Contribution of Individual Disulfide Bonds to the Oxidative Folding of Ribonuclease A†

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ABSTRACT: The eight cysteine residues of ribonuclease A form four disulfide bonds in the native protein. We have analyzed the folding of three double RNase A mutants (C65A/C72A, C58A/C110A, and C26A/C84A, lacking the C65−C72, C58−C110, and C26−C84 disulfide bonds, respectively) and two single mutants (C110A and C26A), in which a single cysteine is replaced with an alanine and the paired cysteine is present in the reduced form. The folding of these mutants was carried out in the presence of oxidized and reduced glutathione, which constitute the main redox agents present within the ER. The use of mass spectrometry in the analysis of the folding processes allowed us (i) to follow the formation of intermediates and thus the pathway of folding of the RNase A mutants, (ii) to quantitate the intermediates that formed, and (iii) to compare the rates of formation of intermediates. By comparison of the folding kinetics of the mutants with that of wild-type RNase A, the contribution of each disulfide bond to the folding process has been evaluated. In particular, we have found that the folding of the C65A/C72A mutant occurs on the same time scale as that of the wild-type protein, thus suggesting that the removal of the C65−C72 disulfide bond has no effect on the kinetics of RNase A folding. Conversely, the C58A/C110A and C26A/C84A mutants fold much more slowly than the wild-type protein. The removal of the C58−C110 and C26−C84 disulfide bonds has a dramatic effect on the kinetics of RNase A folding. Results described in this paper provide specific information about conformational folding events in the regions involving the mutated cysteine residues, thus contributing to a better understanding of the complex mechanism of oxidative folding.

Bovine pancreatic ribonuclease A (EC 3.1.27.5) has been the model protein for folding studies (*1*−*7*) since the landmark discovery by Anfinsen (*8*) that the amino acid sequence provides all the information required for a protein to fold properly. RNase A contains four disulfide bonds (C26−C84, C40−C95, C65−C72, and C58−C110), which are critical to both the function and stability of the native enzyme (*9*, *10*). The two disulfide bonds (C26−C84 and C58−C110) that link a α-helix and a *β*-sheet in the protein core are the most important to conformational stability (*10*). On the other hand, the two disulfide bonds (C40−C95 and C65−C72) that link surface loops are most important to catalytic activity because of their proximity to active site residues (*10*).

The folding of RNase A in the presence of a small-molecule thiol redox couple has been studied in many laboratories, and different mechanisms of folding have been proposed depending on the experimental conditions. Initial folding studies performed by Creighton and co-workers (*11*−*16*) in the presence of reduced and oxidized glutathione suggested that the process proceeds through a single pathway, the rate-determining step being the formation of the C40−C95 disulfide bond. Creighton deserves great credit for having developed the strategy of using disulfide bonds as probes to study the folding of disulfide-containing proteins. Nevertheless, at present, these early studies on the folding of RNase A are subject to criticism because of the inadequate analytical methods used to trap the folding intermediates and to assign the disulfide bonds.

Parallel studies on the folding of RNase A were performed by Scheraga and co-workers (*17*−*29*). These authors suggested that RNase A regenerates, in the presence of DTTred[1] and DTTox, through two parallel pathways involving the formation of two native-like species containing three disulfide bonds. The main pathway produces a species lacking the C40−C95 disulfide bond, in line with the former results of Creighton, while a species lacking the C65−C72 disulfide bond is produced in a minor regeneration pathway. Furthermore, they reported that RNase A mutants C65A/C72A and

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C65S/C72S fold with a rate that is slower than that of the wild-type protein (*29*). It should be considered that although the use of DTT simplifies the folding pathways, because the mixed disulfides with proteins do not tend to accumulate, these folding conditions are quite far from the physiological ones.

Marino and co-workers (*30*−*32*) introduced the use of mass spectrometry in investigating the oxidative folding of RNase A in the presence of the glutathione redox system. These authors showed that the process proceeds through the reiteration of two sequential reactions: (i) reaction with the exogenous glutathione producing a mixed disulfide and (ii) formation of an intramolecular disulfide bond. More recently, they have characterized the population of one-disulfide intermediates occurring in the folding of RNase A under conditions that mimic those present in the ER lumen (*33*). They revealed the presence of only 12 disulfide-bonded species out of the 28 expected, confirming the previous conclusion that the formation of the S−S bonds during the folding of RNase A proceeds through a nonrandom mechanism (*25*). The mass spectrometry approach revealed that at the early stages of the process (i) the native C26−C84 pairing is absent, (ii) disulfide bonds containing C26 are under-represented, and (iii) most of the non-native disulfide bonds are formed by C110. In a collaborative project with Raines and co-workers (*4*), we have studied the folding of three double RNase A mutants (C65A/C72A, C58A/C110A, and C26A/C84A, lacking the C65−C72, C58−C110, and C26−C84 disulfide bonds, respectively) and two single mutants (C110A and C26A), in which a single cysteine is replaced with an alanine and the unpaired cysteine is present in the reduced form. Alanine was selected to replace cysteine in the mutants as it is regarded as the best substitute for cysteine in protein folding studies (*10*, *34*, *35*). We were prompted to undertake this study to shed light, on general grounds, on the role of disulfide bonds in the oxidative folding and, more specifically, to explain some contradictory conclusions about the role of the C65−C72 bond.

The use of mass spectrometry has allowed us (i) to follow the formation of intermediates and thus the pathway of folding of the RNase A mutants, (ii) to quantitate the intermediates that formed, and (iii) to compare the rates of formation of intermediates. By comparison of the folding kinetics of the mutants with that of wild-type RNase A, the relative contribution of each disulfide bond to the folding process has been evaluated. These results then provide information about conformational folding events in the regions involving the mutated cysteine residues.

MATERIALS AND METHODS

Materials. Reduced dithiothreitol, ethylenediaminetetraacetic acid, reduced glutathione, oxidized glutathione, trypsin, and RNase A were obtained from Sigma Chemical Co. Tris and iodoacetamide were purchased from Fluka. The prepacked Sephadex G-25M PD10 column was acquired from Pharmacia. Endoproteinase Asp-N and carboxypeptidase B were acquired from Boehringer Mannheim GmbH. Guanidinium chloride (Gdn) and cyanogen bromide was acquired from Pierce. All other reagents were of the highest grade commercially available. The concentrations of solutions of wild-type and mutant RNase A were determined using an absorption of 0.695 at 278 nm for a 1 mg/mL solution (*36*).

Preparation of C65A/C72A, C58A/C110A, C26A/C84A, C110A, and C26A RNase A. Plasmid DNA encoding RNase A mutants C65A/C72A, C58A/C110A, C26A/C84A, C110A, and C26A was prepared by oligonucleotide-mediated site-directed mutagenesis. Plasmid pBXR was designed to direct the production of RNase A in *Escherchia coli* (*37*). Mutagenesis was performed on plasmid pBXR replicated in *E. coli* strain DH11S or CJ236 (*10*, *38*). RNase A mutant proteins were produced and purified by the methods of Klink et al. (*10*) with the following modifications. After oxidation of the native disulfide bonds of the C26A and C110A mutants, meticulous care was taken to prevent further oxidation of the free sulfhydryl groups. Buffers for all chromatography steps were degassed prior to use. Protein solutions were immediately degassed and purged with Ar(g) after each purification step. After purification, the mutants were dialyzed exhaustively against deionized water under an Ar(g) atmosphere, and then lyophilized.

Peptide Mapping of C65A/C72A, C58A/C110A, C26A/C84A, C110A, and C26A RNase A. C65A/C72A, C58A/C110A, C26A/C84A, C110A, and C26A RNase A were reduced and denatured as previously described (*30*). After reduction and denaturation, the proteins were carboxyamidomethylated with iodoacetamide (10/1 IAM/SH molar ratio) in 0.10 M Tris-HCl containing 1 mM EDTA (pH 7.5) for 15 min at room temperature in the dark. The excess of the reagents was then removed by HPLC desalting using a Vydac T4 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). Samples were desalted from the excess of the reagents using a linear gradient of 5 to 95% solvent B at a flow rate of 1 mL/min. Eluted proteins were monitored at 220 and 280 nm. The protein fraction was recovered and lyophilized. The carboxyamidomethylated proteins were hydrolyzed with cyanogen bromide followed by proteolytic digestion with trypsin. Cyanogen bromide hydrolysis was carried out in 200 μL of 70% TFA using a 10-fold molar excess of reagent on methionine residues for 18 h at room temperature in the dark. The cyanogen bromide reaction was stopped by adding 10 volumes of cold water and lyophilizing the sample. Tryptic digestion was performed on the mixture of peptides obtained from the cyanogen bromide hydrolysis in 0.4% ammonium bicarbonate (pH 8.5) at 37 °C for 4 h using an enzyme/substrate ratio of 1/50.

MALDIMS Analyses. Peptide mixtures were analyzed by MALDI-TOF mass spectrometry. MALDIMS analyses were carried out using a Voyager DE mass spectrometer (PerSeptive Biosystem, Boston, MA). The mass range was calibrated using bovine insulin (average molecular mass of 5734.6 Da) and a matrix peak (379.1 Da) as internal standards. Samples were dissolved in 0.1% TFA at 10 pmol/μL. An 1 μL aliquot was applied to a sample slide and allowed to air-dry before 1 μL of a solution of α-cyano-4-

[1] Abbreviations: DTTox, oxidized dithiothreitol; DTTred, reduced dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ESIMS, electrospray ionization mass spectrometry; ER, endoplasmic reticulum; GSSG, oxidized glutathione; GSH, reduced glutathione; IAM, iodoacetamide; LC−ESIMS, liquid chromatography−electrospray ionization mass spectrometry; MALDIMS, matrix-assisted laser desorption ionization mass spectrometry; MW, molecular mass; RNase A, bovine pancreatic ribonuclease A; TFA, trifluoroacetic acid.

hydroxycinnamic acid (10 mg/mL) in ethanol, acetonitrile, and 0.1% TFA (1/1/1, v/v/v) was applied. The matrix was allowed to air-dry before spectra were collected. Mass spectra were generated from the sum of 50 laser shots.

Folding Reactions. C65A/C72A, C58A/C110A, C26A/C84A, C110A, and C26A RNase A and wild-type RNase A were reduced and denatured as previously described (*30*). The protein solutions were then separated from the excess of DTT and denaturant by HPLC desalting. The protein fraction was recovered, lyophilized, and used within 2 days. The purity of the reduced and denatured proteic samples was checked by ESIMS analysis. Lyophilized reduced and denatured wild-type and mutant RNase A were dissolved to a concentration of approximately 3 mg/mL in 1% acetic acid and then diluted into the folding buffer [0.1 M Tris-HCl and 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 folding; typical final concentrations of the glutathione species were 1.5 mM GSH and 0.3 mM GSSG. The pH of the solution was adjusted to 7.5 with Tris base and the reaction carried out at 25 °C under a nitrogen atmosphere.

Alkylation of Folding Aliquots. The folding was monitored on a time course basis by sampling aliquots of the folding mixture at appropriate intervals. The protein samples were alkylated as described previously (*30*, *39*). IAM was freshly dissolved in 0.1 M Tris-HCl containing 1 mM EDTA (pH 7.5) at 65 °C and cooled to room temperature before being used. During preparation of the reagents, the solution was protected from light to minimize photolytic production of iodine that is a very potent oxidizing agent for thiols. The folding aliquot (50 μL) was added to an equal volume of a 2.2 M IAM solution. Alkylation was performed for 20 s in the dark at room temperature, under a nitrogen atmosphere. After 20 s, 350 μL of 5% acetic acid was added and the sample was desalted on a prepacked PD10 column equilibrated and eluted with 1% acetic acid. The protein fraction was recovered and lyophilized. These alkylation conditions were demonstrated to be effective in providing a rapid quenching of the thiol groups (*30*, *39*).

Electrospray Mass Analysis. ESIMS analyses were carried out using a Bio-Q triple-quadrupole mass spectrometer equipped with an electrospray ion source (Micromass, Manchester, U.K.). The protein samples were dissolved in water containing 2% acetic acid and diluted in a 1/1 ratio with acetonitrile. A 10 μL aliquot of the protein solution (10 pmol/μL) was directly injected into the ion source via loop injection. Data were acquired at 10 s/scan and elaborated by Mass Lynx 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 of 16 951.5 Da). Each set of folding data was obtained as the means of three independent folding experiments. The differences between folding experiments performed completely independent of each other were about 5%.

Identification of Disulfide Bonds in the Folding Intermediates. Aliquots of the folding mixture of wild-type and RNase A mutants were sampled after incubation for 4 min, 30 min, 7 h, and 24 h and alkylated as described. The samples were proteolytically digested with trypsin followed by 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). Endoproteinase-AspN digestion was carried out on the mixture of tryptic peptides in 0.8% ammonium bicarbonate (pH 8) using 10% acetonitrile as the activator at 37 °C for 18 h using an enzyme/substrate ratio of 1/100 (w/w). The peptide mixtures were analyzed by MALDIMS as previously described. To confirm the assignment of mass signals to peptides along the RNase A sequence, carboxypeptidase B hydrolysis was carried out on tryptic/Asp-N peptide mixtures in 0.4% ammonium bicarbonate (pH 8.5) at 37 °C for 15 min. The peptide mixtures were then reanalyzed by MALDIMS. Alternatively, mass signals were confirmed by performing a manual Edman step as described previously (*40*).

Table 1: Properties of Ribonuclease A Mutants

ribonuclease A	measured MW (Da)	expected MW (Da)	redox state
C65A/C72A	13 619.1 ± 0.7	13620.2	3 S−S
C58A/C110A	13 620.5 ± 0.9	13620.2	3 S−S
C58A/C110A	13 621.1 ± 0.8	13620.2	3 S−S
C110A	13 652.0 ± 1.1	13652.2	3 S−S/1 SH
C26A	13 652.2 ± 0.5	13652.2	3 S−S/1 SH

RESULTS

Characterization of C65A/C72A, C58A/C110A, C26A/C84A, C110A, and C26A RNase A. The RNase A C65A/C72A, C58A/C110A, C26A/C84A, C110A, and C26A mutants were analyzed by ESIMS. All the spectra show the presence of a single component exhibiting a molecular mass in agreement with the expected mass values calculated on the basis of their mutated amino acid sequences, as reported in Table 1. A peptide mapping of each mutant was performed to confirm the protein sequence. The RNase A mutants were reduced, denatured, and alkylated as described. The alkylated proteins were hydrolyzed with cyanogen bromide and trypsin, and the peptide mixtures that were obtained were analyzed by MALDIMS. The recorded MALDI signals were associated with the corresponding fragments on the basis of their molecular masses. The MALDI mapping analysis demonstrated that the five mutants do have the expected mutated amino acid sequences (data not shown). This extensive mass spectrometric characterization made us fully confident with all the mutants that were being studied.

Folding of RNase A Mutants. The reduced and denatured proteins were incubated in the presence of a 1.5 mM GSH/0.3 mM GSSG mixture for the oxidative folding. Aliquots of the folding process were withdrawn at different intervals, and the intermediates present in solution were trapped by alkylation of the free thiol groups as described and analyzed by ESIMS to identify the disulfide-bonded intermediates that formed. It is worth underlining that, under the alkylation conditions we set up, the trapping of free thiols is quantitative (*30*−*33*). The carboxyamidomethylation reaction, used to trap the free SH groups, increased the molecular mass of the intermediates by a fixed amount of 57 Da for each free SH group, thus allowing the separation by mass of intermediates containing different numbers of disulfide bonds. In addition, the relative concentration of each intermediate can be determined by measuring the total ion current produced

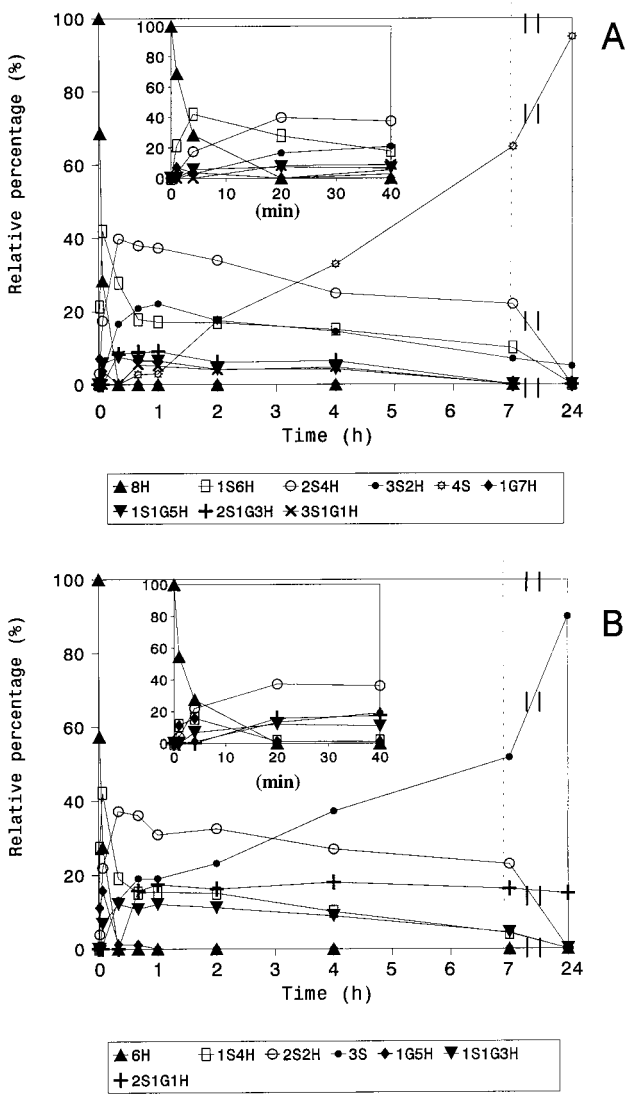

FIGURE 1: Time course analysis of the oxidative folding of wild-type RNase A (A) and the C65A/C72A RNase A mutant (B) in the presence of 1.5 mM GSH and 0.3 mM GSSG. Percentages of intermediates are derived by ESIMS analysis as described in the text. The inset shows early stages of the reaction. *n*S represents intramolecular disulfide bonds. *n*G represents mixed disulfides with the exogenous glutathione. *n*H represents free thiols.

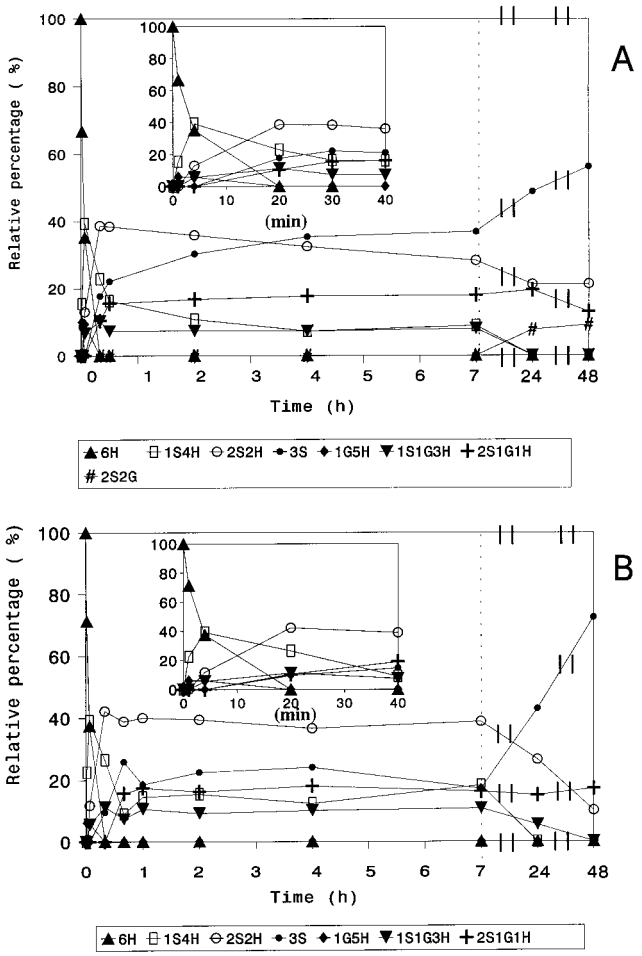

FIGURE 2: Time course analysis of the oxidative folding of C58A/C110A (A) and C26A/C84A (B) ribonuclease A mutants. The inset shows early stages of the reaction. *n*S represents intramolecular disulfide bonds. *n*G represents mixed disulfides with the exogenous glutathione. *n*H represents free thiols.

by each species provided that the different components are endowed with comparable ionization capabilities (*30*, *32*).

The relative intensity of the intermediates formed during the folding process of C65A/C72A RNase A is shown in Figure 1B in comparison with the folding of wild-type RNase A (Figure 1A), described elsewhere (*32*). The reduced species (6H in Figure 1B) rapidly disappears after 20 min; the 2S2H intermediate predominates from 20 min up to about 4 h when the relative concentration of the 3S species increases. At 24 h, the fully oxidized protein accumulates to a concentration of 90%. This analysis shows that species containing mixed disulfide with glutathione do not accumulate to a concentration higher than 15% throughout both the folding processes, confirming that these intermediates constitute transient species rapidly evolving toward the formation of intramolecular disulfide bonds.

The time scale of C65A/C72A RNase A folding is very similar to that observed for the folding of the wild-type protein (compare to Figure 1A). The only marked difference between the two processes is that the appearance of the completely oxidized wild-type species (4S) shows a lag phase at the early stages of the folding while the fully oxidized species (3S) in the folding of C65A/C72A RNase A does not show any initial lag phase.

The time course of the folding of the C58A/C110A mutant of RNase A is shown in Figure 2A. The completely reduced species (6H) disappears within the first 20 min. The 2S2H intermediates predominate until 4 h. From 4 h onward, the 3S species predominates never reaching values higher than 60%, even after 48 h. Finally, at the late stages of the folding process, 2S2G abortive intermediates containing two mixed disulfides with glutathione tend to accumulate at a level of about 10%.

The time course of the folding of the C26A/C84A mutant is reported in Figure 2B. The reduced species (6H) disappears within 20 min, while the 2S2H species rapidly appear in the first minutes and predominate until 7 h. The level of the fully oxidized species (3S) is never higher than 20% until 7 h, increases after incubation for 7 h, and reaches a level of 70% at 48 h. The levels of intermediates containing one mixed disulfide with glutathione are always lower than 20%.

The relative intensity of the disulfide bonded intermediates formed during the folding of the C110A mutant of RNase A is reported in Figure 3A. The time course very clearly

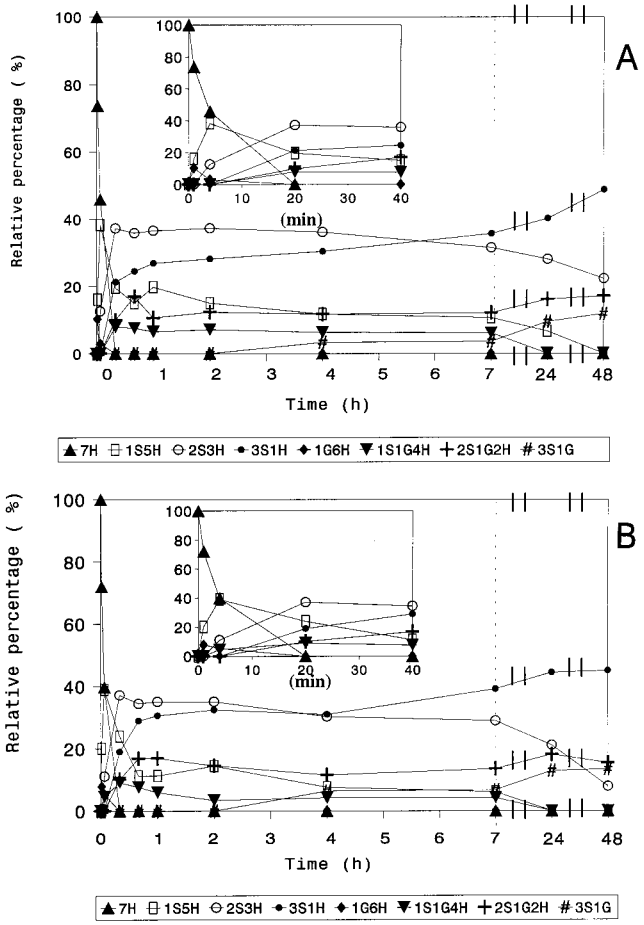

FIGURE 3: Time course analysis of the oxidative folding of C110A (A) and C26A (B) ribonuclease A mutants. The inset shows early stages of the reaction. *n*S represents intramolecular disulfide bonds. *n*G represents mixed disulfides with the exogenous glutathione. *n*H represents free thiols.

shows that intermediates 2S3H and 3S1H are predominant from the beginning of the reaction up to 7 h. A steady state of nearly equal concentration is established between these two intermediates from 40 min onward. The 3S1H species accumulate to a level slightly higher than that of 2S3H species, reaching a value of 45% after only 48 h. A 3S1G species is observed from 120 min onward, indicating that the species 3S1H is further oxidized by glutathione.

The time course of the folding of the C26A mutant of RNase A reported in Figure 3B seems to be very similar to that observed in the case of the C110A mutant. From 40 min up to 7 h, there is a clear equilibrium between the 2S3H and 3S1H species, with the latter accumulating to a concentration higher than 45% only at 48 h. A 3S1G species is also observed in this folding process, indicating that the 3S1H species is further oxidized by glutathione.

Identification of Disulfide Bonds in the Folding of Wild-Type RNase A. Aliquots of the folding mixture were withdrawn at 4 min, 30 min, 7 h, and 24 h and the thiol-containing intermediates present in solution trapped with iodoacetamide. The peptide mixtures, obtained by digesting the folding intermediates with trypsin and endoproteinase Asp-N, were directly analyzed by MALDIMS. The assignments of disulfide bonds in the folding of the wild-type protein are reported in Table 2. Since several mass signals, i.e., those occurring at *m*/*z* 1834.9, 2187.2, 2302.2, 2328.6, 2652.9, and 2772.7, correspond to isobaric disulfide-bridged peptides, an aliquot of the peptide mixtures was then submitted to a single step of Edman degradation (*40*). The shifts of the signals to lower mass values, corresponding to the removal of the two putative N-termini, in most cases confidently allow the bridge assignment. However, in the case of the peptide with the 40−52 disulfide bond, containing a cysteine residue at the N-terminal position, because of the signal collapse after the Edman degradation step, a further experiment was needed. An aliquot of the tryptic peptide mixtures was then incubated with carboxypeptidase B, which shifted the signals to lower mass values corresponding to the removal of the putative basic C-terminus. This procedure confirmed all the disulfide bond assignments and allowed us to establish that the mass signals at *m*/*z* 1834.9, 2187.2, 2302.2, 2328.6, 2652.9, and 2772.7 correspond to peptides linked by different disulfide bonds, as shown in Table 2.

Table 2: Disulfide Bonds Present in the Folding Mixture of Wild-Type RNase A[a]

MALDI (MH+)					
4 min	30 min	7 h	24 h	assignment	S−S bond
1249.1	1249.9	absent	absent	83−85 and 92−98	C84−C95
1508.7	1508.6	absent	absent	53−61 and 62−66	C58−C65
1750.8	1750.3	1750.0	absent	40−52 and SG	C40−SG
absent	1834.9	1835.1	1835.0	40−52 and 83−85	C40−C84
absent	1834.9	absent	absent	53−61 and 92−98	C58−C95
2100.9	2100.8	2100.9	absent	105−120 and SG	C110−SG
2187.2	2187.5	2187.2	absent	67−82 and 83−85	C72−C84
2187.2	2187.5	2187.2	2187.5	83−85 and 105−120	C84−C110
2301.9	**2302.1**	**2302.2**	**2302.3**	**40−52 and 92−98**	**C40−C95**
absent	**absent**	**2302.2**	**2302.3**	**14−31 and 83−85**	**C26−C84**
2328.6	**2328.3**	**2328.3**	**2328.6**	**62−66 and 67−82**	**C65−C72**
2328.6	2328.3	2328.3	absent	62−66 and 105−120	C65−C110
2420.7	2420.4	2420.4	2420.3	40−52 and 53−61	C40−C58
2652.9	2652.8	2652.8	absent	67−82 and 92−98	C72−C95
2652.9	2652.8	2652.8	absent	92−98 and 105−120	C95−C110
2772.7	2772.5	2772.6	absent	53−61 and 67−82	C58−C72
2772.7	**2772.5**	**2772.6**	**2771.4**	**53−61 and 105−120**	**C58−C110**
absent	3239.1	3238.7	3238.1	40−52 and 105−120	C40−C110
absent	absent	3355.9	absent	14−31 and 40−52	C26−C40
absent	absent	3706.2	absent	14−31 and 105−120	C26−C110

[a] Native S−S bonds are in bold. SG represents mixed disulfides with glutathione.

The extensive MALDI analysis revealed after 4 min of folding, the presence of three out of four native disulfide bonds (C40−C95, C65−C72, and C58−C110), together with nine non-native disulfide bonds. After 30 min, the number of non-native disulfide bonds is increased, while the fourth native bond, C26−C84, is still absent. After 7 h, we observed the presence of all four native disulfide bonds, the absence of some non-native couplings, and the presence of two new non-native disulfide bonds involving C26. The MALDI analysis of the sample withdrawn after 24 h showed essentially the native disulfide bonds together with a few weak signals assigned to non-native couplings, which correspond to molecular species that have not yet completed the folding process. It is worth underlining the fact that after 24 h we observed an almost complete (95%) recovery of RNase A activity, thus indicating that our folding conditions generated genuine native RNase A rather than significant amounts of isomers containing a non-native conformation. Finally, it is worth noting the constant occurrence of C40 or C110 involved in mixed disulfides with glutathione.

C65A/C72A

	84-95	84-110	40-58	26-58	**40-95**	**26-84**	**58-110**	95-110	40-110	26-40	26-110	40-SG	110-SG
4 min	+	+	+	a	+	**a**	+	+	a	a	a	+	+
30 min	+	+	+	+	+	+	+	+	+	+	+	+	+
7 h	+	+	+	+	+	+	+	+	+	+	+	+	+
24 h	+	+	+	a	+	+	+	+	+	+	+	+	+

C58A/C110A

	84-95	72-95	72-84	**65-72**	**40-95**	**26-84**	40-84	40-72	26-95	26-40	95-SG	40-SG
4 min	+	a	a	**a**	**a**	**a**	+	a	a	a	a	**a**
30 min	+	+	+	**a**	+	+	+	+	+	a	a	+
7 h	+	+	+	+	+	+	+	+	+	+	a	+
24 h	+	+	+	+	+	+	+	+	+	a	+	+

C26A/C84A

	65-110	72-95	40-58	**65-72**	**40-95**	58-95	**58-110**	95-110	40-110	58-72	72-110	40-SG	110-SG
4 min	a	+	a	+	**a**	a	**a**	+	a	+	a	+	+
30 min	+	+	+	+	+	+	+	+	+	+	+	+	+
7 h	+	+	+	+	+	+	+	+	+	+	+	+	+
24 h	+	+	+	+	+	+	+	+	+	+	+	+	+

FIGURE 4: Disulfide bonds present in the folding process of double ribonuclease A mutants at different times: (+) disulfide present at that time and (a) disulfide absent at that time. SG represents mixed disulfides with the exogenous glutathione. Native S−S bonds are in bold.

Identification of Disulfide Bonds in the Folding of C65A/C72A RNase A. Aliquots of the folding mixture were withdrawn at 4 min, 30 min, 7 h, and 24 h, and treated as described for the wild-type protein. The assignment of the disulfide bonds reported in Figure 4 was based on the same procedure described above. After 4 min of folding, two native disulfide bonds (C40−C95 and C58−C110) together with four non-native couplings were present. It is worth noting the absence of the native disulfide bond (C26−C84) at early stages of the reaction, as observed in the folding of wild-type RNase A (see Tables 2 and refs *31* and *33*). All three native disulfide bonds and a higher number (eight) of non-native ones are present in the folding mixture withdrawn after 30 min. After 7 h, the same disulfide bonds were detected in the folding mixture. At 24 h, the MALDI spectrum revealed the presence of the three expected native disulfide bonds with weak signals corresponding to non-native disulfide bonds. The presence after 24 h of signals corresponding to non-native disulfide bonds demonstrates that at this stage some minor molecular species have presumably not completed the folding process. It is worth noting that the fully oxidized species produced in the folding process was shown to have RNase A catalytic activity (*10*).

Finally, like wild-type RNase A folding, the only cysteine residues involved in the formation of mixed disulfides with glutathione were C40 and C110.

Identification of Disulfide Bonds in the Folding of C58A/C110A RNase A. Aliquots of the folding mixture were withdrawn at 4 min, 30 min, 7 h, and 24 h; the intermediates present in solution were alkylated, hydrolyzed, and analyzed by MALDI mapping as described previously. The identification of disulfide bonds reported in Figure 4 was obtained as described above. After 4 min of folding, the three native disulfide bonds are absent and only two non-native couplings involving C84 are present. In the folding mixture withdrawn after 30 min are present two native disulfide bonds (C26−C84 and C40−C95) and five further non-native bonds. At 7 h, the native disulfide bond (C65−C72) is finally observed along with two additional non-native disulfides involving C26. Finally, after 24 h, the MALDI spectrum reveals the presence of the three native disulfide bonds together with many signals corresponding to non-native disulfide bonds, thus demonstrating that at this stage of the process many molecular species exist that have not yet completed the folding process as clearly shown in Figure 2A.

Finally, C40 was found to be involved in a mixed disulfide with glutathione from 30 min onward. At 24 h, an additional mixed disulfide was observed involving C95, which is probably due to the 2S2G abortive intermediates present at late stages of the folding process as discussed previously (Figure 2A).

Identification of Disulfide Bonds in the Folding of C26A/C84A RNase A. Aliquots of the folding mixture were withdrawn at 4 min, 30 min, 7 h, and 24 h and analyzed as described. The identification of disulfide bonds is reported in Figure 4. After 4 min of folding, only one native disulfide bond (C65−C72) and three non-native couplings are present. From 30 min onward, all three native disulfide bonds are present together with several non-native disulfide bonds. This distribution of disulfide bonds does not change with time. Finally, C40 and C110 were found to be involved in mixed disulfides with glutathione during the whole process.

Identification of Disulfide Bonds in the Folding of C110A RNase A. The same strategy previously described was used for the assignment of disulfide bonds present in the folding of the C110A mutant of RNase A. The results are shown in

C110A

	40-SG	26-40	84-95	72-95	72-84	**65-72**	**40-95**	**26-84**	26-95	40-84	58-95	40-58	58-72	26-58
4 min	+	a	a	+	+	**a**	**a**	**a**	a	a	a	a	+	a
30 min	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7 h	+	+	+	+	+	+	+	+	+	+	+	+	+	+
24 h	+	a	+	+	+	+	+	+	+	+	+	+	+	+

C26A

	40-SG	110-SG	84-95	72-95	72-84	**65-72**	**40-95**	84-110	**58-110**	40-84	58-95	40-58	58-72	65-110	95-110	40-110	72-110
4 min	+	+	a	+	+	+	**a**	+	**a**	a	a	+	+	+	+	+	a
30 min	+	+	a	+	+	+	+	+	+	a	a	+	+	+	+	+	a
7 h	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
24 h	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

FIGURE 5: Disulfide bonds present in the folding process of single ribonuclease A mutants at different times: (+) disulfide present at that time and (a) disulfide absent at that time. SG represents mixed disulfides with the exogenous glutathione. Native S−S bonds are in bold.

Figure 5. After 4 min, only three non-native disulfide bonds were present. After 30 min, the number of disulfide bonds greatly increases with the formation of three native disulfide bonds and 10 non-native couplings. The distribution of disulfide bonds seems to remain unaltered after 7 and 24 h, confirming the steady state observed in the time course of the distribution of intermediates (see Figure 3A). Finally, the constant presence of C40 forming mixed disulfides with glutathione was observed.

Identification of Disulfide Bonds in the Folding of C26A RNase A. The disulfide bonds present in the folding mixture of the C26A mutant of RNase A were identified as described above, and the assignment is reported in Figure 5. After 4 min, only one native disulfide bond (C65−C72) was observed together with eight non-native disulfide bonds. After only 30 min, all three native disulfide bonds are present in the mixture together with the same non-native disulfides detected after 4 min. After 7 h, three additional non-native disulfide bonds are observed (C40−C84, C58−C95, and C72−C110). The distribution of disulfide bonds seems to remain unaltered after 24 h, confirming the steady state observed in the time course of the distribution of intermediates (see Figure 3B). All the analyses revealed that C40 and C110 are involved in mixed disulfides with glutathione.

DISCUSSION

Mass spectrometry was found to be a powerful analytical tool in the description of the folding of RNase A under quasi-physiological conditions (*30*−*33*).

Our previous studies showed that the formation of disulfide bonds during the process occurs through a nonrandom mechanism with a preferential coupling of certain cysteine residues. C110 is involved in many non-native disulfide bonds formed during the acquisition of the native structure. In contrast, C26 is under-represented and the native disulfide bond (C26−C84) is the last to be formed during the folding process (*31*, *33*). The rates of disulfide bond formation basically depend on two factors: (a) local interactions stabilizing the cysteine-related polypeptide chain and (b) intrinsic reactivities of the SH groups. However, the latter factor is by itself related to the evolving intermediate structures, and as a matter of fact, it may vary during the folding process. It is worth noting that in a very detailed study, Scheraga and co-workers (*25*) demonstrated that the preferential couplings of cysteine residues in RNase A refolding do not result from a marked difference in the pK_as of cysteine residues but rather from specific stabilizing interactions. Furthermore, we have demonstrated (*33*) that the nonrandom coupling of cysteine residues is biased in three respects: (i) toward the formation of short-range linkages, (ii) toward the formation of native disulfides, and (iii) toward the formation of disulfides involving cysteines located in the C-terminal region.

In this paper, we have extensively exploited mass spectrometry in investigating the folding of three double RNase A mutants (C65A/C72A, C58A/C110A, and C26A/C84A) and two single mutants (C110A and C26A) to evaluate the role of the disulfide bonds or the single cysteine residues in the folding process. The use of mass spectrometry in this study has allowed us (1) to follow the intermediate formation and folding pathways of RNase A mutants, (2) to quantitate the intermediates that formed, and (3) to compare the rates of formation of intermediates along the folding of RNase A mutants with that of the wild-type protein.

We have actually shown that the mutant proteins fold according to the sequential mechanism proposed for the wild-type protein, under quasi-physiological conditions (*32*). Thus, only a limited number of intermediates are formed in the process as compared to all those expected. Nevertheless, within the frame of this common pathway, differences do exist in the amount and formation rate of individual intermediates arising during the folding of particular mutants.

Folding of the C65A/C72A RNase A Mutant. Our results demonstrate that the removal of the C65−C72 disulfide bond has no effect on the kinetics of folding of RNase A. The folding of the C65A/C72A mutant occurs on the same time

scale as the folding of the wild-type protein (see Figure 1); in fact, the appearance of the fully oxidized species occurs at almost the same rate in both folding processes. Furthermore, the individual native and non-native disulfides, but obviously not those involving the two absent cysteine residues, appear at the same times during the folding processes. These results suggest that the folding processes of the wild-type and mutant RNase A are driven by similar interatomic interactions without any great influence of the C65−C72 bond. Actually, if the formation of the type III β-turn involving residues 66−69 (*17*, *19*, *25*) is energetically favored, these local interactions, independent from the disulfide bond, rule the subsequent folding events. However, it seems likely that the C65A/C72A mutant folds on the same time scale as the wild-type protein, if these local structures remain flexible in the transition state of the process.

Interestingly, the C65−C72 disulfide bond is the only disulfide bond that is not conserved throughout the RNase superfamily (*41*). The atomic resolution structure of RNase A shows that the C65−C72 bond occurs in a double conformation (L. Mazzarella, personal communication). On the other hand, in the structure of a deamidated derivative (N67isoD) of RNase A, which exhibits a higher flexibility in the region of the 65−72 disulfide bond, the disulfide bond adopts a single conformation (*42*). These findings are in line with our results because they confirm that the formation of the type III β-turn precedes that of the disulfide bond.

The above data, although fully confirming the initial hypothesis made by Nemethy and Scheraga that the loop region of the 65−72 disulfide bond is indeed a chain folding initiation site for RNase A (*17*), are in contrast with the more recent studies by the same group (*26*, *29*). They actually claim that the C65A/C72A RNase A mutant folds more slowly than the wild-type protein (*29*) without making any reference to their previous hypothesis. The discrepancy between our results and their results may be due to the lack of a rigorous structural characterization of their folding intermediates; however, the possibility that it may also be ascribed to the use of very different experimental conditions cannot be ruled out.

It is worth noting that the only relevant difference between the folding processes of the wild type and the C65A/C72A mutant is the absence of a lag phase in the appearance of the fully oxidized species during the folding of the mutant RNase A. This absence of a lag phase was also observed in the analysis of the folding of the other mutants (see Figures 2 and 3). The presence of a lag phase in the folding of wild-type RNase A could eventually be interpreted as being due to some misfolded species that can act as kinetic traps. This aspect has been thoroughly investigated by Scheraga and coworkers (*43*), who concluded that the few kinetic traps result from the burial of thiol groups in species with native disulfide bonds and native structure and do not correspond to species with non-native disulfide bonds and misfolded structures. Therefore, our finding could be explained essentially by the occurrence of a lower number of intermediate species in the folding of the mutant proteins. For example, only 15 fully oxidized forms containing three disulfide bonds can be generated in the folding of the mutant proteins versus the 105 possible species containing four disulfide bonds that can be produced during the folding of the wild-type protein.

Finally, the identification of disulfide bonds within each intermediates population accumulating during the folding of the C65A/C72A mutant was out the scope of this study, but it is clear that the definition of such isomers within each population will allow the definitive and complete description of the folding pathways in terms of individual molecular species.

Folding of the C58A/C110A and C26A/C84A Mutants. The C58A/C110A and C26A/C84A mutants fold much more slowly than the wild-type protein or the C65A/C72A mutant (see Figures 1 and 2). The fully oxidized species (3S) does not show any lag phase in its appearance at early stages of the process, but becomes only marginally predominant after a very long period of incubation. After 48 h, the 3S species coexists in solution with intermediates species that are absent or almost absent in the final stages of the folding of the C65A/C72A mutant (see Figures 1 and 2). It is interesting to note that the single mutants, C110A and C26A, have almost the same qualitative behavior (compare Figures 2 and 3), thus indicating that the removal of a single cysteine or both cysteines of a disulfide pair has the same effect on the kinetics of the folding process.

A steady state is established between two- and three-disulfide containing species in the folding processes analyzed above, thus suggesting that the slow step could be due to the formation of the third disulfide bond. This slow step could be due either to a slow isomerization of non-native disulfide bonds or to some steric hindrance inhibiting the formation of the last disulfide bond. The assignments of disulfide bonds during the folding of the C58A/C110A, C26A/C84A, C110A, and C26A mutants (see Figures 4 and 5) show that many non-native disulfide bonds are still present in solution at late stages of the reaction, thus confirming that many scrambled molecular species have not yet completed the folding process. Furthermore, during the folding of the C58A/C110A mutant, we unexpectedly observe at the late stages of the reaction the accumulation of a 2S2G species. It could be that the productive 2S1H1G species accumulate to such an extent that they react with a further molecule of glutathione, producing the 2S2G dead-end species.

The C26A/C84A and C58A/C110A mutants are approximately half-folded under our folding conditions since their T_ms are 23.9 ± 0.2 and 27.2 ± 1.7 °C, respectively (*10*). It can well be that the 3S species produced in the folding because of their relatively unstable tertiary structure can easily react with reduced glutathione, producing 2S1H1G and 2S2H species. Conversely, the formation of C58−C110 or C26−C84 disulfide bonds can function as a lock of the native structure, thus hampering the 3S species reshuffling in the folding of both wild-type RNase A and the C65A/C72A mutant. The stability of large disulfide loops, including the 26−84 and 58−110 disulfide bonds, has been claimed to drive the collapse during the early folding stages of RNase A (*44*). It may be then possible that, if the C26−C84 and C58−C110 disulfide bonds are absent, some disulfide intermediates are not significantly stabilized. As a consequence, a larger number of intermediates with comparable conformational stabilities accumulate and interconvert with each other, leading to the steady state observed in the folding of these mutants (Figure 2). Still, some differences in conformational stability must exist among the different

disulfide intermediates, which result in a nonrandom distribution of disulfide bonds (see Figures 4).

The number and type of intermediates observed during the folding of C58A/C110A and C26A/C84A RNase A are similar to those found during the folding of other three-disulfide proteins such as hirudin (*45*, *46*), tick anticoagulant peptide (*47*), and ω-conotoxins (*48*). In each protein, a steady state is established between many disordered disulfide-containing intermediates, and the slow step is the formation of the 3S species from the 2S2H species. In contrast, the most extensively studied three-disulfide containing protein, BPTI, folds differently with the formation of only three stable one-disulfide intermediates and two stable two-disulfide intermediates (*49*). However, BPTI represents a special case because of its exceptional stability (*49*).

Identification of Disulfide Bonds. The identification of disulfide bonds formed during the folding of RNase A mutants showed that C110 is the most actively engaged cysteine residue in the formation of disulfide bonds, as previously seen in the folding of the wild-type protein (*31*, *33*). It should also be noted that C40 is very reactive. Both C40 and C110 are seen to form several non-native disulfide bonds, and both cysteine residues react to form mixed disulfides with glutathione. On these grounds, it is reasonable to suggest that a smaller number of disulfide bonds are formed during the folding of the C58A/C110A and C110A mutants, which lack the most reactive cysteine residue, namely, C110. These data suggest the hypothesis that C110 can function as an internal catalyst that is able to promote reshuffling of disulfides to accelerate isomerization reactions in the re-establishment of the native state.

The most striking result in the assignment of disulfide bonds during the folding process of the C58A/C110A, C26A/C84A, C110A, and C26A mutants is the slow formation of native disulfide bonds at the very early stages of folding (see Figures 4 and 5). After 4 min, only the native C65−C72 disulfide bond is present in the folding of the C26A/C84A and C26A mutants, while this native disulfide bond is absent at the early stages of the folding of the C58A/C110A and C110A mutants. These results suggest that stable native-like interactions occur slowly along the folding process of these mutants so that the formation of native disulfide bonds is not favored with respect to the formation of non-native ones.

Finally, it is interesting to note in the folding of the mutants the extensive involvement of C26 in the formation of native and non-native disulfide bonds. The native disulfide bond (C26−C84) is formed earlier during the folding of the C65A/C72A, C58A/C110A, and C110A mutants than during the folding of wild-type RNase A (compare Table 2 with Figures 4 and 5). Furthermore, C26 was found to be involved in a larger number of non-native disulfide bonds during the folding of the mutants than during the folding of the wild-type protein. It could be possible that the absence of some disulfides in the RNase A mutants provides higher flexibility to the polypeptide chain, resulting in a higher conformational freedom of the segment containing C26 and bringing this residue in the correct position to form disulfide bonds.

CONCLUSIONS

Present results show that the removal of the C65−C72 disulfide bond has no effect on the kinetics of folding of RNase A, suggesting that the folding processes of the wild-type and mutant RNase A are driven by similar interatomic interactions without any great influence of the C65−C72 disulfide bond. We also first report the analysis of the folding of the C58A/C110A and C26A/C84A mutants, showing that they fold much more slowly than the wild-type protein or the C65A/C72A mutant. The data presented here confirm that C110 may work as an internal catalyst that is able to promote shuffling of disulfides to accelerate isomerization reactions in the establishment of the native state. In conclusion, these data advance the understanding of the complex mechanism of oxidative folding of RNase A by providing some insights into the nature of folding intermediates and their rates of interconversion.

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