Glutathione-Dependent Pathways of Refolding of RNase T_1 by Oxidation and Disulfide Isomerization: Catalysis by Protein Disulfide Isomerase[†,‡]

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ABSTRACT: Protein folding, associated with oxidation and isomerization of disulfide bonds, was studied using reduced and denatured RNase T_1 (rd-RNase T_1) and mixed disulfide between glutathione and reduced RNase T_1 (GS-RNase T_1) as starting materials. Folding was initiated by addition of free glutathione (GSH + GSSG) and was monitored by electrospray mass spectrometry (ES-MS) time-course analysis. This permitted both the identification and quantitation of the population of intermediates present during the refolding process. Refolding experiments were performed in the presence of different absolute concentrations of glutathione species while keeping the redox potential fixed, in order to evaluate the effect of the glutathione concentration on the distribution of the refolding intermediates. All the analyses indicate a pathway of sequential reactions in the formation of native RNase T_1 which occurs via the reiteration of two steps: (i) formation of a species containing both mixed disulfides with glutathione and free protein thiols, and (ii) formation of an intramolecular disulfide via thiol−disulfide interchange reaction betwen them. Refolding of rd-RNase T_1 and GS-RNase T_1 was also performed in the presence of protein disulfide isomerase (PDI). Addition of PDI led to a catalysis of each individual reaction of the entire process without altering the refolding pathway. Refolding reactions carried out at different absolute concentrations of glutathione proved that GSH and/or GSSG participate directly in the reaction catalyzed by PDI. On the basis of these experiments and previous results on the refolding of RNase A [Torella, C., Ruoppolo, M., Marino, G., & Pucci, P. (1994) *FEBS Lett. 352*, 301−306], a hypothesis of a general pathway for folding of S−S containing proteins is proposed.

Folding of disulfide-containing proteins is thermodynamically associated with formation of the unique set of native disulfide bonds (Creighton, 1984). As a result, many theoretical and experimental studies have addressed the characterization of disulfide-bonded intermediates in order to elucidate protein folding pathways by determining the appearance of these intermediates and their interconversion rates (Creighton, 1992; Gilbert, 1994). Some of these studies have focused on the process of initial biosynthetic formation of native disulfides which *in vivo* takes place in the endoplasmic reticulum (ER)[1] (Freedman, 1995). According to present evidence, the major potential source of oxidizing equivalents for protein disulfide formation within the ER is oxidized glutathione (Hwang et al., 1992).

To characterize the detailed mechanism and pathway of protein disulfide formation, it is possible to work with synthetic peptides containing two Cys residues [see, e.g., Darby et al. (1994)] as model systems, but such systems do not have the potential for intramolecular thiol−disulfide interchange leading to disulfide isomerization. Such isomerizations have been found to be significant in the case of the best-studied proteins, namely, bovine pancreatic trypsin inhibitor (BPTI, six Cys residues, three disulfides) and bovine pancreatic ribonuclease A (RNase A, eight Cys residues, four disulfides) as illustrated by previous work (Creighton, 1992; Creighton et al., 1993, 1995; Rothwarf & Scheraga, 1993a, 1993b−c; Weissman & Kim, 1995). The simplest realistic model for studying the folding of disulfide-containing proteins is RNase T_1 since it contains only two disulfide bonds (Cys2−Cys10 and Cys6−Cys103). When the disulfide bonds are reduced, RNase T_1 is essentially unfolded but can adopt compact native-like structure in the presence of high concentrations of NaCl (Oobatake et al., 1979; Pace et al., 1988a). Refolding of reduced and denatured RNase T_1 (rd-RNase T_1) in the presence of reduced and oxidized DTT produces primarily intermediates having a single disulfide bond between any pair of the residues Cys2, Cys6, and Cys10; subsequently, the species Cys6−Cys103 accumulates (Pace & Creighton, 1986). Kinetic analysis of rd-RNase T_1 refolding in the presence of a glutathione redox system was

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[‡] This paper is dedicated to Prof. A. Ballio on the occasion of his 75th birthday.

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[1] Abbreviations: BSA, bovine serum albumin; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DTT, reduced dithiothreitol; EDTA, ethylenediaminetetraacetic acid, disodium salt; ER, endoplasmic reticulum; ES-MS, electrospray mass spectrometry; GSH, reduced glutathione; GSSG, oxidised glutathione; HPLC, high-performance liquid chromatography; GS-RNase T_1, mixed disulfide between glutathione and reduced ribonuclease T_1; IAM, iodoacetamide; PDI, protein disulfide isomerase; rd-RNase T_1, reduced and denatured ribonuclease T_1; TFA, trifluoroacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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based mainly on fluorescence analysis reporting an initial fast-phase in the process (Schönbrunner & Schmid, 1992). Conversely, Mücke and Schmid (1994) reported that the presence of the two native disulfide bonds decreases the rate at which the protein folds when it is diluted from denaturant, even though the disulfide bonds substantially increase the stability of the native state. Finally, the folding of a novel substrate GS-RNase T_1, the product of converting each free Cys residue of reduced and denatured RNase T_1 to a mixed disulfide with glutathione, has been described (Ruoppolo & Freedman, 1994, 1995). GS-RNase T_1 is fully unfolded in "native" conditions but could 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. GS-RNase T_1 refolding was monitored by a number of independent techniques, such as recovery of enzyme activity and intrinsic fluorescence. However, these methods mainly report the formation of the final product, although fluorescence analysis detected partially folded intermediates whose presence was confirmed by HPLC analysis.

In the endoplasmic reticulum of eucaryotic cells, disulfide bond formation is dependent on protein disulfide isomerase (PDI) which is present at high concentrations and functions in the presence of millimolar concentrations of GSH and GSSG (Freedman et al., 1994; Freedman, 1995). Previous studies showed that the addition of PDI catalyzes the refolding both of reduced and denatured RNase T_1 (Schönbrunner & Schmid, 1992) and of GS-RNase T_1 in which refolding involves a series of disulfide isomerizations without net oxidation of protein thiols (Ruoppolo & Freedman, 1995).

In a previous paper (Torella et al., 1994), we described the use of electrospray-mass spectrometry (ES-MS) time-course analysis to obtain significant data for kinetic and structural studies on the refolding pathway of a disulfide-containing protein. The experimental approach is based upon the determination of the molecular weight of the alkylated disulfide-bonded intermediates through a time-course analysis of the refolding process. The alkyl group introduced with the quenching reaction, increased the molecular weight of the intermediates by a fixed mass, 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 study the refolding of reduced and denatured bovine pancreatic RNase A (Torella et al., 1994) providing interesting evidence for the existence of differently balanced populations of disulfide-containing intermediates.

In this paper we describe the application of the ES-MS time-course analysis illustrated above, in the structural characterization of the disulfide-bonded intermediates present in the refolding of rd-RNase T_1 or GS-RNase T_1 in the presence of a glutathione redox system which was shown to be efficient for both refolding processes. Figure 1 represents all possible intermediates and equilibria in the refolding of a two-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 nS), mixed disulfides with glutathione (nG), and free cysteine residues (nH). The scheme shown in Figure 1 is derived from an analogous scheme previously reported for RNase A (Konishi et al., 1981).

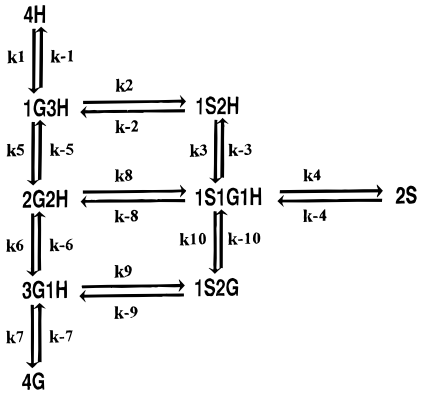

FIGURE 1: Representation of all possible protein intermediates and equilibria in the refolding of a two disulfide bond containing protein in the presence of a glutathione redox system. Intramolecular disulfide bonds are indicated as nS, mixed disulfide with glutathione as nG, and free cysteine residues as nH. Free glutathione (GSH/GSSG) is not represented. Downward vertical arrows represent reactions with GSSG to generate protein−SSG + GSH, and upward vertical arrows represent reactions with GSH to reverse this process. Horizontal arrows to the right represent unimolecular reactions to convert protein−SSG mixed disulfide into protein disulfide + GSH; horizontal arrows to the left represent reduction of protein disulfide by GSH to generate protein−SSG.

We used two derivatives of RNase T_1, rd-RNase T_1 and GS-RNase T_1 (named 4H and 4G, respectively, in Figure 1), in order to evaluate the effect of different starting materials on the refolding pathway and on the distribution of the intermediates along the whole process. The refolding process was also studied in the presence of PDI in order to define the effect of the enzyme as a catalyst on the refolding pathway. The addition of PDI resulted in a catalysis of each individual step of the refolding process. Finally, the refolding experiments were performed at different absolute concentrations of reduced and oxidized glutathione while maintaining a constant redox potential, in order to evaluate the effect of glutathione as a reactant on the distribution of the intermediates and on the catalysis by PDI.

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).

BSA (98−99% pure), DTNB, DTT, EDTA, GSH, GSSG, guanidinium chloride, and hen egg-white lysozyme 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 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) with BSA as a standard.

Preparation of Reduced and Denatured RNase T_1 (rd-RNase T_1). RNase T_1 was reduced at a concentration of about 10 mg/mL in 0.1 M Tris-HCl and 1 mM EDTA (pH 8.5) containing 6 M guanidinium chloride by incubation with reduced DTT (DTT mol/S−S mol = 50/1) for 2 h at 37 °C,

under nitrogen atmosphere. After the addition of 0.2 vol of 1 M HCl, the reaction mixture was desalted on a prepacked PD10 column equilibrated and eluted with 0.01 M HCl. The protein fraction was recovered, tested for the SH content, lyophilized, and stored at −20 °C.

SH Titration. The free SH content of all the modified forms of RNase T_1 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 dianion was measured at 412 nm (extinction coefficient, 13 600 M^{-1} cm^{-1}) (Ellman, 1959).

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 catalyzed by traces of heavy metals.

Lyophilized rd-RNase T_1 or GS-RNase T_1 were dissolved to a concentration of approximately 3 mg/mL in 0.01 M HCl 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 refolding reactions were carried out with different absolute concentrations of GSH and GSSG while maintaining a constant redox potential. The desired amounts of GSH and GSSG stock solutions were added to initiate refolding; typically, final concentrations of the glutathione species were 2 mM GSH/1.5 mM GSSG, 0.5 mM GSH/0.1 mM GSSG. The pH of the solution was adjusted to 7.5 with Tris-base and the reaction carried out at 25 °C under nitrogen atmosphere.

When the refolding was carried out in the presence of PDI, the lyophilized enzyme was dissolved in 0.1 M Tris-HCl 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 rd-RNase T_1 or GS-RNase T_1 and the refolding continued at 25 °C under nitrogen atmosphere, as described.

The refolding was monitored on a time-course basis by sampling aliquots of the refolding mixture at appropriate intervals. The protein samples were alkylated and then removed from the excess of blocking reagent by rapid HPLC desalting. The protein fraction was then recovered and lyophilized.

Alkylation of the Refolding Aliquots. Alkylation of the free SH groups present at any time within the intermediate populations was performed as described (Torella et al., 1994) according to Gray (1993). 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 (50 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 nitrogen atmosphere. After 30 s, 100 μL of 5% TFA was added, and the aliquots were quickly vortexed and stored on ice prior loading on the HPLC.

Desalting by HPLC. The alkylated refolding samples were desalted 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 desalted with a linear gradient of solvent B from 15% to 95% at flow rate of 1 mL/min. Protein monitoring was carried out at 220 nm. When the refolding was carried out in the presence of PDI, the catalyst was eluted in the washing of the column, thus not interfering with the subsequent analysis of the refolding intermediates.

Electrospray Mass Analysis. ES-MS analyses were carried out using a VG-Platform single quadrupole mass spectrometer from Fisons Instruments equipped with an electrospray ion source. The protein samples were dissolved in a mixture of H_2O/CH_3CN (50/50) containing 1% acetic acid. Protein samples (10 μL) in concentrations ranging from 10−20 pmol/μL were introduced into the ion source via loop injection at a flow rate of 10 μL/min. The 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 hen egg-white lysozyme (average molecular mass, 14 305.99 Da).

Kinetic analyses have been performed using the Runge-Kutta method (Forsythe & Malcolm, 1977) and the Symplex algorithm (Nelder & Mead, 1965). Both methods have been implemented in the program MatLab (Teoresi, Torino, Italy).

RESULTS

As reported in the introduction, Figure 1 shows all possible intermediates and equilibria in the refolding of a two-disulfide-containing protein in the presence of a glutathione redox system. Four different kinds of reaction occur in the above scheme of refolding: (i) k_2, k_4, k_8, and k_9 are true first-order rate constants referring to intramolecular reactions $P(SH)_nS-SG \rightarrow P(SH)_{n-1}S-S + GSH$, while the rest of the reactions involve the glutathione as reactant so that (ii) k_1, k_3, k_5, k_6, k_7, and k_{10} refer to reactions involving GSSG:$P(SH)_n + GSSG \rightarrow P(SH)_{n-1}S-SG + GSH$, and the remainder refer to reactions involving GSH as a reactant, (iii) k_{-2}, k_{-4}, k_{-8} and k_{-9} referring to the reverse of (i) and k_{-1}, k_{-3}, k_{-5}, k_{-6}, k_{-7}, and k_{-10} referring to the reverse of (ii). Intramolecular thiol−disulfide isomerizations within the protein are not represented (see below).

Refolding by Disulfide Oxidation. RNase T_1 was reduced in denaturing conditions to generate rd-RNase T_1 which was shown to have 3.95 SH/molecule by DTNB titration. The reduced protein was immediately lyophilized and always used within 1−2 days to avoid any spontaneous reoxidation.

rd-RNase T_1 was incubated in the presence of different concentrations of reduced and oxidized glutathione keeping the value of the redox potential E_0' constant. Typically, the reduced protein was incubated in the presence of 2 mM GSH/1.5 mM GSSG or 0.5 mM GSH/0.1 mM GSSG. Assuming for the glutathione redox system an E_0' standard = −0.25 V (Gilbert, 1990), both redox mixtures give an $E_0' = -0.17$ V. Aliquots of the refolding process were withdrawn at different intervals, trapped by alkylation of the free thiol groups as described, and analyzed by ES-MS to identify the disulfide-bonded intermediates formed. The carboxyamidomethylation 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.

Figure 2 shows the deconvoluted ES spectra of the mixtures of species sampled at time 0, 2 min, 2 h, and 5 h

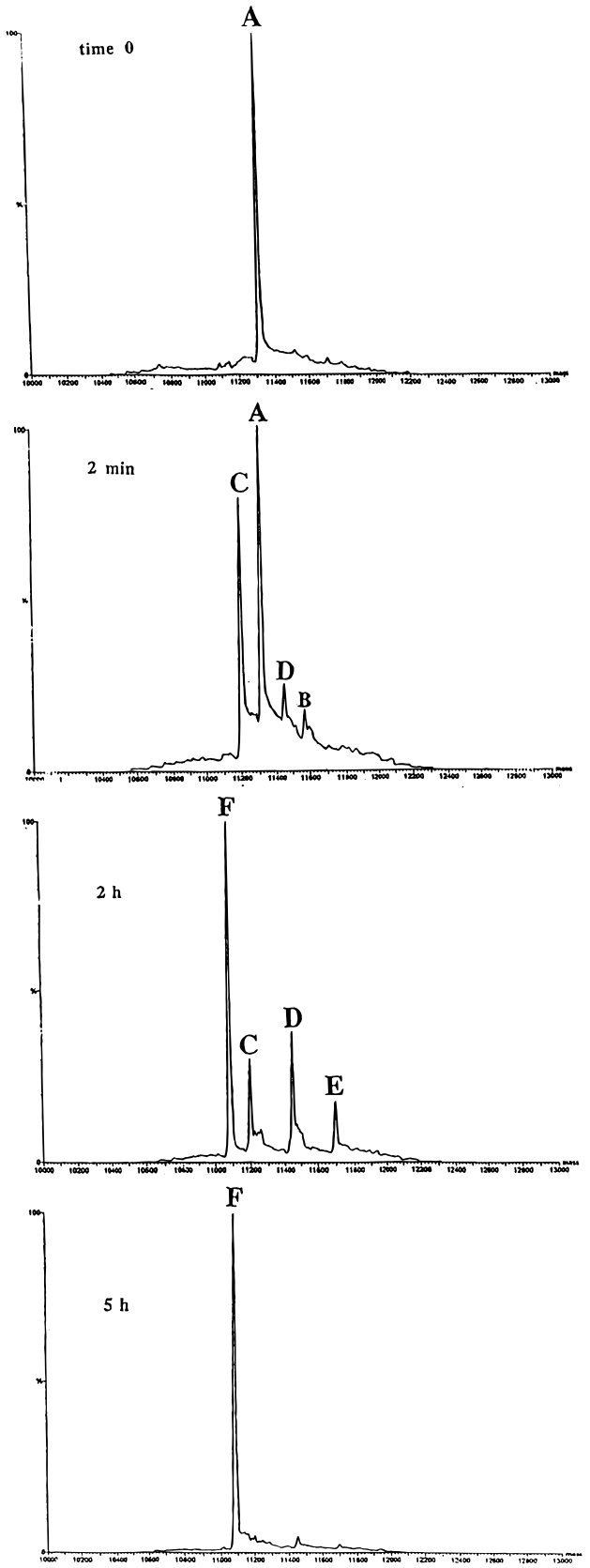

FIGURE 2: ES-MS analysis of rd-RNase T_1 refolding carried out at 1 mg/mL in 0.1 M Tris-HCl and 1 mM EDTA (pH 7.5) at 25 °C in the presence of 2 mM GSH/1.5 mM GSSG. Aliquots were withdrawn from the refolding mixture at time 0, 2 min, 2 h, and 5 h.

from the refolding reaction when the process was performed in the presence of 2 mM GSH/1.5 mM GSSG. At different times, different populations of disulfide intermediates are present which were identified on the basis of their accurate molecular weight. The measured molecular weights, the expected mass values, and the identification of the various intermediates are shown in Table 1. Each population of trapped intermediates is characterized by a different number of intramolecular disulfide bonds (indicated as nS in Table 1), mixed disulfides with the exogenous glutathione (nG) and carboxyamidomethyl groups (nCAM). The number of CAM groups corresponds to the number of free thiols present in the refolding intermediates and are therefore indicated as nH. It is important to emphasize that each observed molecular weight corresponds to a population of isomers, in which the number of protein disulfides, protein-free thiols, and protein−SSG mixed disulfides is defined, but not the positions of the cysteine residues involved. The number of possible isomers within each component defined by mass is indicated in Table 1.

The ES spectrum of the aliquot withdrawn at time 0 (Figure 2) shows the presence of a single component (A), exhibiting a molecular mass of 11 316.75 ± 0.40 Da corresponding to the protein carrying four carboxyamidomethyl groups (expected value 11 316.66 Da). After 2 min of the refolding process, besides the reduced protein (4H), the spectra revealed the simultaneous presence of three species, i.e., B, C, and D. On the basis of their molecular weights, B corresponds to a population of isomers containing one mixed disulfide with the exogenous glutathione and three free SH groups (1G3H), C to intermediates containing an intramolecular disulfide bond and two free SH groups (1S2H), and D to species having an intramolecular disulfide bond, a mixed disulfide with the exogenous glutathione, and a free thiol group (1S1G1H).

The presence of intermediates containing mixed disulfides with the exogenous glutathione confirmed that formation of an intramolecular disulfide bond proceeds via the formation of a protein−glutathione mixed disulfide as shown in the case of RNase A (Torella et al., 1994), of BPTI (Weissman & Kim, 1995) and of a simple model peptide (Darby et al., 1994). The analysis of the sample withdrawn at 2 h reveals, in addition to species C and D already observed, the presence of species F containing two intramolecular disulfides (2S) and E having one intramolecular disulfide and two mixed disulfides with the exogenous glutathione (1S2G); components A and B corresponding to species 4H and 1G3H, respectively, have disappeared at this stage of the process. Finally, after 5 h of refolding only component F was detected in the mixture. Fluorescence and activity analyses (data not shown) indicate an almost complete recovery of the native intrinsic fluorescence and catalytic activity at this stage of the process, thus supporting the conclusion that the refolding generates native RNase T_1 (i.e., component F is predominantly the native species, rather than containing significant amounts of the two alternative 2S isomers).

A further bonus in the use of ES-MS is that each population of intermediates can be quantitated by measuring the total current of ions produced by each species (Miranker et al., 1993). The time course plotted in Figure 3A shows very clearly that intermediates 1S2H and 1S1G1H are predominant from the beginning of the reaction up to about 1 h when the relative concentration of the species 2S increases. A steady state is established between intermediates 1S2H and 1S1G1H; from 30 min onward, the concentration of these species is approximately equal. The com-

Table 1: ES-MS Identification of the Disulfide-Bonded Intermediates Formed during the Refolding of rd-RNase T_1 and of GS-RNase T_1

component	measured molecular mass (Da)	identification[a]	expected molecular mass (Da)	possible isomers
A	11 316.75 ± 0.40	4CAM (4H)	11 316.66	1
B	11 564.05 ± 0.88	1G 3CAM (1G3H)	11 564.97	4
C	11 200.51 ± 0.17	1S 2CAM (1S2H)	11 200.66	6
D	11 448.76 ± 0.46	1S 1G 1CAM (1S1G1H)	11 448.97	12
E	11 697.07 ± 0.28	1S 2G (1S2G)	11 697.28	6
F	11 084.33 ± 0.15	2S (2S)	11 084.66	3
J	12 061.77 ± 0.33	3G 1CAM (3G1H)	12 061.60	4
K	12 309.45 ± 0.37	4G (4G)	12 309.91	1

[a] Abbreviations: CAM, carboxyamidomethyl groups; G, mixed disulfide with glutathione; S, intramolecular disulfide; H, free thiol.

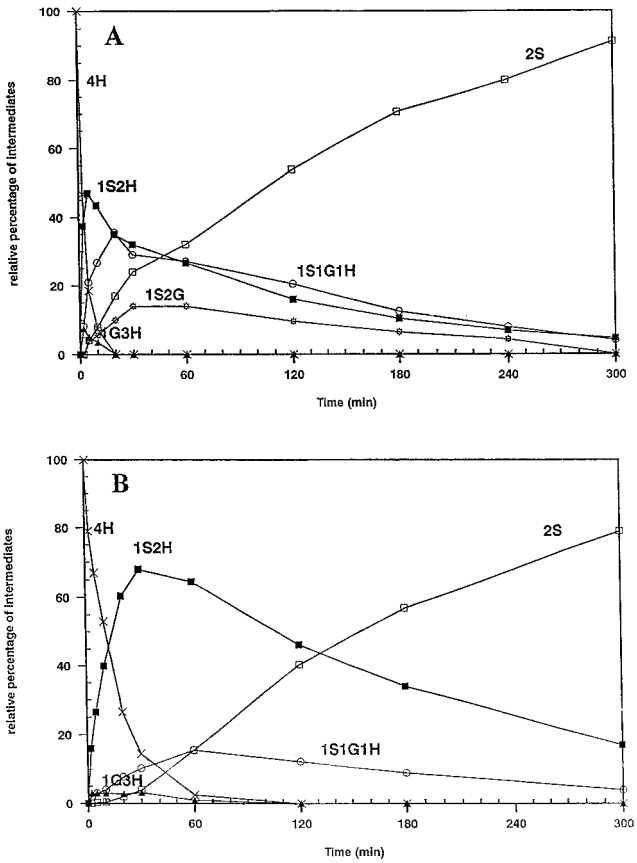

FIGURE 3: Time-course analysis of the refolding of rd-RNase T_1 in the presence of 2 mM GSH/1.5 mM GSSG (panel A) or 0.5 mM GSH/0.1 mM GSSG (panel B). (×) 4H; (▲) 1G3H; (■) 1S2H; (○) 1S1G1H; (☆) 1S2G; (□) 2S.

ponent 1G3H is present only in the first 20 min of the reaction and it rapidly disappears. The species 1S2G is present at very low concentration at the early stages of the reaction, reaching a constant concentration (less than 20%) and disappearing at the late stages of the refolding.

Kinetic analysis of this experiment was performed following the reaction scheme shown in Figure 1; each mass species was treated as a single kinetic species, making the assumption that isomerization reactions are either nonexistent or faster than the reactions reported in the scheme in Figure 1 (see Discussion). This assumption seems to be validated by the fact that there is a good fit between the experimental data and the simulation used, as shown in Figure 4 for the species detected. The derived rate constants for the individual steps are reported in Table 2. This analysis shows that the formation of species 1G3H, 1S2H, and 2S seems to be practically irreversible ($k_1' \gg k_{-1}'$, etc.), the rate-limiting step of the whole process being the formation of species 2S. The rate constants k_3' and k_{-3}', are of similar magnitude, consistent with the equal concentration of 1S2H and 1S1G1H in the steady state (see above).

The refolding experiment was also carried out in the presence of a lower absolute concentration of reduced and oxidized glutathione, namely, 0.5 mM GSH/0.1 mM GSSG, but keeping fixed the redox potential, $E_0' = -0.17$ V, in order to evaluate the effect of the glutathione concentration on the distribution of the intermediates; the corresponding time-course analysis is shown in Figure 3B. Under these conditions, no changes in the nature of S−S-containing intermediates was observed, but they accumulated at different rates and at different levels. The initial species 4H disappears slower, the appearance of the species 2S is not as fast as that observed in the previous conditions, and a lower yield of 2S is obtained (80% compared to 91%). Furthermore, it is clearly evident that there is a net decrease of the relative amount of all the intermediates containing mixed disulfides with glutathione. The population of intermediate 1S2H is now much greater than that of 1S1G1H and is predominant from the beginning of the reaction up to 2 h when the species 2S increases. It is also worth noting that species 1S2G is not formed in these conditions. No kinetic analysis was performed in these conditions since the oxidized glutathione concentration was equivalent to that of the substrate protein so that a simple pseudo-first-order reaction cascade scheme could not be employed. However, the overall picture is consistent with the direct involvement of glutathione in the pathway so that at this lower level of GSH + GSSG the concentrations of species containing −S−SG mixed disulfides are reduced and the overall reaction is slowed.

Refolding by Disulfide Isomerization. The unfolded mixed disulfide derivative GS-RNase T_1 can be refolded to native RNase T_1 in the presence of different ratios of GSH/GSSG, and the refolding is strictly linked to disulfide isomerization (Ruoppolo & Freedman, 1995). GS-RNase T_1 was incubated in the presence of the same redox buffer 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 ES-MS, as described.

Figure 5A shows the time course of the GS-RNase T_1 refolding in the presence of 2 mM GSH/1.5 mM GSSG. The spectrum of the aliquot withdrawn at time 0 shows the presence of a single component K, exhibiting a molecular mass of 12 309.45 ± 0.37 Da (Table 1) corresponding to the protein carrying four exogenous glutathione groups (4G). During refolding with this substrate, the same species were detected as in the refolding of rd-RNase T_1, with the addition of a new component J (Table 1) corresponding to the protein with three mixed disulfides with glutathione and a single protein thiol (3G1H). The intermediate 3G1H is present at

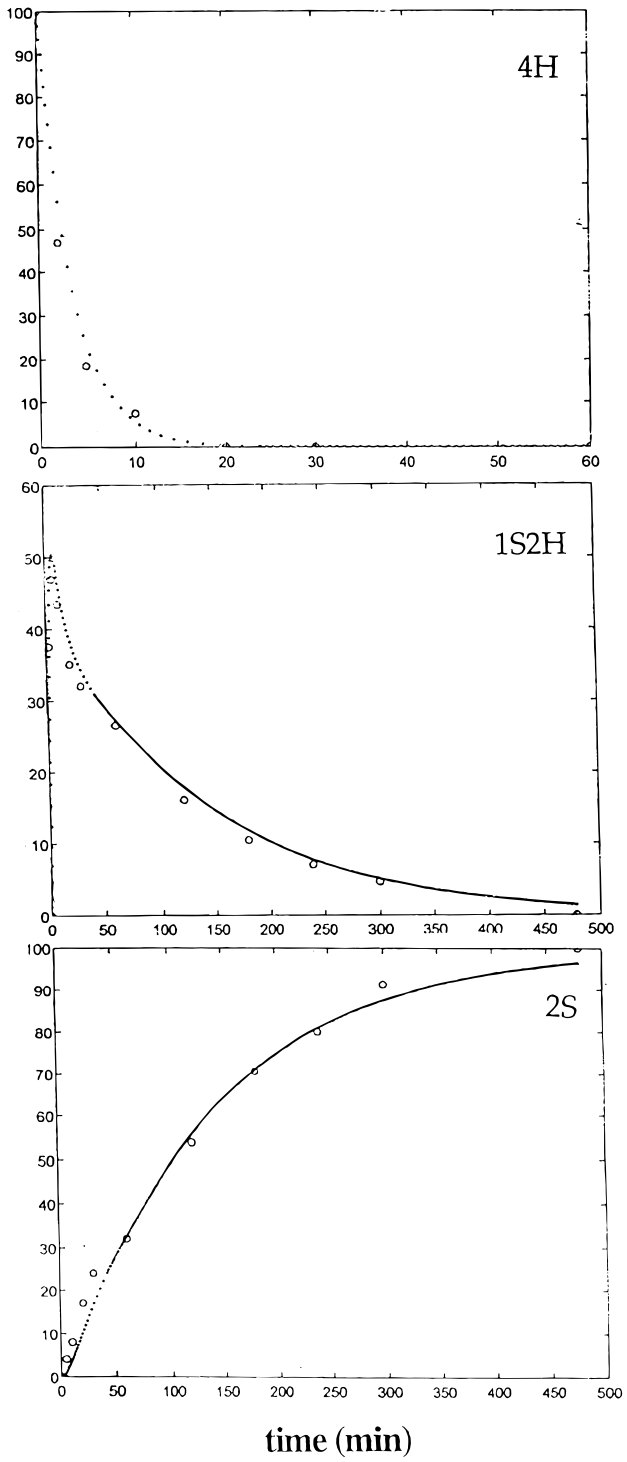

FIGURE 4: Fitting between the experimental data and the simulation used for species detected in the refolding of rd-RNase T_1 in the presence of 2 mM GSH/1.5 mM GSSG.

very low concentration only at the early stages of the reaction and it then disappears. After 5 min, the concentrations of species 4G and 1S2G are of similar magnitude while species 1S1G1H and 3G1H are present at much lower concentration. The time-course analysis shows clearly that the population of intermediates 1S2G is predominant from the beginning of the reaction up to 2 h when the concentration of the species 2S increases. The concentration of the other major population of intermediates, 1S1G1H, remains lower than that of the species 1S2G throughout. Finally, the species 1S2H is present at very low concentration, never greater than 10%,

Table 2: Kinetics of Nonassisted and PDI-Assisted rd-RNase T_1 Refolding in the Presence of 2 mM GSH/1.5 mM GSSG

rate constant (min^{-1})[a]	−PDI	+PDI	catalysis[b]
k_1'	0.34	ND	
k_{-1}'	<0.03	ND	
k_2	2.61	ND	
k_{-2}'	<0.08	ND	
k_3'	0.25	7.87	31
k_{-3}'	0.26	5.59	21
k_{10}'	(0.06)[c]	(0.58)[c]	
k_{-10}'	(0.11)[c]	(0.63)[c]	
k_4	0.018	0.57	31
k_{-4}'	<0.001	ND	

[a] k_2 and k_4 are true first-order rate constants, k_1', k_3', and k_{10}' are pseudo-first-order constants equivalent to k_1[GSSG], k_3[GSSG], and k_{10}[GSSG], respectively, while k_1, k_3, and k_{10} are second-order rate constants; k_{-1}', k_{-2}', k_{-3}', k_{-4}', and k_{-10}' are pseudo-first order constants equivalent to k_{-1}[GSH], k_{-2}[GSH], k_{-3}[GSH], k_{-4}[GSH], and k_{-10}[GSH], respectively, while k_{-1}, k_{-2}, k_{-3}, k_{-4}, and k_{-10} are second-order rate constants. [b] Catalysis is the ratio of the rate constant of the PDI-assisted refolding versus the corresponding rate constant of the nonassisted process. [c] These values are derived from the kinetic data, but their precision is limited by the low level to which the corresponding intermediates accumulate.

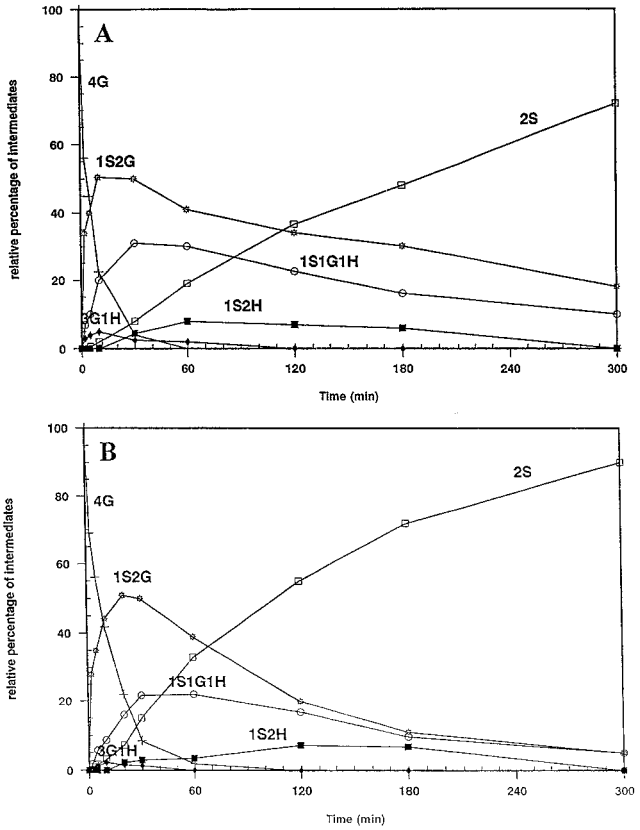

FIGURE 5: Time-course analysis of the refolding of GS-RNase T_1 in the presence of 2 mM GSH/1.5 mM GSSG (panel A) or 0.5 mM GSH/0.1 mM GSSG (panel B). (+) 4G; (◆) 3G1H; (☆) 1S2G; (○) 1S1G1H; (■) 1S2H; (□) 2S.

and it disappears at 5 h. At 5 h, species 2S is predominant (75%) with a minor presence of species 1S2G and 1S1G1H. Fluorescence and activity data (Ruoppolo & Freedman, 1995) indicate after 5 h a recovery of about 80% of the native intrinsic fluorescence and catalytic activity thus supporting the conclusion that species 2S is only native RNase T_1. As underlined for the rd-RNase T_1 refolding, it has to be noted that each observed molecular weight intermediate could correspond to a population of disulfide isomers.

Table 3: Kinetics of Nonassisted and PDI-Assisted GS-RNase T_1 Refolding in the Presence of 2 mM GSH/1.5 mM GSSG

rate constant (min^{-1})[a]	−PDI	+PDI	catalysis[b]
k_{-7}'	0.18	ND	
k_7'	0.08	ND	
k_9	10.6	ND	
k_{-9}'	0.44	ND	
k_{-10}'	0.13	7.4	57
k_{10}'	0.19	10.4	55
k_{-3}'	(0.02)[c]	(0.35)[c]	
k_3'	(0.06)[c]	(3.0)[c]	
k_4	0.010	0.38	38
k_{-4}'	< 0.001	ND	

[a] k_4 and k_9 are true first-order rate constants, k_3', k_7', and k_{10}' are pseudo-first-order constants equivalent to k_3[GSSG], k_7[GSSG], and k_{10}[GSSG], respectively, while k_3, k_7, and k_{10} are second-order rate constants; k_{-3}', k_{-4}', k_{-7}', k_{-9}', and k_{-10}' are pseudo-first-order constants equivalent to k_{-3}[GSH], k_{-4}[GSH], k_{-7}[GSH], k_{-9}[GSH], and k_{-10}[GSH], respectively, while k_{-3}, k_{-4}, k_{-7}, k_{-9}, and k_{-10} are second-order rate constants. [b] Catalysis is the ratio of the rate constant of the PDI-assisted refolding versus the corresponding rate constant of the nonassisted process. [c] These values are derived from the kinetic data, but their precision is limited by the low level to which the corresponding intermediates accumulate.

Kinetic analysis of this experiment was performed following the reaction scheme shown in Figure 1; the rate constants for the individual steps are reported in Table 3. Formation of species 2S, seems to be practically irreversible ($k_4 \gg k_{-4}'$), this reaction being the rate-limiting step of the whole process.

The refolding experiment was also performed in the presence of a lower concentration of reduced and oxidized glutathione, namely, 0.5 mM GSH/0.1 mM GSSG, in order to evaluate the effect of the glutathione concentration on the distribution of the intermediates. Figure 5B shows the time-course analysis of this experiment. No changes in the nature of disulfide-containing intermediates were observed. Species 1S2G is still predominant up to 1 h when component 2S becomes predominant. Conversely, species 1S1G1H is present at a lower concentration than that observed in the previous experiment. The appearance of the species 2S is faster than that observed in the previous redox conditions, and an increase in its yield is observed (90%). It is worth noting that the appearance of the minor species 1S2H does not seem to be affected by the different refolding conditions. No kinetic analysis was performed for this experiment as the oxidized glutathione concentration was equivalent to that of the substrate protein so that a simple pseudo-first-order reaction cascade scheme could be not employed.

Refolding Assisted by PDI. When refolding was performed in the presence of protein disulfide isomerase (PDI), the enzyme was preincubated in the redox buffer and then added to the rd-RNase T_1 or GS-RNase T_1 solution at a molar ratio of 0.25 = [PDI]/[substrate]. The PDI-assisted refolding reactions were followed by ES MS analysis as described.

Figure 6 shows a comparison between the time-course analyses of nonassisted and PDI-assisted rd-RNase T_1 refolding in the presence of 2 mM GSH/1.5 mM GSSG. Addition of PDI led to a net catalysis of the refolding reaction. The time-course analysis shows the absence of the intermediate 1G3H detected in the uncatalyzed reaction; in the presence of such a high concentration of PDI, it is possible that the reaction is too fast at the early stages to allow detection of species 1G3H even when the reaction

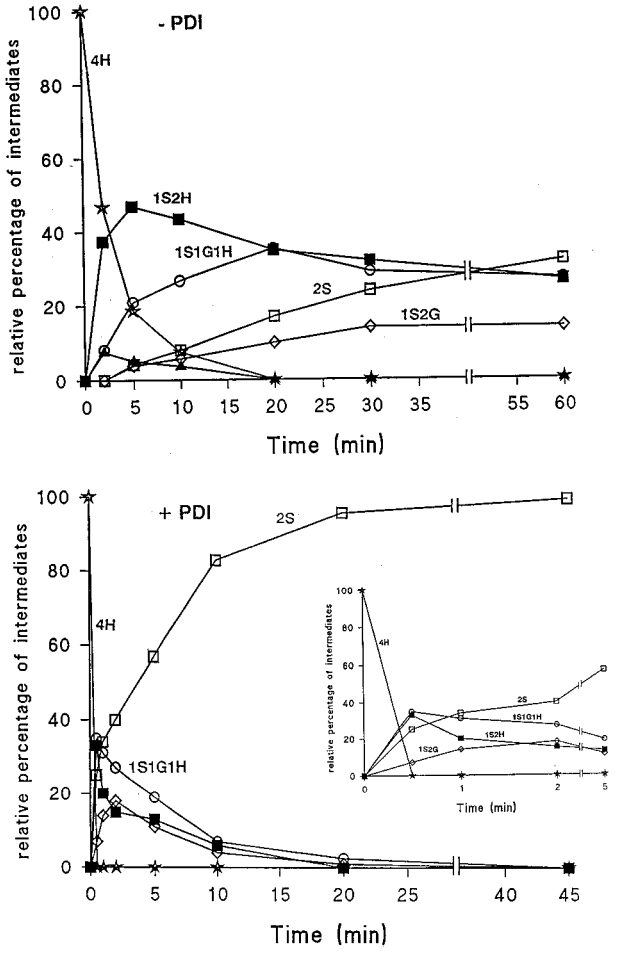

FIGURE 6: Time-course analysis of the refolding of rd-RNase T_1 in the absence and in the presence of PDI. PDI was incubated with 2 mM GSH/1.5 mM GSSG for 10 min at 25 °C and then added to rd-RNase T_1 (1 mg/mL) at a molar ratio of 0.25 = [PDI]/[rd-RNase T_1]. (☆) 4H; (▲) 1G3H; (■) 1S2H; (○) 1S1G1H; (◇) 1S2G; (□) 2S.

mixture was sampled after only 30 s. The reduced protein disappears after only 30 s when species 1S2H, 1S1G1H, and 2S are present to a similar extent. At the one disulfide level, the species 1S2G is present at a concentration never greater than 20% and the steady-state approximately equal concentration of species 1S2H and 1S1G1H is established more rapidly in the presence of PDI than in its absence. The catalyzed refolding was completed after only 45 min.

Kinetic analysis (Table 2) of the PDI-assisted rd-RNase T_1 refolding shows that addition of PDI led to a 20−30-fold increase in the measured rate constants for the three steps which could be analyzed both in absence and presence of the enzyme. As discussed above, the species 1G3H was not observed, so that the rate constants k_1', k_{-1}', k_2, and k_{-2}' could not be calculated. However, a hypothetical rate constant of 21.9 min^{-1} was estimated for the direct conversion of species 4H into 1S2H (4H + GSSG → 1S2H + 2GSH). This is 25 times the value of the product of the rate constants k_1' and k_2 for the uncatalyzed reactions, thus supporting the conclusion that the addition of PDI also catalyzes the early stages of the refolding.

The PDI-assisted refolding experiment was also performed in the presence of a lower concentration of reduced and oxidized glutathione, namely, 0.5 mM GSH/0.1 mM GSSG, in order to evaluate the effect of the glutathione concentration at a fixed E_0' on the catalysis by PDI. Again, addition of

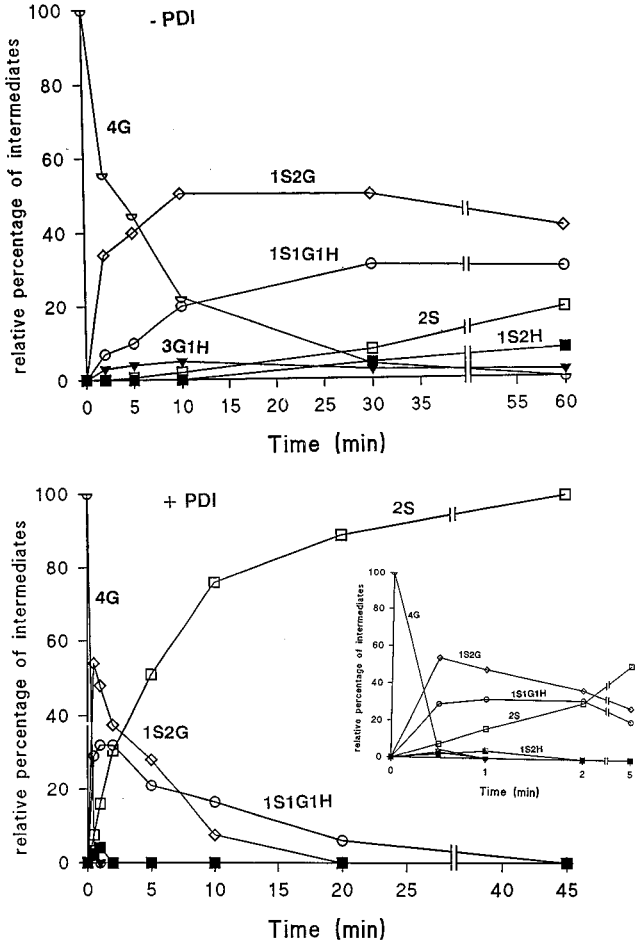

FIGURE 7: Time-course analysis of the refolding of GS-RNase T_1 in the absence and in the presence of PDI. PDI was incubated with 2 mM GSH/1.5 mM GSSG for 10 min at 25 °C and then added to GS-RNase T_1 (1 mg/mL) at a molar ratio of 0.25 = [PDI]/[GS-RNase T_1]. (open half-circles) 4G; (▼) 3G1H; (◇) 1S2G; (○) 1S1G1H; (■) 1S2H; (□) 2S.

PDI led to a net catalysis of the refolding process. The only intermediate that accumulates to a significant level is 1S2H, and the rates of both its appearance and disappearance are significantly reduced in the presence of lower glutathione concentration. Similarly, both the catalyzed and uncatalyzed rates of appearance of 2S are reduced at the lower glutathione level. A comparison between the time-course analysis of nonassisted and PDI-assisted rd-RNase T_1 refolding in the presence of 0.5 mM GSH/0.1 mM GSSG is shown in Figure 1 of the Supporting Information.

The effect of PDI on the refolding of GS-RNase T_1 was also investigated. PDI was incubated in the presence of the redox buffer, 2 mM GSH/1.5 mM GSSG or 0.5 mM GSH/0.1 mM GSSG, and then added to the GS-RNase T_1 at a molar ratio of 0.25 = [PDI]/[GS-RNase T_1]. Figure 7 shows a comparison between the time-course analysis of nonassisted and PDI-assisted GS-RNase T_1 refolding in the presence of 2 mM GSH/1.5 mM GSSG. Addition of PDI led to a net catalysis of the refolding reaction, without altering substantially the distribution of the intermediates along the refolding pathway (note that the distribution after 2 min in the presence of PDI is closely comparable to that after 60 min in the absence of PDI). GS-RNase T_1 disappears after only 1 min when species 1S2G and 1S1G1H constitute the two major populations of intermediates. Species 2S is predominant after only 5 min, and its appearance is much faster than in the absence of PDI, as expected. Finally, species 3G1H and 1S2H are present at very low concentration only at the first stages of the refolding. Kinetic analysis (Table 3) of the PDI-assisted GS-RNase T_1 refolding in the presence of 2 mM GSH/1.5 mM GSSG shows that addition of PDI led to an approximately 50-fold increase in the rate constants where they could be measured in both conditions. As discussed above, the species 3G1H is present at very low concentration only at the early stage of the refolding so that the rate constants k_{-7}', k_7', k_9, and k_{-9}' could not be calculated and a hypothetical rate constant for the direct conversion of species 4G into species 1S2G was estimated (4G → 1S2G + GSSG). The measured value was 13.1 min^{-1}. This is 7 times the value of the product of the rate constants k_{-7}' and k_9 for the uncatalyzed reaction, thus supporting the conclusion that the addition of PDI also catalyzes the first stages of the refolding process. In our previous study of the PDI-catalyzed refolding of GS-RNase T_1 (Ruoppolo & Freedman, 1995) we monitored formation of native protein by intrinsic fluorescence and observed a 7-fold acceleration by PDI under conditions comparable to those used here.

The PDI-assisted GS-RNase T_1 refolding experiment was also performed in the presence of a lower absolute concentration of reduced and oxidized glutathione, namely, 0.5 mM GSH/0.1 mM GSSG keeping E_0' constant (= −0.17 V). Addition of PDI led to a net catalysis of the overall process. A comparison between the time-course analysis of the nonassisted and PDI-assisted GS-RNase T_1 refolding in these conditions is shown in Figure 2 of the Supporting Information. The intermediate 1S2G is predominant up to 2 min when the species 2S increases. The species 3G1H is present at a very low concentration only in the early stages of the process while the minor intermediate 1S2H is not formed under these conditions. The appearance of species 2S, which reaches 100% after 45 min, is faster than that observed in the PDI-assisted GS-RNase T_1 refolding carried out at higher glutathione concentration.

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 (Creighton, 1986; Gething & Sambrook, 1992), where the major thiol redox system is provided by glutathione (Hwang et al., 1992). 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 (Creighton et al., 1995). Most studies focused on the refolding of fully reduced, denatured proteins taking these derivatives as a model of the denatured state. In this work we have studied the refolding of RNase T_1 starting from both the reduced protein and a mixed disulfide between the protein and glutathione, with the aim of investigating the effect of different denatured starting materials on the refolding pathways and on the relative abundance of refolding intermediates. It seems conceivable that mixed disulfides between proteins and glutathione are important folding intermediates *in vivo*. Since the nascent chain emerges into the ER lumen into conditions of high oxidized glutathione concentration, it may well be that unpaired cysteine residues are converted to mixed disulfides before the complete translocation.

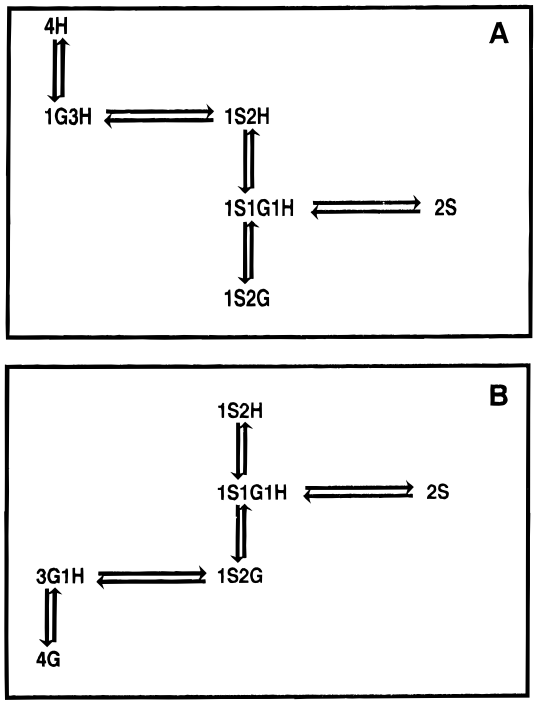

FIGURE 8: Refolding pathway of reduced and denatured RNase T_1 (A) and GS-RNase T_1 (B) in the presence of a glutathione redox system.

We employed a novel approach based on ES-MS time-course analysis which provides crucial data for kinetic and structural studies of the refolding processes. The definition of the precise molecular weight of the refolding intermediates allowed us to establish the isomeric species formed during the process and to determine a pathway of sequential reactions for both refolding processes (Figure 8). All the analyses demonstrate that only a limited number of intermediates is formed in the process as compared to all those theoretically possible (see Figures 1 and 8). In particular one population of intermediates containing more than one mixed disulfide with glutathione was detected in folding from the reduced species and one population of intermediates with more than one free -SH was detected in folding from GS-RNase T_1. These observations allowed us to exclude some possible refolding pathways for both rd-RNase T_1 and GS-RNase T_1, thus significantly decreasing their number.

The first step in the rd-RNase T_1 refolding is the formation of a mixed disulfide, producing species 1G3H while the first step in the GS-RNase T_1 refolding is a reduction reaction producing species 3G1H. Then, both species, 1G3H and 3G1H, undergo an internal attack by a free SH group to form an intramolecular disulfide. 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: a species 2G2H was in fact never found. The time-course analysis clearly indicates that species 1S1G1H is the immediate precursor of species 2S from both starting materials, but interconversion between species 1S2H, 1S1G1H, and 1S2G is observed whether the refolding starts from the 4H state or from the 4G state. Species 1S2G and 1S2H are dead-end intermediates in the refolding of rd-RNase T_1 and GS-RNase T_1, respectively; they, in fact, have to be transformed back to 1S1G1H to form the final product 2S. Formation of species 2S from 1S1G1H is the rate-limiting step for all the processes studied.

It should be noted that the intermediates identified by the ES-MS 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 bond isomeric species. However, we cannot exclude the possibility that only a limited number of isomers is formed within an intermediate population due to kinetic or thermodynamic constraints, thus further restricting the possible folding pathways. In an earlier study of refolding of rd-RNase T_1, using oxidized DTT as oxidant, it was shown that a number of different isomeric species containing a single disulfide bond are formed (Pace & Creighton, 1986); these were isomers of 1S2H since mixed disulfides with DTT are relatively unstable. The identification of disulfide bonds within each intermediate population was out of the scope of the present study, but it is clear that the definition of such isomers within a population will allow the definitive and complete description of the folding pathway in terms of individual molecular species.

Notwithstanding this limitation, we investigated whether the overall kinetics of the folding pathway could be modeled by a simple scheme, such as that in Figure 1. The overall kinetics were effectively simulated by such a scheme (Figure 4). This may reflect limitations in the data, relative to the number of steps in the model, but it suggests that each intermediate population is kinetically homogeneous, a condition which will be met if each intermediate population contains a single isomer, or if all the isomers in a structurally diverse population interconvert rapidly. The effective simulation by the simple scheme permits detailed consideration of the derived rate constants (Tables 2 and 3).

For both folding substrates, the fastest step in the pathway is the formation of the first disulfide bond (k_2 and k_9, generating species 1S2H and 1S2G, respectively). The preceding steps to form 1G3H and 3G1H (represented by k_1' and k_{-7}') are significantly slower in these conditions so that these intermediates do not accumulate significantly, and the potential competing reactions of 1G3H and 3G1H to give 2G2H (defined by k_5' and k_{-6}', respectively) are not detected. In principle, k_5' might be expected to be similar to k_1', since they are chemically similar processes, and likewise k_{-6}' may be similar to k_{-7}'. These steps are bimolecular reactions involving either GSSG or GSH as reactants, and so they could be accelerated if the concentrations of these reactants were increased. From the values of the accessible pseudo-first-order rate constants shown in Tables 2 and 3 it is clear that the concentrations of GSH and GSSG would have to be increased 10−100-fold over those used here in order for the formation of 2G2H from either starting material to compete with intramolecular disulfide bond formation. [Note that the synthesis of GS-RNase T_1 (4G) from rd-RNase T_1 (4H) requires the presence of 0.2 M GSSG and denaturing conditions (Ruoppolo & Freedman, 1994).]

After formation of the first disulfide bond there is interconversion between the three possible "one-disulfide" intermediate populations (1S2H, 1S1G1H, 1S2G), and the rate constants for all these steps (k_3', k_{-3}', k_{10}', k_{-10}') are comparable (0.13−0.26 min^{-1}). These rate constants are all significantly faster than that for the unimolecular step in which the second disulphide is formed (k_4), and hence significant concentrations of all three "one-disulfide" intermediates accumulate independent of the starting substrate. The rate constant for the formation of the second disulfide

bond (k_4) is 2−3 orders of magnitude smaller than that for the formation of the first disulfide (k_2, k_9) presumably because the formation of structure (folding) in the "one-disulfide" intermediate restricts the mobility required for formation of the second disulfide. This interpretation is supported by a comparison of these constants with those in a simpler system, the formation of a single disulfide in a small unstructured 28-residue peptide (Darby et al., 1994). In that system, the unimolecular rate constants for the formation of the 1S species from the two possible 1G1H isomers were 0.0082 and 0.012 s^{-1}, i.e., in the range of 0.5−0.7 min^{-1}. These values for the unstructured peptide are slightly smaller than those for the formation of the first disulfide in our protein system, k_2 and k_9 (where there are three possible ways in which intermediates 1G3H or 3G1H can form an intramolecular disulfide), but significantly larger than that for the formation of the second disulfide, k_4, for which similar values are found in the independent experiments with the different initial unfolded substrates. Similarly, the difference of 2−3 orders of magnitude between the intramolecular steps leading to formation of the first and second intramolecular disulfide observed here can be compared with the case of BPTI. The final intramolecular step to displace glutathione and form the third native intramolecular disulfide in BPTI is 400-fold slower than the average rate of the intramolecular step in formation of first intramolecular disulfide (Weissman & Kim, 1995).

The refolding experiments were performed using the same approach in the presence of PDI to understand the role of PDI catalysis in the formation of disulfide bonds. The addition of PDI results in a net catalysis of the entire process without altering the refolding pathway but causing small changes in the relative distribution of the various population of intermediates. All the experiments suggest that the addition of PDI results in a catalysis of each individual reaction of the folding processes; thus PDI seems to catalyze: (i) formation of mixed disulfide with glutathione (e.g., k_3'), (ii) reduction of mixed disulfide (e.g., k_{-10}'), and (iii) formation of intramolecular disulfide bonds (e.g., k_4). These results are not surprising considering the broad range of activities shown by PDI (Freedman et al., 1984, 1994). The small changes in relative concentrations of intermediate found in the presence of PDI indicate that direct transfer of disulfide from PDI active site to protein dithiol (followed by reoxidation of PDI by GSSG) can only be a very small component of the overall PDI-catalyzed reaction (cf. Darby et al., 1994; Ruddock et al., 1996). It is important to underline that by this approach we cannot detect any effect of PDI on the isomerization of disulfide bonds within a single population of intermediates. Actually, PDI could possibly catalyze the formation of only native disulfide bonds in both processes, bypassing disulfide rearrangements thus further restricting the folding pathways (Creighton et al., 1993).

Uncatalyzed and PDI-catalyzed refolding of both starting materials was also studied in presence of lower concentrations of GSH and GSSG while maintaining the same initial value of $[GSH]^2/[GSSG]$ to fix the redox potential and hence the redox status of PDI active site. This would be expected to influence the rates of bimolecular reactions differentially since the concentration of GSH was diminished 4 times (from 2 to 0.5 mM) while the concentration of GSSG was reduced 15 times (from 1.5 to 0.1 mM). Hence it is not surprising that the glutathione conditions had effects on the overall rates of formation of the final 2S product which depends on the nature of the initial substrate (Table 4). For folding from 4H, where two of the forward reactions are depending on [GSSG] and all the reverse reactions are depending on [GSH] (see Figure 1), the net effect is a greater overall rate in the high glutathione conditions. For folding from 4G, where two of the forward reactions and two of the reverse reactions are depending on [GSH], while two of the reverse reactions are depending on [GSSG], the net effect is a greater overall rate in the low glutathione conditions. These changes are observed both in absence and presence of PDI. For the same reason, the steady-state levels of some intermediates differ between the two glutathione concentrations, and indeed formation of the "dead-end" intermediate 1S2G was not significant when the refolding was performed from rd-RNase T_1 in the presence of lower concentrations of glutathione.

Table 4: Half-Time of Appearance of 2S Product in Various Conditions[a]

substrate	4H		4G	
PDI	− (min)	+ (min)	− (min)	+ (min)
high GSH/GSSG	110	4	190	5
low GSH/GSSG	160	21	100	3

[a] Derived from data in Figures 3 and 5−7. See the figure legends for detailed conditions.

The elucidation of the catalytic mechanism of PDI was outside the scope of this study, but some conclusions can be drawn. In the redox conditions used, PDI should be present with equal concentrations of dithiol or disulfide form (Lundström & Holmgren, 1993) or mainly in the reduced form (Hawkins et al., 1991). In either case, the formation of mixed disulfides between PDI and glutathione or PDI and the substrate protein should be facile, and such species are potential intermediates in the PDI-catalyzed process observed here. This hypothesis is supported by recent data on the catalysis of oxidative folding of RNase A by mutant forms of PDI, where covalent PDI−RNase mixed disulfide species were detected (Walker et al., 1996). It is also consistent with work on the catalysis of the renaturation of bovine pancreatic trypsin inhibitor (BPTI) (Zapun et al., 1993, 1994) and hirudin (Wunderlich et al., 1995) by DsbA, the periplasmic catalyst of disulfide formation from *E. coli*, where a DsbA−glutathione mixed disulfide was observed at the early stages of the process. With the analytical method and PDI concentration employed here, it was not possible to detect or quantitate such transient intermediates species.

As stated, RNase T_1 is the simplest model disulfide-containing protein for refolding studies; nevertheless, the results presented in this paper may lead to more general conclusion on refolding mechanisms. In quasiphysiological 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, and (ii) internal attack of a free SH group to form an intramolecular disulfide bond. This hypothesis is supported by our data on the refolding of RNase A which seems to refold via a similar mechanism (Torella et al., 1994) and the recent results obtained by Weissman and Kim (1995) on the refolding of bovine pancreatic trypsin inhibitor (BPTI) where they demonstrate by using a different analytical approach that the formation of an intramolecular disulfide

bond occurs via a protein−glutathione mixed disulfide. The proposed mechanism predicts that only a limited number of intermediates actually accumulate and that isomerization between species with the same number of disulfides is only extensive at late stages of the process where slow conformational transitions become significant.

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SUPPORTING INFORMATION AVAILABLE

Two figures with the following figure legends showing the time-course analysis of rd-RNase T_1 and GS-RNase T_1 (3 pages). Ordering information is given on any current masthead page.

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