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Early intermediates in the PDI-assisted folding of ribonuclease A

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Abstract

The oxidative refolding of ribonuclease A has been investigated in several experimental conditions using a variety of redox systems. All these studies agree that the formation of disulfide bonds during the process occurs through a nonrandom mechanism with a preferential coupling of certain cysteine residues. We have previously demonstrated that in the presence of glutathione the refolding process occurs through the reiteration of two sequential reactions: a mixed disulfide with glutathione is produced first which evolves to form an intramolecular S-S bond. In the same experimental conditions, protein disulfide isomerase (PDI) was shown to catalyze formation and reduction of mixed disulfides with glutathione as well as formation of intramolecular S-S bonds.

This paper reports the structural characterization of the one-disulfide intermediate population during the oxidative refolding of Ribonuclease A under the presence of PDI and glutathione with the aim of defining the role of the enzyme at the early stages of the reaction. The one-disulfide intermediate population occurring at the early stages of both the uncatalyzed and the PDI-catalyzed refolding was purified and structurally characterized by proteolytic digestion followed by MALDI-MS and LC/ESIMS analyses. In the uncatalyzed refolding, a total of 12 disulfide bonds out of the 28 theoretical possible cysteine couplings was observed, confirming a nonrandom distribution of native and nonnative disulfide bonds. Under the presence of PDI, only two additional nonnative disulfides were detected. Semiquantitative LC/ESIMS analysis of the distribution of the S-S bridged peptides showed that the most abundant species were equally populated in both the uncatalyzed and the catalyzed process.

This paper shows the first structural characterization of the one-disulfide intermediate population formed transiently during the refolding of ribonuclease A in quasi-physiological conditions that mimic those present in the ER lumen. At the early stages of the process, three of the four native disulfides are detected, whereas the Cys26–Cys84 pairing is absent. Most of the nonnative disulfide bonds identified are formed by nearest-neighboring cysteines. The presence of PDI does not significantly alter the distribution of S-S bonds, suggesting that the ensemble of single-disulfide species is formed under thermodynamic control.

Keywords: disulfide bond; mass spectrometry; PDI; refolding intermediates; RNase A

Oxidative refolding of reduced bovine pancreatic ribonuclease A (RNase A) has been studied using different approaches and a variety of different redox systems; several folding pathways were deduced depending essentially on the E_0' value of the redox buffer (Anfinsen, 1973; Hantgan et al., 1974; Creighton, 1979a; Scheraga et al., 1984; Rothwarf & Scheraga, 1993a, 1993b, 1993c, 1993d; Torella et al., 1994; Li et al., 1995; Ruoppolo et al., 1996b, 1997; Xu et al., 1996; Rothwarf et al., 1998a, 1998b; Iwaoka et al., 1998). Using reduced and oxidized DTT (DTTred/DTTox) Scheraga and coworkers (Rothwarf & Scheraga, 1993a, 1993b, 1993c, 1993d; Li et al., 1995) showed that the intermediates containing one and two disulfide bonds are largely conformationally disordered and that the formation of further disulfide bonds constitutes the rate-limiting steps in the regeneration of fully native RNase A. More recently, the same authors (Xu et al., 1996) showed that the distribution of the disulfide bonds within the one-disulfide intermediates population is nonrandom and thermodynamically controlled. They found that all the four native disulfide pairings (Cys26–

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Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; BSA, bovine serum albumin; CM, carboxymethyl cysteine; DTTox, oxidized dithiothreitol; DTTred, reduced dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ER, endoplasmic reticulum; ESIMS, electrospray ionization mass spectrometry; FPLC, fast protein liquid chromatography; GSH, reduced glutathione; GSSG, oxidized glutathione; IAA, iodoacetic acid; LC/ESIMS, liquid chromatography/electrospray ionization mass spectrometry; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry; M.W., molecular weight; PDI, protein disulfide isomerase; RNase A, bovine pancreatic ribonuclease A; RP-HPLC, reversed-phase high performance liquid chromatography; TFA, trifluoroacetic acid; TIC, total ion current; TOF, time of flight.

Cys84, Cys40–Cys95, Cys58–Cys110, and Cys65–Cys72) have populations greater than those predicted by loop entropy calculations, with the disulfide bond Cys65–Cys72 representing 40% of the entire one-disulfide population. It should be considered that although the use of DTT simplifies the refolding pathways because the mixed disulfides with proteins do not tend to accumulate, these refolding conditions are patently nonphysiological. During protein folding in the endoplasmic reticulum (ER), in fact, disulfide bond formation is dependent on protein disulfide isomerase (PDI), which is present at high concentration and functions in the presence of millimolar concentrations of reduced glutathione (GSH) and oxidized glutathione (GSSG) (Freedman et al., 1994, 1995). PDI has been shown to catalyze a broad range of reactions, such as disulfide bond formation, reduction, and rearrangement by thiol/disulfide exchange on a wide range of substrates (Gilbert, 1990, 1997; Freedman et al., 1995). During protein folding, PDI can catalyze disulfide rearrangements in a process that eventually leads to a native structure that is resistant to further rearrangements (Weissman & Kim, 1993; Gilbert, 1994; Laboissière et al., 1995). Other members of the thioredoxin superfamily, glutaredoxin or DsbA (Bardwell et al., 1991; Lundstrom-Ljung & Holmgren, 1995), which have only one thioredoxin homologous redox-active domain are poor catalysts of disulfide rearrangements in protein folding, suggesting that domains other than the redox-active domains contribute significantly to substrate interactions that are important for disulfide rearrangements (Klappa et al., 1998). The challenge is therefore to understand the mechanism of PDI-assisted folding in great detail at the molecular and structural levels and to discover how assisted folding pathways are orchestrated in the cell.

We have introduced the use of mass spectrometry to study the oxidative folding under quasi-physiological conditions and to investigate the effect of folding factors on the kinetics and on the mechanism of the refolding process (Torella et al., 1994; Ruoppolo et al., 1996a, 1996b, 1997, 1998). Our studies showed that the process proceeds through the reiteration of two sequential reactions: (1) reaction with the exogenous glutathione producing a mixed disulfide and (2) formation of an intramolecular disulfide bond. This mechanism predicts that only a limited number of intermediates are formed compared to all those theoretically possible. In the same experimental conditions, a preferential coupling of certain cysteine residues has been observed in the refolding of RNase A, which leads initially to the formation of some well-defined nonnative disulfide bonds, together with the native ones (Ruoppolo et al., 1996b). More recently, we have shown that PDI can catalyze formation and reduction of mixed disulfides with glutathione as well as formation of intramolecular disulfides in the PDI assisted refolding of RNase A (Ruoppolo et al., 1997). However, these studies did not allow detection of any effect of PDI on the distribution of disulfide bonds within a single population of intermediates.

Here, for the first time, we have purified and structurally characterized the population of one-disulfide intermediates that form transiently in the refolding of RNase A in the presence of PDI and glutathione under conditions that mimic those present in the ER lumen, with the aim to investigate the impact of PDI catalysis at the early stages of the process. The one-disulfide intermediates constitute the only species in which the assignment of disulfide bonds corresponds to the identification of the isomeric components present in the population of intermediates. We have shown that a total of 12 one-disulfide species out of 28 possible isomers are formed in the uncatalyzed refolding of RNase A, thus confirming that the distribution of S-S bonds does not take place at random. In the presence of PDI, two additional nonnative disulfides were observed. Semi-quantitative analyses of the distribution of the S-S bonds showed that different species accumulate at the same level in the presence and in the absence of PDI. The effect of PDI catalysis is therefore to catalyze the formation and further conversion of one-disulfide intermediates without significant impact on the identity of the one-disulfide intermediate population.

Results

Refolding of RNase A

The refolding of denatured and reduced RNase A was carried out as described. After incubation at 25 °C under nitrogen atmosphere for 4 min, the refolding intermediates were trapped in a stable form by alkylation with 0.5 M iodoacetic acid. Although iodoacetate quenching should be used cautiously (Xu et al., 1996), an appropriate concentration of iodoacetate, such as the 0.5 M used in our studies, provides rapid quenching of the thiol groups as clearly stated by Weissman and Kim (1991) and confirmed by kinetic analysis performed by Gray (1993). In our previous paper (Torella et al., 1994), we obtained, by using the conditions suggested by Gray, the same distribution of disulfide bonded species when trapping intermediates with iodoacetic acid or iodoacetamide thus demonstrating that the distribution of disulfide bonded species is not an artefact due to the kind of reagent used to block the free cysteine residues. The refolding incubation time of 4 min was chosen so that the one-disulfide intermediate was the most abundant population in the refolding mixture at that stage of the process, as previously demonstrated (Ruoppolo et al., 1997). The electrospray ionization mass spectrometry (ESIMS) spectrum of the refolding mixture after 4 min of incubation showed in fact the presence of three major components (Fig. 1A). Species A corresponds to intermediates having one intramolecular S-S bond and six carboxymethyl cysteines (1S6CM) (measured M.W. = 14,035.92 ± 0.91 Da; calculated M.W. = 14,036.2 Da), B to the fully reduced and carboxymethylated protein (8CM) (measured M.W. = 14,153.97 ± 1.32 Da; calculated M.W. = 14,154.2 Da), and C to a population of species having two intramolecular disulfides and four carboxymethyl cysteines (2S4CM) (measured M.W. = 13,917.63 ± 1.4 Da; calculated M.W. = 13,918.2 Da). Two very minor components were also detected; D corresponds to species containing one mixed disulfide with the exogenous glutathione and seven carboxymethyl cysteines (1G7CM) (measured M.W. = 14,402.31 ± 0.87 Da; calculated M.W. = 14,401.87 Da) while E contains one mixed disulfide, one intramolecular S-S bond, and five carboxymethyl cysteines (1S1G5CM) (measured M.W. = 14,284.15 ± 0.97 Da; calculated M.W. = 14,283.37 Da). The other peaks labeled as A1, B1, and C1 in Figure 1A correspond to species containing an extra carboxymethyl group, which was observed previously, following alkylation with IAA and arises from modification of a group other than cysteine (Torella et al., 1994).

We have previously shown that ESIMS analysis can be used to accurately quantify each population of intermediates by measuring the total ion current produced by each species provided that the different components are endowed with comparable ionization capabilities (Ruoppolo et al., 1997, 1998). The species present in the ES spectrum were then quantified by measuring the total ion current produced by each species showing that the one-disulfide intermediate represented 46% of the total species, the fully reduced

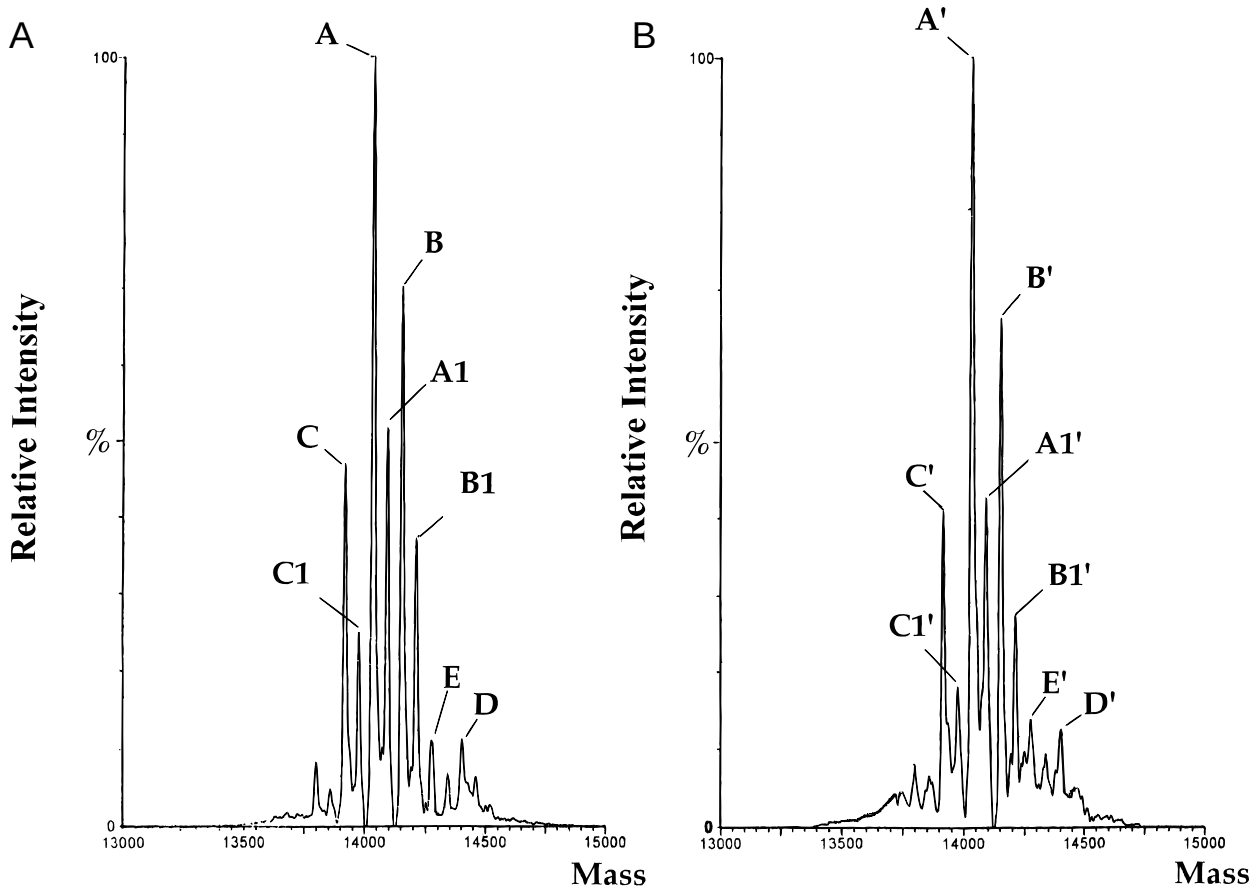

Fig. 1. ESIMS analysis of refolding intermediates. ESIMS analysis of the refolding mixture of RNase A blocked after 4 min of incubation (**A**) in the absence and (**B**) in the presence of PDI. Refolding was carried out at 1 mg/mL in 0.1 M Tris-HCl, 1 mM EDTA (pH 7.5) at 25 °C in the presence of 1.5 mM GSH/0.3 mM GSSG. PDI was incubated in the same refolding buffer for 10 min and then added at a concentration of 1 μM. Intermediates were blocked by alkylation with IAA. Peaks present in the spectra are described in the text.

and carboxymethylated protein 31%, the two-disulfide intermediates 17% and each of the two mixed disulfide species 3%, thus confirming that the one disulfide intermediate constitutes the most abundant population at this stage of refolding.

The refolding reaction was also performed in the presence of PDI at a final concentration of 1 or 10 μM, as described. Previous results (Ruoppolo et al., 1997) showed that the addition of 1 or 10 μM PDI determined a fourfold and a 13-fold catalysis, respectively, of the overall rate of refolding reaction.

The refolding intermediates produced after 4 min of incubation in the presence of 1 μM PDI were alkylated and analysed by ESIMS. The corresponding spectrum (Fig. 1B) is very similar to that obtained for the uncatalyzed process showing that the one-disulfide intermediate is the most abundant population at this time point also in the PDI-assisted refolding. This experiment does not provide a clear evidence of a net PDI catalysis of the formation of one-disulfide intermediates. The refolding reaction was then carried out in the presence of 10 times higher concentration of PDI to provide a net catalysis of the formation of one-disulfide intermediates. Samples from the uncatalyzed and PDI-catalyzed reaction were withdrawn after 1 min and trapped as described. The ESIMS analysis of these samples revealed that the fully reduced and carboxymethylated protein and the one disulfide-intermediates represented 74 and 18%, respectively, in the uncatalyzed reaction, while the same species accounted for 35 and 44%, respectively, in the catalyzed reaction, thus showing that under these experimental conditions the one-disulfide intermediate accumulates more rapidly in the presence of PDI. In both cases, minor components corresponding to differently oxidized form of RNase A were observed.

It should be underlined again that this kind of analysis allowed only the detection of population of isomers characterized by the same content of disulfide bonds but not the definition of the cysteine residues involved in the S-S bonds. These analyses therefore did not allow detection of any effect of PDI on the distribution of disulfide bonds within a single population of intermediates.

Purification of one-disulfide intermediate population

The alkylating reaction, used to block the refolding process, introduces an additional negative charge for each free cysteine thus allowing the disulfide containing intermediates to be separated according to their charge using anion-exchange chromatography (Creighton, 1992). Figure 2A shows a typical anion-exchange chromatogram of the intermediates trapped after 4 min of the uncatalyzed refolding. The chromatographic profile shows the presence of six peaks labeled A, B, C, D, E, and R with B, C, D, and E only

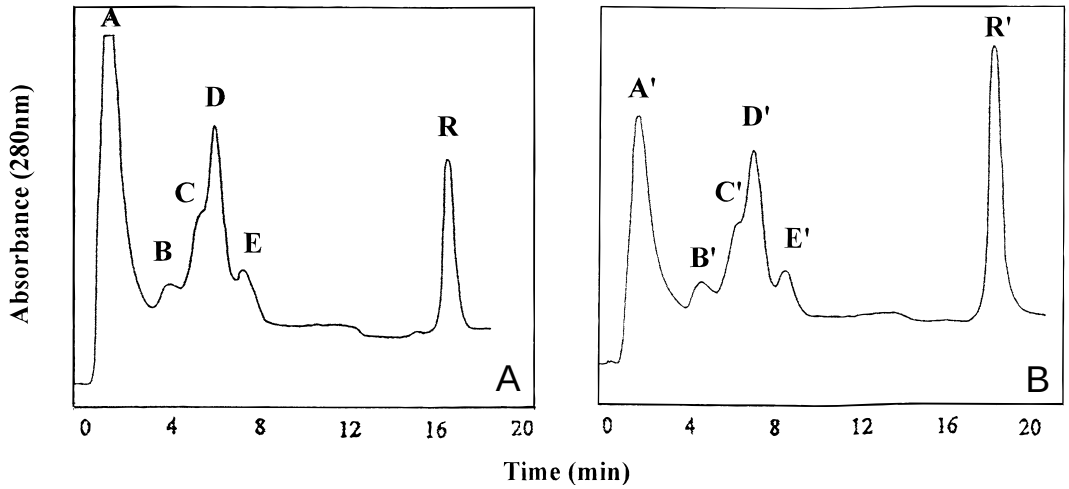

Fig. 2. Anion-exchange separation of refolding intermediates. Anion-exchange chromatogram of the refolding mixture of RNase A blocked after 4 min of incubation (**A**) in the absence and (**B**)in the presence of 1 μM PDI. A and A′ represent the void volume that contains the two disulfides species, peaks B-E and B′-E′ represent subfractions of the one-disulfide intermediates, and R and R′ represent the reduced and alkylated protein. Experimental conditions used in the chromatographic separation are described in the text.

partially resolved. Peaks were collected, desalted, and analyzed by ESIMS. Their mass values indicate that each of the four partially resolved peaks (B–E) corresponds to one-disulfide species, peak R to the reduced and carboxymethylated protein and peak A (void volume) contains the two disulfides species. The very minor mixed disulfides species are probably lost in the baseline noise. The separation of the one-disulfide species in different subsets is probably due to the acquisition of different conformations by the isomeric components after formation of the first disulfide bond. However, even these subfractions might not necessary represent pure species, because different one-disulfide intermediates could elute at the same retention time.

The same strategy was employed to purify the one-disulfide intermediate population formed in the presence of PDI. Figure 2B shows the anion-exchange chromatogram of samples withdrawn after 4 min of the reaction in the presence of 1 μM PDI. The ESIMS analysis showed that the partially resolved B′–E′ peaks correspond to one-disulfide intermediates, peak R′ to the reduced and carboxymethylated species, and peak A′ (void volume) contains the two disulfide species.

Assignment of S-S bonds within one-disulfide intermediates produced in the uncatalyzed refolding

An aliquot of the unfractionated population of one-disulfide intermediates produced during the uncatalyzed refolding experiment was doubly digested with trypsin and endoproteinase Asp-N to isolate each Cys residue within an individual peptide. The resulting peptide mixture was separated by RP-HPLC and analyzed "on-line" by ESIMS. Alternatively, the peptide mixture was directly analyzed by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). Reduced and carboxymethylated RNase A was also hydrolyzed with trypsin and endoproteinase Asp-N and the peptide mixture analyzed by liquid chromatography/electrospray ionization mass spectrometry (LC/ESIMS) and MALDI-MS. The digestion map of the reduced and carboxymethylated RNase A was necessary to simplify the assignment of the S-S bonded peptides in the one-disulfide species digestion map, since the latter one contains all the fragments from the digestion of reduced and blocked protein in addition to fragments linked by S-S bonds.

Figure 3A shows the total ion current (TIC) profile of the mixture of peptides derived from the one-disulfide intermediates produced after 4 min of the uncatalyzed refolding, whereas Figure 3B reports the same analysis carried out on the reduced and carboxymethylated RNase A. The comparative analysis of the ESI spectra of the two chromatograms immediately led to the identification of the peptides containing reduced Cys residues that occurred in both digests. The assignment of these peptides is reported in Figure 3B. In addition, a series of fragments corresponding to peptides linked by S-S bonds were detected in the population of one-disulfide intermediates and are labeled with numbers in Figure 3A. The S-S bonded peptides identified in the analyses are listed in Table 1. As shown in Table 1, a single TIC fraction (Fig. 3A) can contain more than one species characterized by different disulfide bonds. Moreover, different fragments linked by the same S-S bond were observed as a consequence of nonspecific cleavages occurring at Glu49 and Tyr76 and incomplete cleavage at Lys91. However, data from LC/ESIMS analysis provided unambiguous identification of all the peptides occurring in the digests. MALDI-MS analysis (data not shown) revealed the presence of all the disulfides identified by LC/ESIMS with the exception of the S-S bond Cys58–Cys65 corresponding to mass value 1,508.8 determined by ESIMS analysis.

LC/ESIMS and MALDI analyses show that, after 4 min of the uncatalyzed refolding, three out of four native disulfide bonds (Cys65–Cys72, Cys58–Cys110, and Cys40–Cys95) are already present together with nine nonnative disulfides. The absence of further disulfide bridges, including the native Cys26–Cys84 pairing, indicates that only a limited number of S-S bonds, and hence of one-disulfide intermediates, are formed at the early stages of the refolding. Alternatively, these species might be present at very low level thus escaping detection by the mass spectrometer. Nevertheless, even if they are present, they should not be largely repre-

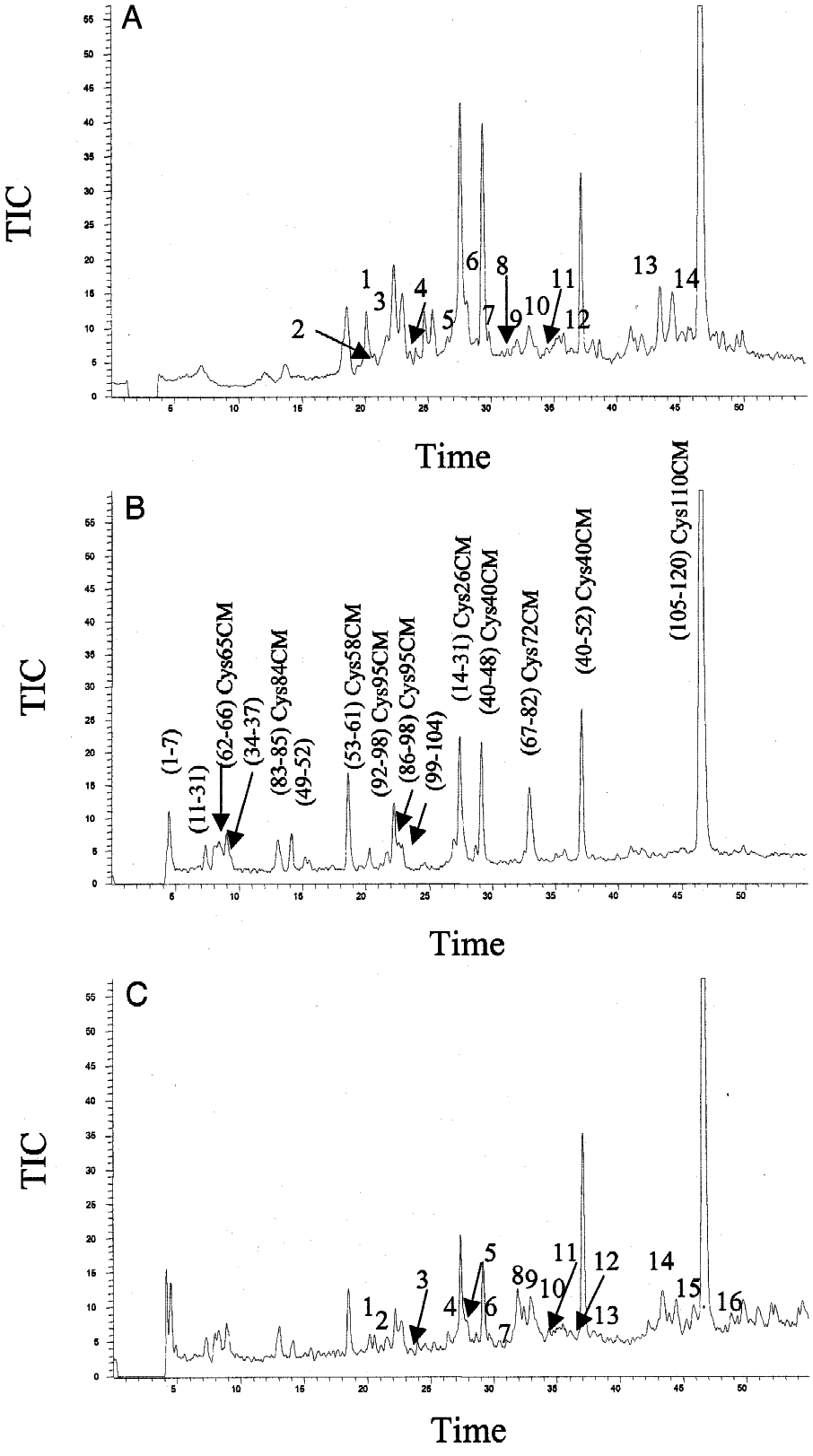

Fig. 3. LC/ESIMS analyses of peptide mixtures. Total ion current (TIC) profile of the peptide mixture derived by the tryptic and endoproteinase Asp-N hydrolysis of the unfractionated population of one-disulfide intermediates produced (**A**) in the noncatalyzed and (**C**) in the 1 μM PDI catalyzed refolding of RNase A. Fractions containing disulfide bonded peptides are labeled with numbers. For the assignment of S-S bonds, refer to Table 1. (**B**) The total ion current (TIC) profile of the reduced and carboxymethylated RNase A digested with trypsin and endoproteinase Asp-N. The assignment of peptides to the corresponding peaks is shown; CM indicates carboxymethyl cysteine.

Table 1. *Assignment of S-S bonds in the LC/ESIMS analysis of peptide mixture derived by the doubly digested unfractionated population of one-disulfide intermediates produced in the uncatalyzed and 1 μM PDI catalyzed refolding of RNase A*[a]

Molecular mass	− PDI fraction	+ PDI fraction	Assignment	S-S bond
1,248.0 ± 0.4	1	1	(83–85) + (92–98)	Cys84–Cys95
1,508.8 ± 0.7	2	2	(53–61) + (62–66)	Cys58–Cys65
1,568.2 ± 0.8	3		(67–76) + (83–85)	Cys72–Cys84
1,709.6 ± 0.3	4	3	(62–66) + (67–76)	**Cys65–Cys72**
2,153.4 ± 0.2	5	4	(53–61) + (67–76)	Cys58–Cys72
2,886.2 ± 0.2	6	5	(40–52) + (86–98)	**Cys40–Cys95**
2,018.8 ± 0.3	7	6	(40–48) + (53–61)	Cys40–Cys58
1,899.4 ± 0.1	8	7	(40–48) + (92–98)	**Cys40–Cys95**
2,189.3 ± 0.9		8	(67–82) + (83–85)	Cys72–Cys84
2,329.9 ± 0.1	9	8	(62–66) + (67–82)	**Cys65–Cys72**
2,772.8 ± 0.2	10	9	(53–61) + (67–82)	Cys58–Cys72
2,953.8 ± 0.8	**Absent**	10	(14–31) + (40–48)	Cys26–Cys40
2,654.4 ± 0.4	11	11	(67–82) + (92–98)	Cys72–Cys95
2,184.8 ± 0.3	12	14	(83–85) + (105–120)	Cys84–Cys110
2,326.6 ± 0.3	12	14	(62–66) + (105–120)	Cys65–Cys110
2,419.6 ± 0.1	12	12	(40–52) + (53–61)	Cys40–Cys58
3,352.9 ± 0.7	**Absent**	13	(14–31) + (40–52)	Cys26–Cys40
2,768.2 ± 1.2	13	14	(53–61) + (105–120)	**Cys58–Cys110**
2,650.2 ± 0.7	14	15	(92–98) + (105–120)	Cys95–Cys110
3,590.9 ± 1.0	**Absent**	16	(67–82) + (105–120)	Cys72–Cys110

[a]Native S-S bonds are in bold.

sented populations, and therefore we can confidently assume that they do not markedly affect the results.

To obtain a better identification of the S-S bonds formed at the early stages of refolding, the single subsets of the population of one disulfide intermediates separated by anion-exchange chromatography (Fig. 2A) were hydrolyzed as described for the unfractionated population and the resulting peptide mixtures analyzed by MALDI-MS. As an example, Figure 4 shows the partial MALDI spectrum of the double digest of peak D. The recorded mass signals were assigned to individual peptides on the basis of their mass values. The signals at *m/z* 3,243.2, 2,774.1, 2,330.6, and 2,189.1 correspond to peptide pairs linked by an S-S bond, as illustrated in Figure 4. Assignments were confirmed by a single step of manual Edman degradation followed by re-analysis by MALDI-MS (Morris & Pucci, 1985). On the basis of these results, the peak D is characterized by the presence of one-disulfide isomers in which all the S-S bonds involve Cys72.

Table 2 summarizes the disulfide bonds identified in each subset of the population of one-disulfide intermediates. From these data, it is clear that the individual subfractions are characterized by the presence of a specific cysteine residue involved in different couplings. Subfraction B is characterized by the presence of S-S bonds involving Cys110; in the subfraction C all disulfides involve Cys95; subfraction D shows S-S bonds involving Cys72 and finally, in the subfraction E, only disulfides involving Cys 40 were identified. These findings support the hypothesis that the chromatographic separation of the one-disulfide intermediate population is due to the acquisition of different conformations by the isomeric components after the formation of local structure (Montelione & Scheraga, 1989; Lustig & Fink, 1992).

These data essentially confirm the LC/ESIMS analysis carried out on the unfractionated population of one-disulfide isomers, the only difference being the disulfide Cys58–Cys65 detected in fraction 2 of the TIC profile (Fig. 3A; Table 1) and not observed in the analysis of the subfractions.

Assignment of S-S bonds within one-disulfide intermediates produced in the PDI catalyzed refolding

The same strategy was employed to structurally characterize the population of one-disulfide intermediates produced when the refolding process was carried out in the presence of 1 μM PDI. The unfractionated population of isomers was proteolytically digested with trypsin and Asp-N endoproteinase and the resulting peptide mixture analyzed by LC/ESIMS and MALDI-MS. Figure 3C shows the corresponding TIC profile in which peaks containing S-S bonded peptides are labeled with numbers. The identification of the disulfide containing peptides is reported in Table 1. This analysis essentially revealed the presence of the S-S bonds already detected in the noncatalyzed refolding. However, two further nonnative S-S bonds, Cys26–Cys40 and Cys72–Cys110, could be observed in the TIC fraction 10, 13, and 16. MALDI-MS analysis (data not shown) revealed the presence of the same S-S bonds identified by LC/ESIMS with the exception of the coupling Cys58–Cys65 identified by the mass value 1,508.8 determined by ESIMS analysis.

Individual subfractions of the population of one-disulfide isomers produced in the 1 μM PDI assisted refolding and separated by anion-exchange chromatography (Fig. 2B) were digested as described above. The resulting peptide mixtures were analyzed by MALDI-MS, and the results are reported in Table 3. As observed in the noncatalyzed refolding, each subfraction was characterized by the occurrence of disulfide bonds, and hence intermediates, involving a specific cysteine residue. Accordingly, the two addi-

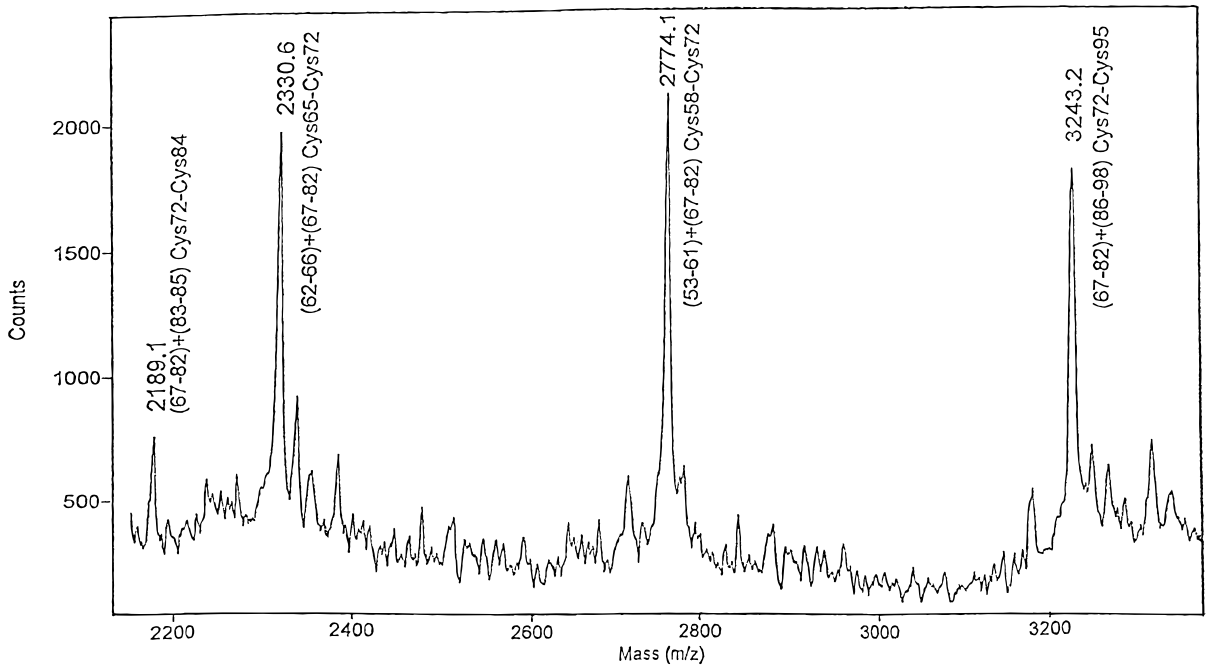

Fig. 4. MALDI-MS analysis of peptide mixture. Partial MALDI spectrum of the peptide mixture derived by the tryptic and endoproteinase Asp-N hydrolysis of the peak D of the anion-exchange chromatogram shown in Figure 2A. The assignment of peptides to the corresponding peaks is shown.

tional disulfides detected, Cys72–Cys110 and Cys26–Cys40, were identified in the subset B′, characterized by S-S bonds involving Cys110 and in the subfraction E′ containing disulfides involving Cys40, respectively.

Again, a remarkable agreement between the LC/ESIMS analysis of the peptides from the unfractionated population of one-disulfide intermediates and the MALDI analysis of the fragments from the single subfractions purified by ion-exchange chromatography was observed. The only difference consisted in the absence of the S-S bond Cys58–Cys65 in the analysis of the subfraction.

Table 2. *Assignment of S-S bonds following MALDI-MS analysis of the peptide mixtures derived by the doubly digested chromatographic subfractions of the one-disulfide intermediate detected in the noncatalyzed refolding of RNase A (Fig. 2A)*[a]

Peak	MALDI (MH+)	Assignment	S-S bond
B	2,771.4	(53–61) + (105–120)	**Cys58–Cys110**
	2,327.4	(62–66) + (105–120)	Cys65–Cys110
	2,184.8	(83–85) + (105–120)	Cys84–Cys110
C	3,241.4	(86–98) + (105–120)	Cys95–Cys110
	2,652.2	(92–98) + (105–120)	Cys95–Cys110
	1,249.1	(83–85) + (92–98)	Cys84–Cys95
D	3,243.2	(67–82) + (86–98)	Cys72–Cys95
	2,774.1	(53–61) + (67–82)	Cys58–Cys72
	2,330.6	(62–66) + (67–82)	**Cys65–Cys72**
	2,189.1	(67–82) + (83–85)	Cys72–Cys84
E	2,889.0	(40–52) + (86–98)	**Cys40–Cys95**
	2,421.3	(40–52) + (53–61)	Cys40–Cys58

[a]Native S-S bonds are in bold.

Table 3. *Assignment of S-S bonds following MALDI-MS analysis of the peptide mixtures derived by the doubly digested chromatographic subfractions of the one-disulfide intermediate detected after 4 min of the 1 μM PDI catalyzed refolding of RNase A (Fig. 2B)*[a]

Peak	MALDI (MH+)	Assignment	S-S bond
B′	3,593.1	(67–82) + (105–120)	Cys72–Cys110
	2,972.3	(67–76) + (105–120)	Cys72–Cys110
	2,771.8	(53–61) + (105–120)	**Cys58–Cys110**
	2,327.7	(62–66) + (105–120)	Cys65–Cys110
	2,184.3	(83–85) + (105–120)	Cys84–Cys110
C′	3,241.9	(86–98) + (105–120)	Cys95–Cys110
	2,652.8	(92–98) + (105–120)	Cys95–Cys110
	1,248.7	(83–85) + (92–98)	Cys84–Cys95
D′	3,243.4	(67–82) + (86–98)	Cys72–Cys95
	2,773.9	(53–61) + (67–82)	Cys58–Cys72
	2,652.9	(67–82) + (92–98)	Cys72–Cys95
	2,330.4	(62–66) + (67–82)	**Cys65–Cys72**
	2,189.1	(67–82) + (83–85)	Cys72–Cys84
E′	3,356.5	(14–31) + (40–52)	Cys26–Cys40
	2,955.3	(14–31) + (40–48)	Cys26–Cys40
	2,888.7	(40–52) + (86–98)	**Cys40–Cys95**
	2,420.8	(40–52) + (53–61)	Cys40–Cys58

[a]Native S-S bonds are in bold.

A control experiment was carried out on the unfractionated population of isomers produced in the presence of 10 μM PDI following the above-described disulfide mapping strategy. The MALDI-MS analysis revealed the presence of the same S-S bonds detected in the presence of 1 μM PDI (data not shown). Since 10 μM PDI does provide a net catalysis of the formation of one-disulfide intermediates, the above results strengthen the conclusion that the enzyme does not significantly affect the distribution of one-disulfide intermediates.

Distribution of one-disulfide intermediates

An estimation of the relative abundance of each disulfide was calculated from the LC/ESIMS analysis by extracting the ion current associated with individual disulfide bonded peptides. Since for any given disulfide, different peptide fragments exist due to nonspecific cleavages, the ion current for each disulfide was obtained by summing the ion current relative to all peptides containing that particular disulfide. The relative abundance of each disulfide was then obtained by dividing the ion current associated with each disulfide by the total ion current produced by all the S-S bonded peptides. The results are reported in Table 4; each value is the mean of two independent LC/ESIMS experiments. The standard deviation was found to be 3%.

Being aware of the fact that different peptides can have different ionization efficiencies, it is clear that this procedure it is not a good way to estimate the relative abundance of the individual disulfide bonded species but we use the value reported in Table 4 only to estimate any possible difference between the nonassisted and the PDI-assisted refolding. The two mixtures are in fact constituted by the same peptides, characterized by the same ionization properties, and are therefore affected by the same errors in calculating the relative percentage of the S-S bonds. Table 4 shows very clearly that no marked differences exist in the abundance of S-S bonds detected in the noncatalyzed and in the PDI-catalyzed process, suggesting that PDI is not altering the steady state distribution of the one-disulfide intermediates at the very early stages of the process.

Table 4. *Relative percentage of ion current associated with disulfide bonded peptides*[a]

Disulfide bond	Relative percentage (%) of ion current		Ratio
	− PDI	+ 1 μM PDI	
Cys58–Cys110	8.2	8.1	1.0
Cys84–Cys110	11.8	12.2	1.0
Cys65–Cys110	8.8	10.6	0.8
Cys95–Cys110	17.6	18.7	0.9
Cys65–Cys72	15.7	14.3	1.1
Cys58–Cys72	5.7	N.D.[b]	N.D.
Cys72–Cys95	N.D.	N.D.	N.D.
Cys72–Cys84	N.D.	N.D.	N.D.
Cys40–Cys95	6.0	5.8	1.0
Cys40–Cys58	12.0	9.0	1.3
Cys58–Cys65	8.0	7.0	1.1
Cys84–Cys95	N.D.	N.D.	N.D.
Cys26–Cys40		N.D.	
Cys72–Cys110		N.D.	

[a]Native S-S bonds are in bold.
[b]N.D., not detectable.

Discussion

We investigated for the first time the distribution of the S-S bonds within the population of one-disulfide intermediates produced during the PDI-catalyzed refolding of RNase A in the presence of the glutathione redox system with the aim of answering a long-standing question as to whether PDI is mainly catalyzing SS bond formation or isomerization. Knowledge of the steady-state distribution of one-disulfide intermediates formed during the refolding of RNase A can provide important insights about early folding events, since these intermediates form first during the process. In addition, these intermediates constitute the only population of species in which the definition of the S-S bonds formed is directly related to a description of the conformation of individual components.

The structural characterization of the population of one-disulfide intermediates was carried out by using state of the out sophisticated MS techniques and revealed the presence of a limited number of S-S bonds. A total of 12 disulfide bonds, and hence 12 isomeric structures was, in fact, observed out of the 28 expected possible cysteine couplings. These results confirm that the formation of the S-S bonds during the refolding of RNase A proceeds through a nonrandom mechanism (Ruoppolo et al., 1996b; Xu et al., 1996) strongly influenced by a proximity effect (see below).

The distribution of different species in the steady-state one-disulfide population is dependent on their rate of formation by oxidation or isomerization and their rate of disappearance by reduction, isomerization, or further oxidation. It would be expected that abundant species that accumulate and that can be observed are a combination of those that form most rapidly and those that are somehow conformationally stabilized and then slowly evolving. On this ground, species absent from the one disulfide population would be those devoid of particular stability and/or those that cannot readily be formed because of some conformational barrier leading to a high kinetic activation energy for the formation of the S-S bond. A detailed examination of the 12 intermediates detected in the one-disulfide population provides possible explanations of the nonrandom couplings of cysteines.

Figure 5 represents all the possible one-disulfide pairs in RNase A and indicates those experimentally observed in the one-disulfide intermediate population. It is noteworthy that of the 12 individual disulfides observed in the uncatalyzed process, 6 represent "nearest-neighbor" pairings (adjacent to the diagonal in Fig. 5). Such pairings are expected to be both kinetically and thermodynamically favored (relative to longer-range pairings) because of the minimal loss of chain entropy involved in their formation. Of the six observed nearest-neighbor pairings, five are nonnative disulfide bonds that must isomerize later on along the folding pathway. The only native nearest-neighbor bond is Cys65–Cys72; the early formation of this native disulfide bond had already been reported and it was claimed that the 65–72 disulfide loop is located in one of the chain folding initiation sites (CFIS) of RNase A (Xu et al., 1996; Iwaoka et al., 1998; Rothwarf et al., 1998a, 1998b).

The remaining six disulfides detected include three bonds that are relatively short range, although not nearest-neighbor, and three longer range bonds including the two native disulfides Cys40–Cys95 and Cys58–Cys110. A possible explanation for their early appearance is that the formation of each of these bonds endows some stabilizing structural features, which overcome the entro-

26								
40	(-/+)							
58	-	+						
65	-	-	+					
72	-	-	+	+ *				
84	- *	-	-	-	+			
95	-	+ *	-	-	+	+		
110	-	-	+ *	+	(-/+)	+	+	
	26	40	58	65	72	84	95	110

Fig. 5. Summary of observed disulfide bonds: +, disulfide bond observed; −, disulfide bond not observed; ±, disulfide bond observed only in the presence of PDI; *, native disulfide bond.

pically unfavorable component of the formation of long-range cross-links.

It should be noted that the only nearest-neighbor disulfide not observed is the nonnative bond Cys26–Cys40, and the only native disulfide not observed is Cys26–Cys84, both involving Cys26. These results confirm previous data that showed that Cys26 is less involved than other cysteines in the formation of S-S bonds during early stages of the refolding of RNase A (Creighton, 1979b; Ruoppolo et al., 1996b). This might reflect a generally lower reactivity of Cys26 in the unfolded protein, possibly due to the electrostatic nature of its microenvironment. In this respect, it is noteworthy that the cysteine residues located toward the C-terminus of the protein are generally found to be involved in a higher number of S-S bonds within the one-disulfide population than those at the N-terminus. Altogether, in fact, Cys84, Cys95, and Cys110 occur in 8 out of 12 total disulfides with Cys110 involved in 4 of the most abundant single-disulfide isomers (Table 4). On the other hand, the segment of the polypeptide chain containing Cys26 might be endowed with less conformational freedom, thus making it difficult to bring this cysteine in the correct position to form S-S bonds.

In summary, the nonrandom one disulfide intermediate population is biased in three respects (1) toward the formation of short-range linkages, (2) toward the formation of native disulfides, and (3) toward the formation of disulfides involving cysteines located in the C-terminal rather than N-terminal portion of the molecule. While the first two of these trends are relatively easy to rationalize, the last one is more difficult to interpret. It could reflect a more complete collapse of the C-terminal portion of the molecule, allowing facile disulfide interchange within a restricted volume or it could reflect formation of some stable structures around Cys26, which inhibits its interaction with other portions of the chain. Clearly further structural analysis is required here.

The structural characterization of the population of one-disulfide intermediates produced in the PDI-catalyzed refolding of RNase A provided a direct evaluation on whether the catalyst is able to alter the distribution of isomeric species within this population. The overall effect of PDI on the population of one-disulfide isomers, in fact, would be expected to be to bias the population toward molecular species relatively more stable but relatively slower to form. This effect could lead, in principle, to a broadening in the population (if the intermediates are similar in stability but have very different uncatalyzed rates of formation) or to a narrowing of the population (if the catalyst drives the process toward the formation of specific intermediates endowed with particular stability, i.e., those containing native disulfides), or to little effect on the distribution of isomers within the population (if the uncatalyzed process is effectively under thermodynamic control).

The results presented in this paper revealed that the PDI-catalyzed refolding generates essentially the same disulfide bonds and hence the same intermediates identified in the noncatalyzed reoxidation. Moreover, semiquantitative analysis of S-S bonds indicates that they accumulate at the same level in the two conditions. The only detectable effect of PDI consists of the formation of two additional nonnative disulfide bonds, Cys26–Cys40 and Cys72–Cys110. The former constitutes the only "nearest-neighbor" pairing not detected in the uncatalyzed process while the latter involves Cys110 located in the C-terminal portion of RNase A. Formation of these two SS bonds is then in agreement with the trend described above for the nonrandom couplings of cysteine residues. The occurrence of these two further nonnative disulfide bonds in the presence of PDI might be due to the enhancement of their rates of formation, which are relatively slow in the noncatalyzed process because of unidentifiable conformational barriers. It should be emphasized that disulfide bonds involving Cys26 were detected only in the presence of PDI; however, the native disulfide bond Cys26–Cys84 was not observed even in the presence of the catalyst.

Data presented in this paper prove, for the first time, the hypothesis that at the early stages of refolding of RNase A formation and interconversion of intermediates are essentially under thermodynamic control and hence the folding pathway is a function of the protein itself. These results obtained under quasi-physiological conditions are in good agreement with those reported by Scheraga et al. using the nonphysiological DTT redox system (Xu et al., 1996). The similar distribution of isomeric species within the pop-

ulation of one-disulfide intermediates in the uncatalyzed and in the PDI-assisted process indicates that at the early stages of the refolding, catalysis of the isomerization of disulfide bonds has no significant effect on the folding pathway. The fact that the population of one-disulfide intermediates is hardly affected by the presence of PDI further proves that thermodynamic control operates at this stage of RNase A refolding.

Since nonnative disulfide bonds need to isomerize to produce native RNase A, PDI-dependent catalysis of the rearrangements of SS bonds presumably become significant for the overall pathway at later stages of the refolding process, as previously suggested (Walker & Gilbert, 1997). At that stage, the intermediates would already have acquired some tertiary structure and the important function of PDI should be to catalyze disulfide rearrangements within kinetically-trapped, structured folding intermediates as previously reported for the PDI-assisted refolding of BPTI (Weissman & Kim, 1993). In this respect, it should be underlined that at the early stages of the RNase A refolding process, the one and two disulfide intermediates are largely conformationally disordered (Li et al., 1995), and therefore there are no significant conformational barriers to disulfide isomerization.

Materials and methods

Materials

Dithiothreitol (DTT), ethylenediamine tetraacetic acid (EDTA), Bis-tris, reduced glutathione (GSH), oxidized glutathione (GSSG), iodoacetic acid (IAA), trypsin, and RNase A were obtained from Sigma Chemical Co. (St. Louis, Missouri); Tris was purchased from Fluka (Buchs, Switzerland). Pre-packed Sephadex G-25M PD10 and Mono Q HR 5/5 Column were acquired from Pharmacia (Uppsala, Sweden). Endoproteinase Asp-N was purchased from Boehringer Mannheim GmbH (Mannheim, Germany). Guanidinium chloride (Gdn) was acquired from Pierce (Rockford, Illinois). All other reagents were of the highest grade commercially available.

The concentration of native RNase A solutions was determined using an 1% extinction coefficient of 0.695 at 278 nm (Schaffer et al., 1975).

PDI was purified from bovine liver as previously described (Freedman et al., 1995). Concentrations of PDI were determined by the method of Bradford (Bradford, 1976) with bovine serum albumin (BSA) as a standard.

Refolding reactions

RNase A was reduced and denatured as previously described (Torella et al., 1994). The purity of the reduced and denatured protein was checked by ESIMS analysis. No oxidized protein was detected even after months of storage.

Lyophilized reduced and denatured RNase A was dissolved to a concentration of ~3 mg/mL in 1% acetic acid and then diluted into the refolding buffer (0.1 M Tris-HCl, 1 mM EDTA pH 7.5) to a final concentration of 1 mg/mL. The desired amounts of GSH and GSSG stock solution (25 mM made fresh daily) were added to initiate refolding; typically, final concentrations of the glutathione species were 1.5 mM GSH/0.3 mM GSSG. The pH of the solution was adjusted from about 7.0 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 enzyme was dissolved in 0.1 M Tris-HCl, 1 mM EDTA pH 7.5 and preincubated in the presence of 1.5 mM GSH/0.3 mM GSSG redox buffer for 10 min at 25 °C. PDI concentration was fixed to 1 or 10 μM.

The refolding reaction was quenched after 1 or 4 min by alkylation with iodoacetic acid (IAA) (Torella et al., 1994). IAA was freshly dissolved in 0.1 M Tris-HCl, containing 1 mM EDTA and buffered with NaOH to pH 7.5. During preparation of the reagents, the solutions were protected from light to minimize photolytic production of iodine that is a very potent oxidizing agent for thiols. The refolding samples (500 μL) were added to an equal volume of 1 M IAA solution. Alkylation was performed for 2 min in the dark at room temperature, under nitrogen atmosphere. The sample was then desalted on a prepacked PD10 column equilibrated and eluted with Bis-Tris 20 mM pH 6.14, EDTA 0.2 mM. The protein fraction was then recovered and analyzed by FPLC.

Isolation of one-disulfide intermediate population

The population of one-disulfide intermediates was separated from the other refolding intermediates by anion-exchange chromatography on a Mono Q HR 5/5 column using a Pharmacia FPLC system. The elution system consisted of Bis-Tris 20 mM pH 6.14/EDTA 0.2 mM (solvent A) and Bis-Tris 20 mM pH 6.14/EDTA 0.2 mM/NaCl 0.1 M (solvent B). The system was equilibrated at 0% of solvent B for 15 min. Different disulfide species were separated using a linear gradient of solvent B from 0 to 100% over 120 min at flow rate of 1 mL/min. Eluted species were monitored at 280 nm, recovered, and lyophilized.

The lyophilized species were desalted by RP-HPLC using a Vydac C4 column (5 μm, 0.46 $\times$ 25 cm). The elution system consisted of 0.1% TFA (solvent A) and 0.07% TFA in 95% acetonitrile (solvent B). Refolding intermediates were desalted with a linear gradient of solvent B from 15 to 95% at a flow rate of 1 mL/min. Eluted proteins were monitored at 220 nm, recovered, and lyophilized.

Peptide mapping

Both carboxymethylated one-disulfide intermediates and the reduced and carboxymethylated RNase A were doubly digested with trypsin and endoproteinase Asp-N to ensure cleavage between the eight cysteine residues. Tryptic hydrolysis was carried out in 0.4% ammonium bicarbonate pH 8.5 at 37 °C for 8 h using an enzyme: substrate ratio of 1:50 (w/w). The sample was then boiled at 100 °C for 2 min and lyophilized. Endoproteinase Asp-N hydrolysis was carried out on the mixture of tryptic peptides in 0.8% ammonium bicarbonate pH 8 using 10% acetonitrile as activator at 37 °C for 18 h using an enzyme: substrate ratio of 1:100 (w/w).

MALDI-MS analyses

Peptide mixtures were analyzed by MALDI-TOF mass spectrometry using a Voyager DE mass spectrometer (PerSeptive Biosystems, Boston, Massachusetts). The mass range was calibrated using bovine insulin (average molecular mass 5,734.6 Da) and a matrix peak (379.1 Da) as internal standards. Samples were dissolved in 0.1% TFA at 10 pmol/μL. One microliter was applied to a sample slide and allowed to air-dry, before applying 1 μL of α-cyano-4hydroxycinammic acid (10 mg/mL) in ethanol/acetonitrile/0.1% TFA 1:1:1 (v:v:v). The matrix was allowed to air-dry before col-

lecting spectra. Mass spectra were generated from the sum of 50 laser shots.

LC/ESIMS analyses

Peptide mixtures were analyzed "on-line" by ESIMS using a LCQ Finnigan (Thermo Quest, Finnigan Corp., San Jose, California) mass spectrometer with an ion-trap analyzer. Fifty microliters of the peptide mixture (1 μg/μL) was loaded on a Vydac C-18 reversed-phase column. The mobile phase A consisted of 0.1% TFA; solvent B was 0.1% TFA in 95% acetonitrile. The separation was carried out using a gradient of 5–40% B in 58 min. The column effluent (1 mL/min) was split 2:1 to give a flow rate of 330 μL/min into the mass spectrometer connected "on-line." The remaining part was spectrophotometrically detected at 220 nm. Spectra were acquired from 150–2,000 Da. Data were acquired and elaborated by LCQ Navigator software provided by the manufacturer. The mass range was calibrated using a mixture of caffeine, Met-Arg-Phe-Ala peptide, and Ultramark 1621.

ESIMS analyses

ESIMS analyses were carried out using a BIO-Q triple quadrupole mass spectrometer equipped with an electrospray ion source (Micromass, Manchester, UK). Ten microliters of the protein solution (10 pm/μL) were directly injected into the ion source via loop injection. Data were acquired at 10 s/scan and elaborated by the MassLynx software provided by the manufacturer. Mass scale calibration was performed by means of multiply charged ions from a separate injection of horse heart myoglobin (average molecular mass 16,951.5 Da).

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

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