding was monitored in the absence of PDI [(○) $k_{obs} = 0.66 \times 10^{-3}$ s^{-1}] and at [PDI]/[GS-RNase T_1] ratio of 0.025 [(▼) $k_{obs} = 0.76 \times 10^{-3}$ s^{-1}] and 0.25 [(◇) $k_{obs} = 0.79 \times 10^{-3}$ s^{-1}]. For comparison, data are shown in the absence of both PDI and added NaCl [(□) $k_{obs} = 0.25 \times 10^{-3}$ s^{-1}].

The effect of PDI in catalyzing the refolding of GS-RNase T_1 was also analyzed in presence of a high concentration of NaCl. GS-RNase T_1 was incubated in the presence of 1.5 M NaCl for 6 or more hours, PDI was preincubated in the presence of the redox buffer (2 mM GSH/1.5 mM GSSG) containing 1.5 M NaCl for 10 min, and the refolding reaction was then initiated by adding the solution of PDI plus redox buffer to the GS-RNase T_1 solution. The refolding reaction in the presence of 1.5 M NaCl and PDI was followed by recovery of ribonuclease activity. As shown in Figure 6, addition of PDI at a ratio of 0.025 ([PDI]/[GS-RNase T_1]) did not produce any increase in the measured rate of refolding nor did a further increase in PDI concentration to a ratio of 0.25 ([PDI]/[GS-RNase T_1]); derived values of rate constants are quoted in the legend to Figure 6. As a control, the activity of PDI in catalyzing the reactivation of s-RNase A was used as a sensitive assay of the enzyme (Freedman et al., 1995) to detect any direct effects of high NaCl concentrations on PDI. In this assay, the activity of PDI was found to be unaffected by the presence of NaCl; PDI activity in the presence of 1.5 M NaCl was 98% of the activity measured in standard conditions. It therefore appears that PDI does not further catalyze the rapid reactivation of GS-RNase T_1 produced by nonenzymatic disulfide interchange

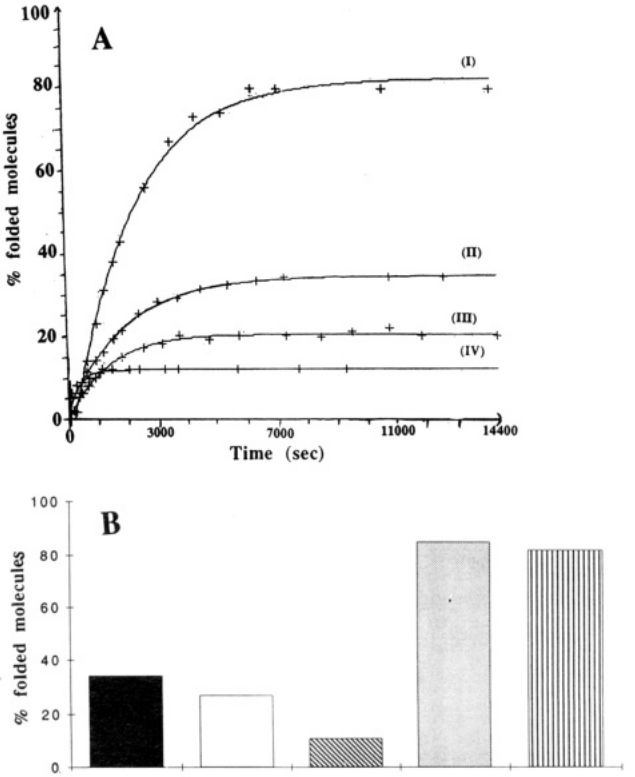

FIGURE 7: (A) Refolding reaction of GS-RNase T_1 in the presence of BiP followed by fluorescence analysis. BiP was incubated with GS-RNase T_1 at a ratio ([BiP]/[GS-RNase T_1]) of 5/1 (IV), 1/1 (III), and 0.5/1 (II) for 30 min at 25 °C. The refolding reaction was then started by adding thiol compounds (2 mM GSH/1.5 mM GSSG). The reaction in absence of BiP but in the presence of a control protein, lysozyme ([lysozyme]/[GS-RNase T_1] = 1/1), is also shown (I). (B) Regain of the native enzymatic activity of GS-RNase T_1 in the presence of BiP ± ATP after 4 h of the refolding reaction. [BiP]/[GS-RNase T_1] = 0.5/1 (closed bar), [BiP]/[GS-RNase T_1] = 1/1 (open bar), [BiP]/[GS-RNase T_1] = 5/1 (hatched bar), and [BiP]/[GS-RNase T_1] = 1/1, 10 mM ATP, 10 mM $MgCl_2$ (dotted bar). For comparison, data are shown in the absence of both BiP and added ATP-$MgCl_2$ (stripped bar).

in high salt conditions despite being fully active in these conditions. Possibly, in the compact, high-salt state of GS-RNase T_1, the catalysis by PDI of disulfide interchange was limited by the relatively poor accessibility to the enzyme of the mixed disulfide groups.

Refolding in the Presence of BiP. For experiments in the presence of BiP, the chaperone was incubated with GS-RNase T_1 directly in the fluorimeter cuvette for 30 min at 25 °C to permit formation of complex between the chaperone and the unfolded protein. The refolding reaction was then initiated by adding the glutathione species (2 mM GSH/1.5 mM GSSG). As shown in Figure 7A, the presence of BiP strikingly reduced the conversion of the unfolded mixed disulfide to native RNase T_1, as expected in the absence of ATP. The yield decreased to 20% at equimolar concentrations of BiP and GS-RNase T_1, whereas an equimolar concentration of a control protein (lysozyme) had no effect on the yield of GS-RNase T_1 refolding. A further increase in the BiP concentration led to an almost complete blockage of the refolding reaction.

Refolding in the Presence of BiP and ATP. The refolding experiments in the presence of BiP were also monitored by following the regain of ribonuclease activity. The chaperone was incubated with GS-RNase T_1 directly in the spectrophotometer cuvette for 30 min at 25 °C to permit formation of the complex. The refolding reaction was then initiated

by adding 2 mM GSH/1.5 mM GSSG. For the experiment in the presence of ATP, BiP was incubated with GS-RNase T_1 at equimolar concentrations. The refolding was then initiated by adding the glutathione species (2 mM GSH/1.5 mM GSSG) and ATP and $MgCl_2$ at 10 mM final concentrations.

As shown in Figure 7B, enzyme activity data on refolding in presence of BiP and in absence of ATP, demonstrated a BiP-dependent block on refolding, comparable to that observed directly by fluorescence (Figure 7A). The yield of active RNase T_1 after 4 h of refolding decreased as the ratio of [BiP]/[GS-RNase T_1] increased (0.5/1, yield 34%; 1/1, yield = 27%; 5/1, yield = 11%). When the refolding was performed in the presence of BiP + ATP, recovery of enzyme activity was 85%, slightly higher than that observed in absence of both BiP and ATP. Hence ATP relieves the BiP-dependent block on refolding. (The high concentration of ATP did not affect the ribonuclease activity in this assay.)

DISCUSSION

Most studies on protein folding linked to the formation of native disulfide bonds have used fully reduced proteins as the starting point. In this work we have employed a mixed disulfide between a protein and the major cellular thiol compound glutathione. Apart from its possible physiological relevance (see below), this substrate has a number of advantageous features, defined in our previous study (Ruoppolo & Freedman, 1994). It is a single chemical species which is stable, soluble, and fully unfolded in native conditions; on the other hand, the presence of the glutathione groups is not a major limitation on the flexibility of the molecule, since in high ionic strength conditions it can fold reversibly into a native-like conformation. The mixed disulfide is also an interesting substrate for conversion to the native disulfide-bonded protein, in that folding to the native state is induced by a series of disulfide isomerizations, with no net oxidation.

The data presented here demonstrate that GS-RNase T_1 can be refolded in high yield to native RNase T_1 in conditions which permit thiol−disulfide interchange, i.e., in the presence of a thiol−disulfide redox buffer comprising GSH ± GSSG. Refolding was monitored by a number of independent techniques. The recovery of enzyme activity in high yield confirmed that the predominant product was native RNase T_1, and kinetic analysis indicated that the appearance of native enzyme could be described by a single first-order rate constant. However, this method only reports on the formation of the final product. Intrinsic fluorescence is a sensitive and convenient continuous assay for kinetic analysis of refolding; in some conditions it indicated that the process involved sequential reactions. Hence the fluorescence analysis was detecting inactive intermediate species whose fluorescence signal was distinct from that of the initial unfolded starting material. The presence of partly folded intermediates was confirmed directly by HPLC analysis; the structures of these intermediates have not been defined in this work but their disulfide status is currently under study. The HPLC analysis also confirmed that the final product of reaction was indistinguishable from native RNase T_1, and this was further supported by nonreducing SDS−PAGE. The folding to form native RNase T_1 was wholly dependent on thiol−disulfide interchange with the added redox buffer components; in their absence, the mixed disulfide GS-RNase T_1 was entirely stable in all the conditions used.

Folding to generate native RNase T_1 from the mixed disulfide occurred over a wide range of redox conditions. The most reducing conditions were those where thiol−disulfide interchange was initiated by addition of 5 mM GSH. The protein substrate itself contributed only between 0.01 and 0.12 mM disulfides (as protein-SSG), so that the redox conditions established were as reducing as those found within the cytosol. Nevertheless, in these conditions, the mixed disulfide was converted rapidly and in high yield to the native disulfide-bonded protein, with no accumulation of reduced protein, indicating the great stability of the native disulfide bonds. The most oxidizing conditions employed (2 mM GSH + 1.5 mM GSSG) closely model the concentrations of these species within the lumen of the ER, the site at which native disulfide bonds form during biosynthesis. Interestingly, the overall kinetics of formation of the native protein were slowest in these conditions. A more detailed kinetic analysis of the influence of redox conditions on individual steps in the process would be interesting but requires the resolution and structural characterization of the intermediate species.

The interaction between a protein-SS-glutathione mixed disulfide substrate and a GSH/GSSG redox buffer, as used in these studies, may be an instructive model of the cellular situation. The nascent chain emerges into the lumen into conditions of high glutathione concentration, and it may be that unpaired Cys residues are converted to mixed disulfides before the complete translocation of a domain permits the cooperative formation of native disulfide bonds. This suggestion is supported by the observation that a mutant lysozyme, lacking a single Cys residue, is secreted from yeast with the partner Cys residue linked to glutathione as a mixed disulfide (Hayano et al., 1993).

Since it is known that the formation of native disulfide bonds is catalyzed by PDI (Freedman et al., 1994; Freedman, 1995), we tried to influence the refolding process enzymatically. Under our conditions, PDI at substoichiometric concentrations catalyzed formation of the native disulfide-bonded protein product. When intermediates in the folding process are better defined, it will be possible to analyze the action of PDI in greater detail.

The results described here on kinetics of formation of native disulfide-bonded RNase T_1, starting from its mixed disulfide with glutathione, can be compared with studies by Schönbrunner and Schmid (1992) in which refolding to yield the native disulfide-bonded protein was initiated by adding a glutathione redox system to fully reduced RNase T_1. The two studies with the different starting materials were carried out with similar protein concentrations and at similar pH and temperature. In the work of Schönbrunner and Schmid (1992), the redox conditions were 4 mM GSH + 0.4 mM GSSG, close to the intermediate redox conditions used in our work, and the kinetic analysis of refolding was mainly based on changes in intrinsic fluorescence. Hence the studies are closely comparable except for the difference in the initial state of the protein. As in our studies starting from the mixed disulfide, the kinetics of refolding of reduced protein, monitored by fluorescence, indicate an additional fast phase not detected by other methods, implying the formation of transient nonnative intermediates, and (again as in our studies) the concentrations of such intermediates, monitored by gel electrophoresis, are low throughout the refolding process.

Interestingly, the rates of refolding of the two forms of the protein are very similar, but the catalytic effect of PDI is more marked with the mixed disulfide substrate, in which refolding involves a series of disulfide isomerizations without net oxidation of protein thiols. Thus, in the absence of any catalyst of refolding, the overall time constant for refolding determined by Schönbrunner and Schmid (4300 s) corresponds to a rate constant of 0.23×10^{-3} s^{-1}, which is very close to our values in similar redox conditions ($0.25-0.28 \times 10^{-3}$ s^{-1}; see Table 1). However, the catalysis of refolding by PDI reported by Schönbrunner and Schmid (1992) is 2.9-fold at a PDI/reduced RNase T_1 ratio of 0.64:1, increasing to 7-fold in presence of a 4-fold molar excess of PDI. In our work starting with the mixed disulfide (Table 3), 4-fold catalysis of refolding is observed at a PDI/substrate ratio of 0.1, and catalysis is almost 7-fold at a ratio of 0.25:1. Under redox conditions comparable to those of Schönbrunner and Schmid (1922), we find 2-fold catalysis of refolding of the mixed disulfide at a stoichiometry of 0.025:1 (Table 4). Thus, unless there is a major discrepancy in the specific activities of the PDI samples used in the two studies, the mixed disulfide appears to be a preferred substrate for PDI.

BiP is known to bind short peptides and unfolded proteins (but not native proteins), to show ATPase activity *in vitro* which is specifically stimulated by peptides and unfolded proteins, and to be required *in vivo* for the correct folding and assembly of many secretory and cell-surface proteins. Predictably, we found in previous work that GS-RNase T_1 stimulated the ATPase activity of BiP whereas native RNase T_1 had no such effect (Ruoppolo & Freedman, 1984). We have now shown that the binding of GS-RNase T_1 to BiP (in absence of ATP) blocks its refolding to native RNase T_1 by disulfide interchange. This implies that the binding to BiP restricts the conformational flexibility of the unfolded mixed disulfide such that Cys residues of the bound protein are sterically prevented from approaching and forming intramolecular protein disulfide bonds. In the presence of adenine nucleotides, the unfolded protein is released and refolding resumes; activity data in fact showed a recovery of 85% of native catalytic activity.

The concentrations of folding catalysts and molecular chaperones in the ER are much greater than those used in the experiments performed here, so the concentrations used may have been insufficient to produce the maximum effect on the rate of refolding of GS-RNase T_1. Furthermore, RNase T_1 has been extensively employed in protein refolding studies precisely because the denatured and reduced forms are relatively soluble. Effects of PDI and/or BiP on the rate or yield of refolding might be more dramatic with other protein substrates.

In the presence of 1.5 M NaCl, GS-RNase T_1 can adopt a compact, native-like structure (Ruoppolo & Freedman, 1994). Initiation of thiol−disulfide interchange in these conditions has shown that the rate of formation of native disulfide bonds is significantly enhanced. It appears that the compact conformation limits the possibilities for disulfide interchange, favoring the formation of the native intramolecular disulfides. These conformational constraints must nevertheless permit GSH to have initial access to buried protein-SSG groups since the initiation of disulfide interchange in this substrate requires the removal of a bound GS group (P-SSG + GSH → P-SH + GSSG) prior to an intramolecular step of thiol−disulfide interchange (P-SH + P-SSG → P-SS-P + GSH). In the presence of 1.5 M NaCl, PDI at almost stoichiometric concentrations did not catalyze the reaction; hence the active site dithiol−disulfide groups of the enzyme (Freedman et al., 1994) were presumably not able to access RNase T_1 thiols or mixed disulfides in the compact native-like conformation present in high ionic strength conditions.

The data presented here indicate that the GS-S-protein mixed disulfide derivatives are ideal model unfolded proteins that can be used as substrates for detailed studies on secretory protein folding *in vitro* and on the interaction between unfolded proteins and facilitators of protein folding.

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