Regeneration Studies of an Analog of Ribonuclease A Missing Disulfide Bonds 65−72 and 40−95[†]

Cathy C. Lester,[‡] Xiaobing Xu,[§] John H. Laity,[||] Sakurako Shimotakahara,[||] and Harold A. Scheraga*

Baker Laboratory of Chemistry, Cornell University, Ithaca, New York 14853-1301

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ABSTRACT: Mutants of bovine pancreatic ribonuclease A (RNase A) that contain four of the eight cysteine residues found in the wild-type protein were prepared. Cysteine residues 40, 65, 72, and 95 were replaced by serine to form [C40S,C65S,C72S,C95S] RNase A or by alanine to form [C40A,C65A,C72A,C95A] RNase A, which contain the following four cysteine residues: 26, 58, 84, and 110. The substitutions resulted in deletion of wild-type disulfide bonds, 65−72 and 40−95. These mutants were prepared to investigate interactions that may be important for the folding and unfolding of the wild-type protein. The mutant protein was expressed and purified in an unfolded sulfonated form. Upon regeneration of the native form from the reduced mutant with DTT^{ox}, all three of the possible two-disulfide pairings, including the native one, formed. One-dimensional ^1H NMR spectra demonstrated that the conformations of these three species are similar and are predominantly disordered; however, there is evidence of local structure in the vicinity of one histidine residue. It was also shown that disulfide pairing is not completely random and that both entropic factors and enthalpic interactions contribute to the formation of the native-disulfide bonds. The presence of more than a statistical population of native-disulfide pairings indicates that specific local interactions present in the reduced protein direct the preferential formation of native-disulfide bonds in the two-disulfide mutant.

Considerable insight into protein folding has been acquired by studying the disulfide-coupled oxidative-folding and reductive-unfolding pathways of ribonuclease A (RNase A)[1] (Hantgan et al., 1974; Ahmed et al., 1975; Takahashi & Ooi, 1976; Creighton, 1977a,b, 1979; Konishi & Scheraga, 1980a,b; Konishi et al., 1981, 1982a,b; Wearne & Creighton, 1988; Rothwarf & Scheraga, 1991, 1993a−d; Li et al., 1995; Rothwarf et al., 1997). Recent progress in understanding these pathways has been achieved with studies utilizing the cyclic redox reagent dithiothreitol (DTT) (Rothwarf & Scheraga, 1991, 1993a−d; Li et al., 1995; Rothwarf et al., 1997). During oxidation of the fully-reduced protein, a large number of intermediates formed which could be divided into groups according to the number of disulfide bonds that they contained (e.g. one, two, three, and four disulfide bonds). Interconversion among species within a group was significantly faster than interconversion between groups, and regeneration was shown to proceed through parallel pathways that ultimately involved the formation of two three-disulfide-containing intermediates, des-[65−72] and des-[40−95], which contain the native-disulfide bonds 26−84 and 58−110 but are missing the disulfide bonds 65−72 and 40−95, respectively (Rothwarf & Scheraga, 1991a; Rothwarf et al., 1995, 1997).

Recombinant mutants of RNase A were produced to probe these three-disulfide-containing intermediates (Laity et al., 1993). NMR structural studies of [C65S,C72S] RNase A and [C40A,C95A] RNase A, which were produced to model des-[65−72] and des-[40−95], respectively, demonstrated that the predominant conformations of these mutants are similar to that of the wild-type protein with small perturbations in the local structure in the vicinity of the sites of the mutations (Shimotakahara et al., 1997; Laity et al., 1997). Mutants [C26S,C84S] RNase A and [C58S,C110S] RNase A were also prepared and were found to be thermally unstable and enzymatically inactive at room temperature, demonstrating that disulfide bonds 26−84 and 58−110 are important for stabilizing the native conformations of the folding intermediates des-[65−72] and des-[40−95].

* Author to whom correspondence should be addressed.

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[‡] N.I.H. Postdoctoral Fellow (1992−1994).

[§] Leukemia Society of America Special Fellow (1995−1997).

[||] NIH Predoctoral Trainee.

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[1] Abbreviations: RNase A, bovine pancreatic ribonuclease A; DTT^{ox}, oxidized dithiothreitol; DTT^{red}, reduced dithiothreitol; GSSG, oxidized glutathione; GSH, reduced glutathione; AEMTS, 2-aminoethylmethanethiosulfonate [$(NH_2)C_2H_5SSO_2CH_3$]; des-[40−95], des-[65−72], three-disulfide-containing intermediates of RNase A with native-disulfide bonds 26−84 and 58−110 and missing disulfide bonds 40−95 or 65−72, respectively; [C65S,C72S] RNase A, three-disulfide mutant of RNase A with replacement of cysteines 65 and 72 by serine; [C26S,-C84S] RNase A, three-disulfide mutant of RNase A with replacement of cysteines 26 and 84 by serine; [C58S,C110S] RNase A, three-disulfide mutant of RNase A with replacement of cysteines 58 and 110 by serine; [C40S,C95S] RNase A, [C40A,C95A] RNase A, three-disulfide mutant of RNase A with replacement of cysteines 40 and 95 by serine or alanine, respectively; [C40S,C65S,C72S,C95S] RNase A, [C40A,C65A,C72A,C95A] RNase A, two-disulfide mutant of RNase A with replacement of cysteines 40, 65, 72 and 95 by serine or alanine, respectively; DDS, disulfide detection system; MALDI-TOF, matrix-assisted laser desorption ionization−time of flight; EDTA, ethylenediaminetetraacetic acid; GdnSCN, guanidine thiocyanate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; TFA, trifluoroacetic acid; NTB, 2-nitro-5-thiobenzoic acid; cCMP, cytidine 2′,3′-cyclic phosphate.

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Another study identified intermediates belonging to the one-disulfide-containing group (Xu et al., 1996). In that study, one-disulfide-containing intermediates were isolated and characterized by peptide mapping. It was shown that the populations of all four species containing the native-disulfide pairings 26−84, 40−95, 58−110, or 65−72, respectively, were greater than those expected if disulfide pairing was completely random, indicating that certain local interactions promote the formation of these native-disulfide bonds. Of the total one-disulfide-containing species, 40% were found to contain the native-disulfide bond 65−72.

In contrast to species populating the one- and three-disulfide-containing groups of intermediates, little is known about the distribution of disulfide bonds in the two-disulfide group. It is not known if the preference for the formation of disulfide bond 65−72 persists throughout the folding process. While it has been proposed that intermediates containing 65−72 may be important for the formation of des-[40−95] (Xu et al., 1996), other intermediates missing the 65−72 disulfide bond must also exist which can lead to the formation of des-[65−72]. Since the oxidative-folding of RNase A proceeds through multiple pathways involving a large number of intermediates, no members of the 210 possible species in the two-disulfide-containing ensemble have yet been isolated and characterized. Because disulfide bonds 26−84 and 58−110 are important for stabilizing des-[65−72] and des-[40−95], an important two-disulfide intermediate that would populate both of the major folding pathways would also contain these disulfide bonds.

This two-disulfide intermediate, containing native-disulfide bonds 26−84 and 58−110, is believed to form during the reductive unfolding of the wild-type protein (Li et al., 1995). It was shown that reduction of wild-type RNase A with DTT^{red} also proceeds along two parallel pathways involving reduction of disulfide bonds 65−72 or 40−95 to form the three-disulfide-containing intermediates des-[40−95] and des-[65−72], respectively (Li et al., 1995). Formation of these three-disulfide-containing intermediates constitutes the rate-limiting step for unfolding, and it was suggested that local unfolding events were involved in the reduction process instead of a global unfolding of the entire protein. The reduction of des-[65−72] with DTT^{red} at 15 °C was believed to proceed through the reduction of disulfide bond 40−95. According to this mechanism, after reduction of these two disulfide bonds, a two-disulfide-containing species, containing the 26−84 and 58−110 disulfides, must be formed; this species was then reduced further to produce the fully-reduced protein since no other intermediates were observed.

The purpose of the present study is to investigate two-disulfide-containing species of RNase A. Since no two-disulfide-containing intermediates have been isolated from the 210 possible species that can form during the oxidative-folding process, recombinant methods have been applied to investigate possible intermediates. Disulfide bonds 65−72 and 40−95 were removed by replacing the specific cysteine residues by serine or alanine, resulting in species with disulfide pairings between cysteine residues 26, 58, 84 and 110. This particular mutant was prepared for the following reasons: (1) Since des-[65−72] and des-[40−95] have stable conformations which are similar to that of wild-type RNase A, studies of this mutant will elucidate the effects of deleting both disulfide bonds 65−72 and 40−95 on the stability and structure of the wild-type protein. (2) Since a two-disulfide intermediate containing disulfide bonds 26−84 and 58−110 has been suggested to be a necessary intermediate along the unfolding pathway of native RNase A and is likely to populate the two-disulfide group of intermediates formed during the oxidation process, studies of this mutant will provide insight into the folding/unfolding processes. (3) Since the regeneration of this mutant will result in the formation of only three possible two-disulfide species, the distribution of these species can be used to probe local interactions in the unfolded protein that promote the formation of specific disulfide bonds. Comparison of the experimental results with predictions based on loop-entropy considerations will reveal the effects of enthalpic contributions in stabilizing certain species.

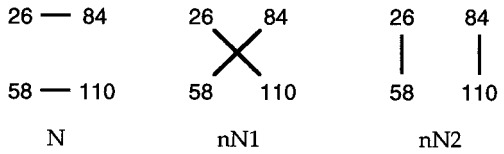

FIGURE 1: Three ways to pair the four half-cystine residues, 26, 58, 84 and 110, of mutant RNase A. The solid line represents a disulfide bond between the labeled residues. N is the native pairing, and nN1 and nN2 are non-native pairings.

In this study, cysteines 40, 65, 72, and 95 were replaced by serine or alanine to form [C40S,C65S,C72S,C95S] RNase A and [C40A,C65A,C72A,C95A] RNase A, respectively. Each mutant was expressed and purified in an unfolded form in which the cysteine residues were sulfonated or blocked with AEMTS. Upon regeneration with DTT^{ox}, all three of the possible two-disulfide species shown in Figure 1 form. The populations of these species are compared with the populations expected for a statistically coiled peptide based only on loop entropy factors; the populations observed for the species containing the native-disulfide pairings are greater than predicted by loop entropy considerations, indicating that enthalpic interactions must also contribute to the folding process. The presence of more than a statistical population of native-disulfide pairings indicates that specific local interactions present in the reduced protein direct the preferential formation of native-disulfide bonds in the two-disulfide mutant. Evidence for the presence of local structure is also demonstrated by one-dimensional NMR spectra of the two-disulfide species. It is shown that the conformations of all three two-disulfide species are similar and are predominantly disordered; however, evidence exists for the presence of local structure in the vicinity of one of the four histidine residues.

MATERIALS AND METHODS

Preparation of mutant ribonuclease A is described elsewhere (Laity et al., 1993). Site-directed mutagenesis was carried out with the T7-Gen *In Vitro* Mutagenesis Kit (United States Biochemical). Replacement of Cys 65 and Cys 72 with serine was carried out using the vector M13sjc4095s.21 (Laity et al., 1993), which contained the Cys 40→Ser and Cys 95→Ser mutation, and replacement of Cys 65 and Cys 72 with Ala was carried out using the vector M13sjc4095a.1b, which contained the Cys 40→Ala and Cys 95→Ala mutation. Both vectors were then subcloned into the pSJIIGEM-2 vector and expressed as fusion proteins with Gene 10 in *Escherichia coli* strain HMS174(DE3)-pLys(S). The isolated fusion protein present in inclusion bodies was dissolved in

7 M guanidine·HCl, 25 mM tris, 5 mM EDTA at pH 8.0, and the cysteine residues were sulfonated following the procedure of Thannhauser and Scheraga (1985). Sulfonated mutant RNase A was then cleaved from Gene 10 with Factor Xa (Boehringer-Mannheim).

Purification. Cation-exchange chromatography on a Mono-S HR5/5 column (Pharmacia) with 50 mM formic acid, 3 M urea at pH 4.0, and a NaCl gradient was used for the first stage of purification of the sulfonated mutants. The collected fractions were exchanged into 0.1 M acetic acid using a Pharmacia HR 16/50 column packed with G25 superfine resin and lyophilized. Further purification was carried out by reducing the sulfonated protein with 50 mM DTT^{red} and 4 M GdnSCN at pH = 7.5, blocking the free thiols with AEMTS and by fractionating the mixture using cation-exchange chromatography with a Rainin Hydropore SCX 21 × 10 cm column in 25 mM HEPES and 1 mM EDTA at pH 7.0; the resulting protein was exchanged into 0.1 M acetic acid with the Pharmacia HR 16/50 column described above.

Folding. Because oxidized and reduced glutathiones (GSSG and GSH) often promote the formation of stable intermolecular disulfide bonds between a protein and a glutathione molecule, oxidized and reduced DTT (DTT^{ox} and DTT^{red}) were used to promote disulfide-bond formation for these mutants. Sulfonated [C40S,C65S,C72S,C95S] RNase A and [C40A,C65A,C72A,C95A] RNase A were reduced by allowing a solution containing about 75 μM protein, 40 mM DTT^{red}, 4 M GdnSCN, 0.1 M Tris, and 3 mM EDTA to incubate at 25 °C and pH 8.0 for 1 to 2 h. The solution was exchanged into 0.1 M Tris, 3 mM EDTA, pH 8.1 (at 4 °C) with the Pharmacia HR 16/50 column described above to a final protein concentration of 5−15 μM. DTT^{ox} was added to the solution to a concentration of 120−200 mM, and disulfide-bond formation was allowed to proceed at 4 °C under a blanket of Argon for 24−72 h. Analysis of the mixture of disulfide-bonded species was facilitated by blocking any free sulfhydryl groups with AEMTS. The blocking reaction was initiated by the addition of aliquots of a solution containing 0.2 M AEMTS, 0.1 M Tris, 3 mM EDTA at pH 7.5 (at 25 °C) to the folding mixture to a final concentration of 0.01 M AEMTS. After the reaction had proceeded for 5 min, the pH of the solution was lowered to between 4.8 and 5.2 with 1 M HCl.

Fractionation of Disulfide-Containing Species. The mixture was desalted, and the disulfide-containing species were fractionated by loading the sample directly onto a Rainin Hydropore SCX 21 × 10 cm cation-exchange column in 50 mM acetate buffer at pH 5.0. After desalting with acetate buffer, separation was achieved at pH 7.0 in 25 mM HEPES with 1 mM EDTA and a gradient of 5−15% NaCl in 45 min. The disulfide content of the species in the mixture was determined using a sensitive HPLC assay for disulfide detection developed in this laboratory and described elsewhere (Thannhauser et al., 1985). With this disulfide-detection system (DDS), the area of the peak produced by detecting the concentration of NTB produced and monitored at 410 nm is directly proportional to the number of disulfide bonds present. Fractions were desalted, either by reversed-phase HPLC on a Waters C_{18} column in 0.1% (v/v) TFA with a linear gradient of 0.1% (v/v) TFA in acetonitrile or by dialysis against 0.1 M acetic acid at 4 °C, and lyophilized.

Peptide Mapping. Identification of the disulfide bonds present in the fractionated species was achieved by digesting the proteins with trypsin and chymotrypsin and using the disulfide-detection system (DDS) to identify disulfide-containing peptide fragments (Xu et al., 1996). The protein was incubated for 1 h at room temperature at a concentration of 1 mg/mL with an enzyme:substrate ratio of 1:100 for trypsin and of 1:25 for chymotrypsin. Digestion was quenched by dropping the sample pH to 2.0 with 5% TFA, and samples were loaded onto a reversed-phase YMC C_{18} minibore column (ODS AQ 120A S3 2 × 150 mm) for fractionation. Fractions were separated with an increasing gradient from 6% to 65% of 0.095% TFA in 50% acetonitrile in 100 min. Disulfide-containing peptides were identified by directing the eluant from the reversed-phase column and detector to the DDS. These disulfide-containing fractions were also collected and lyophilized, and their masses were identified with a MALDI-TOF mass spectrometer from Finniganmat (Lasermat model 2000), supplemented by amino acid analysis.

Enzymatic Activity. The enzymatic activities of the isolated two-disulfide-containing fractions of [C40S,C65S, C72S,C95S] RNase A and [C40A,C65A,C72A,C95A] RNase A were evaluated at 25 °C and pH 7.0 in the presence of cytidine 2′,3′-cyclic phosphate (cCMP) according to the method of Crook et al. (1960) with modifications described by Rothwarf and Scheraga (1993a). Protein concentrations were estimated using an extinction coefficient of 8600 M^{-1} cm^{-1} at 275 nm, which is the value determined for reduced wild-type RNase A (Rothwarf & Scheraga, 1993a).

NMR Spectroscopy. One-dimensional ^1H NMR spectra were recorded on a Varian Unity 500 spectrometer. Fractions isolated by ion-exchange chromatography were buffer exchanged into 0.1 M acetic acid with a Pharmacia PD10 column and lyophilized. Wild-type RNase A was dissolved in D_2O (99.96% isotopic purity, Cambridge Isotopes Inc.) containing 0.1 M acetic acid, heated to 65 °C for 15 min to expose buried amide protons allowing for exchange with solvent deuterons, and then lyophilized. For NMR spectroscopy, all lyophilized samples were dissolved in D_2O (99.99% isotopic purity, Cambridge Isotopes Inc.) containing 0.050 M phosphate buffer adjusted to pH 6.2 ± 0.1 (uncorrected for isotope effects), and data were collected at 10 °C. Phosphate buffer was used because it was shown that the native conformation of the wild-type protein is stabilized in the presence of phosphate (Hermans & Scheraga, 1961). Presaturation at the HOD resonance frequency was applied for 1.0 s before acquisition of the spectrum. Spectra were referenced by assigning a value of 4.92 ppm for the chemical shift of the HOD resonance at 10 °C (Lutz et al., 1993). One-dimensional TOCSY spectra of fraction 2S2 and the wild-type protein at 10 °C were also collected (data not shown). For this experiment, the peak at 7.4 ppm was selectively irradiated with a 28 ms. Gaussian pulse. A 7.4 kHz spin lock field was then applied for a duration of 100 ms.

RESULTS

Regeneration of Mutant Protein

A typical ion-exchange chromatogram for the fractionation of species produced in 41 h during the regeneration of

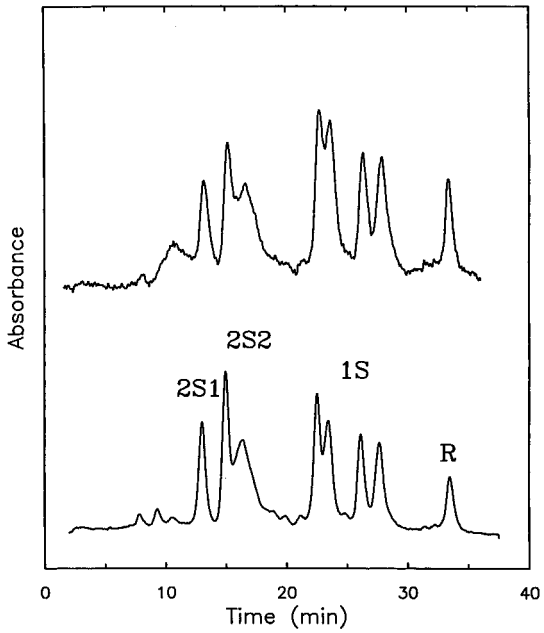

FIGURE 2: Ion-exchange (bottom) and DDS (top) chromatograms of the regeneration mixture of [C40S,C65S,C72S,C95S] RNase A. Regeneration was initiated with 200 mM DTT^{ox} at 4 °C, pH 8.1. The reaction was quenched, and the free thiols were blocked, with AEMTS after 41 h. R and 1S refer to the fully-reduced and one-disulfide-containing fractions, respectively. 2S1 and 2S2 refer to the two fractions of two-disulfide-containing species.

[C40S,C65S,C72S,C95S] RNase A with DTT^{ox} and then blocked with AEMTS is shown in Figure 2. The distribution of disulfide-containing species is assumed to be at a steady-state after this time period because no change in peak area was observed after 36 h. An AEMTS molecule will react with any free sulfhydryl groups and form a disulfide bond between a cysteamine group ($^{+}NH_3CH_2CH_2S$) and the protein thiol. Therefore, blocking with AEMTS contributes an additional net charge of +4 to the fully-reduced mutant and of +2 to one-disulfide-containing species. As labeled in Figure 2, two-disulfide-containing fractions elute from the column first, followed by one-disulfide-containing groups and finally by the fully-reduced and blocked mutant.

The chromatogram from the DDS is also shown in Figure 2. The areas of the DDS peaks are directly proportional to the number of disulfide bonds present; therefore, by comparing the ratio of peak areas from the DDS and from the cation-exchange chromatogram, the relative number of disulfide bonds can be determined. A normalization constant was calculated from the ratio of the peak areas for the fully-reduced and blocked protein which contained four disulfide bonds. The ratios of peak areas from the DDS relative to that from the cation-exchange chromatogram were 3 for the one-disulfide-containing species (labeled 1S in Figures 2 and 3) and 2 for the two-disulfide-containing species (labeled 2S1 and 2S2 in Figures 2 and 3), the extra disulfide bonds in R and 1S coming from the reaction with the blocking agent.

The three small peaks appearing between 8 and 12 min in the ion-exchange chromatogram of Figure 2 represent a portion of the two-disulfide-containing fractions that have undergone a modification which reduces the net charge of the protein. It is well-known that unfolded forms of RNase A readily undergo deamidation of Asn 67 at alkaline pH (Thannhauser & Scheraga, 1985). Because the reduction and

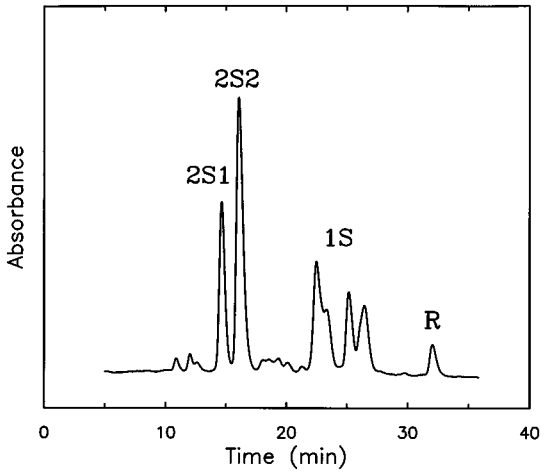

FIGURE 3: Ion-exchange chromatogram of the regeneration mixture of [C40A,C65A,C72A,C95A] RNase A. Regeneration was initiated with 200 mM DTT^{ox} at 4 °C, pH 8.1. The reaction was quenched, and the free thiols were blocked, with AEMTS after 41 h. R and 1S refer to the fully-reduced and one-disulfide-containing fractions, respectively. 2S1 and 2S2 refer to the two fractions of two-disulfide-containing species.

regeneration reactions were carried out at pH 8.0 and 8.1, respectively, these small fractions probably result from deamidation at this site.

Deamidation is a sequence-dependent process that results in the replacement of asparagine with an aspartic acid residue with either an α- or β-linkage to the preceding amino acid (Meinwald et al., 1986). It has been shown that the rate of modification depends strongly on the type of amino acid following asparagine, with the fastest rate occurring for glycine followed by histidine, serine, and alanine (Tyler-Cross & Schirch, 1991; Wright, 1991). Under identical conditions, asparagine undergoes modification at the sequences Asn-His, Asn-Ser, and Asn-Ala at rates that are approximately 4, 6, and 11 times slower, respectively, than the rate for Asn-Gly (Tyler-Cross & Schirch, 1991). Wild-type RNase A contains 10 asparagine residues; however, only Asn 67 is followed by a Gly residue and none of the remaining asparagine residues are followed by histidine, serine or alanine. For the mutants considered here, however, deamidation at sites in addition to Asn 67 is possible because [C40S,C65S,C72S,C95S] RNase A contains two Asn-Ser sequences at Asn 71 and Asn 94 and [C40A,C65A,C72A, C95A] RNase A contains Asn-Ala at these two additional sites. To ensure that the starting material for the regeneration of both mutants contained as little deamidation as possible, the reduced forms of the mutant proteins were blocked with AEMTS and then purified by ion-exchange chromatography before initiating the final reduction and regeneration procedures outlined above.

To minimize the effect of deamidation, [C40A,C65A, C72A,C95A] RNase A was prepared. Treatment of this mutant with DTT^{ox} produced similar mixtures of disulfide-containing species. Fractionation of the regeneration mixture of this mutant using cation-exchange chromatography is displayed in Figure 3. Disulfide-bond formation was allowed to proceed for approximately 41 h at 4 °C after which all free sulfhydryl groups of the mixture were blocked with AEMTS. The cation-exchange chromatogram is similar to that for [C40S,C65S,C72S,C95S] RNase A displayed in Figure 2; however, the second two-disulfide-containing peak

Table 1: Percentage of Total Protein for Disulfide-Containing Fractions Formed under Steady-State Conditions During the Regeneration of Mutant RNase A and Isolated by Cation-Exchange Chromatography

mutant	2S1 (%)	2S2 (%)	1S (%)	R (%)
[C40S,C65S,C72S,C95S] RNase A	11 ± 1[a]	35 ± 4	48 ± 2	6 ± 1
[C40A,C65A,C72A,C95A] RNase A	18 ± 4	34 ± 2	44 ± 6	4 ± 1

[a] Errors are calculated at the 95% confidence limit.

labeled 2S2 is not split into two peaks as observed in Figure 2. Three peaks are observed for the two-disulfide-containing fractions in Figure 2 probably because a slightly lower flow rate was used to record this chromatogram than that used for the chromatogram of Figure 3. This lower flow rate was not used for the Ala mutant to minimize the effects of diffusion on the ion-exchange column. The percentage of total protein for the fractions grouped according to the number of intramolecular disulfide bonds is approximately the same for the two mutants as displayed in Table 1; however, the population of fraction 2S1 is slightly higher for [C40A,C65A,C72A,C95A] RNase A than for [C40S, C65S,C72S,C95S] RNase A. For both mutants, about 50% of the total protein contains two intramolecular disulfide bonds in the steady-state.

The small peaks that elute before the major two-disulfide-containing peaks are also present in approximately the same ratios for this mutant; therefore, these peaks must result from deamidation of Asn 67 rather than from modification of Asn 71 or Asn 94. Based on deamidation rates for peptides, modification of Asn-Ser should occur at a rate that is about two times faster than that for the Asn-Ala sequence. If these peaks resulted from modification of Asn residues preceding the genetically engineered sites, we would expect the peak area for these modified peaks to be greater for [C40S,C65S, C72S,C95S] RNase A than for [C40A,C65A,C72A,C95A] RNase A.

Peptide mapping was used to identify the disulfide bonds present in the two-disulfide-containing fractions, 2S1 and 2S2 for both mutants. The three ways to pair the four remaining half-cystine residues in the mutant proteins are displayed in Figure 1, and all three were identified in different ratios that are reported in Table 2. For both mutants, fraction 2S1 contained the following disulfide pairings: 26−110 and 58−84, which are the non-native-disulfide pairings referred to as nN1 in Figure 1. Fraction 2S2 consisted of the remaining two-disulfide species that contained both the native-disulfide pairings, 26−84 and 58−110, and the non-native pairings 26−58 and 84−110, which are labeled in Figure 1 as N and nN2, respectively. The tryptic/chymotryptic peptide maps for the fractions 2S2 for both mutants are displayed in Figure 4. Disulfide-containing peptides were identified with the DDS and were assigned to specific protein fragments based on their mass. Each disulfide-containing peptide in Figure 4 is labeled, and the corresponding two protein fragments that are joined by the disulfide bond are listed in the Figure legend. Because the absorbance measured with the DDS is directly proportional to disulfide-bond concentration, the relative DDS peak areas were used to assess the relative concentrations of the two-disulfide species contained within fraction 2S2 shown in Figures 2 and 3. The relative percentages of N and nN2 contained in this fraction are 58% and 42%, respectively, for [C40S,C65S,C72S,C95S] RNase A, and 36% and 64%, respectively, for [C40A,C65A, C72A,C95A] RNase A.

Table 2 displays the relative populations of the three possible two-disulfide species. For [C40S,C65S,C72S,C95S] RNase A, the species containing the native-disulfide pairings, 26−84 and 58−110 is the most highly populated followed by the species containing the non-native pairings. For [C40A,C65A,C72A,C95A] RNase A, on the other hand, the most highly populated species contains the non-native pairings 26−58 and 84−110, and the species containing the native-disulfide pairings is the least populated. These differences in populations for the two mutants must result from interactions in the vicinity of the sites of mutation, i.e. interactions that involve the Ser or Ala residues which replaced the four Cys residues of the wild-type protein. These interactions must influence disulfide-bond formation resulting in the different distributions of two-disulfide-containing species for the two mutants.

Loop Entropy Calculations

If the mutant proteins behaved as statistical coils, disulfide-bond formation would proceed in a random fashion, and the populations of the three possible two-disulfide-containing species would depend on the number of residues separating the paired half-cystine residues. For statistically coiled polypeptide chains, the reduction in loop entropy introduced by the formation of an intramolecular disulfide bond can be predicted using the model of overlapping and dependent loops (Poland & Scheraga, 1965; Lin et al., 1984). According to this model, the reduction in loop entropy produced by the formation of two overlapping disulfide bonds for a statistical coil is given by

$$\Delta S = -R[6.94 + 1.5 \ln(N_1 N_2 - N_o^2)] \qquad (1)$$

where R is the gas constant, N_1 and N_2 are the number of residues between the paired half-cystine residues for disulfide bonds 1 and 2, respectively, of a given two-disulfide species, and N_o is the number of residues that are contained within

Table 2: Experimentally Determined Relative Steady-State Populations of the Three Two-Disulfide Combinations[a] Formed During the Regeneration of Mutant RNase A, and the Reduction of Loop Entropy and Relative Populations Calculated for Random Pairings of Half-Cystine Residues of Mutant RNase A

property	26−84;58−110 (N)	26−110;58−84 (nN1)	26−58;84−110 (nN2)
measured relative populations for [C40S,C65S,C72S,C95S] RNase A (%)	44 ± 6[b]	24 ± 5	32 ± 6
measured relative populations for [C40A,C65A,C72A,C95A] RNase A (%)	24 ± 7	34 ± 3	42 ± 8
calculated relative populations[c]	13	25	62
ΔS (cal/mol K)	−36.9	−35.6	−33.8

[a] Fraction 2S1 contains the 26−110 and 58−84 disulfide pairings; fraction 2S2 contains both the native 26−84 and 58−110 disulfide pairings and the non-native 26−58 and 84−110 disulfide pairings. [b] Errors are calculated at the 95% confidence limit. [c] From eqs 1 and 2.

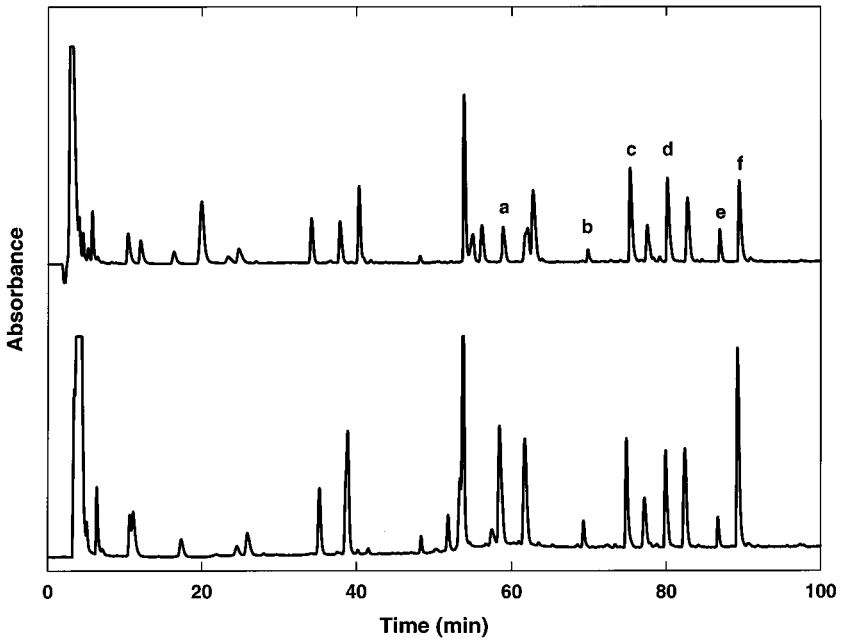

FIGURE 4: Reverse-phase HPLC chromatogram of the tryptic−chymotryptic digestion of the 2S2 peaks of [C40A,C65A,C72A,C95A] RNase A (upper trace) and [C40S,C65S,C72S,C95S] RNase A (lower trace). The labeled fractions represent disulfide-containing peptides which were found to consist of the following fragments: a, (26−31)−(80−85); b, (26−31)−(77−85); c, (26−31)−(47−61); d, (80−85)−(105−115); e, (77−85)−(105−115); f, (47−61)−(105−115).

both disulfide loops. The equilibrium concentrations for the two-disulfide species are

$$\frac{[2S]}{[Red]} = \exp\left(\frac{\Delta S}{R}\right) \quad (2)$$

where [2S] is the equilibrium concentration of a given two-disulfide species ([N], [nN1], or [nN2]), and [Red] is the equilibrium concentration of the fully-reduced protein. Using these equations, the percentage of a specific disulfide species expected if disulfide-bond formation were completely random was calculated from the ratio of [2S] to the total concentration of two-disulfide species ([N] + [nN1] + [nN2]). The calculated reduction in loop entropy and the relative populations predicted for the three two-disulfide-containing proteins are listed in Table 2.

The results presented in Table 2 demonstrate that, if the two-disulfide pairings were completely random, the most highly populated species would contain the disulfide pairings 26−58 and 84−110, which consists of the combination with the shortest disulfide loops, and the least populated species would contain the native-disulfide pairings, 26−84 and 58−110. However, the difference in entropy loss for the three species is only 3.1 cal/mol K which is only 0.86 kcal/mol at 4 °C. The theoretical relative populations listed in Table 2 are different from those determined experimentally for [C40S,C65S,C72S,C95S] RNase A but are in the same order as those for [C40A,C65A,C72A,C95A] RNase A. For both mutants, the species containing the native-disulfide pairings is more highly populated than predicted for a statistical coil. For the species containing non-native pairings, nN2 is more highly populated than nN1 for both mutants, which is in the same order as the predictions from the loop entropy calculations.

Enzymatic Activity

The isolated two-disulfide fractions, 2S1 and 2S2 displayed no measurable enzymatic activity against cCMP at 25 °C and pH 7.0. This result is not surprising because the activities measured for the three-disulfide mutants, [C65S, C72S] RNase A and [C40S,C95S] RNase A are 22% and 5%, respectively, of that for the wild-type protein (Laity et al., 1993), and the activities for the three-disulfide alanine mutants are the same (J. H. Laity and H. A. Scheraga, unpublished data). Therefore, the two-disulfide fraction 2S2, which contains species N and nN2 in the ratios of 58% to 42%, would display only 58% of 5% (or 2.9%) the activity of the wild-type protein if it is assumed that only N is enzymatically active and has the same enzymatic activity as the three-disulfide mutant [C40S,C95S] RNase A. More likely, N lacks the native structure (see below), and hence does *not* have the same enzymatic activity as the three-disulfide mutant. However, very low levels of enzymatic activity against cCMP, about 0.04% of that for the native RNase A, were detected for the fully-reduced wild-type protein (Garel 1978). Therefore, substitution of cysteine residues with Ser or Ala in addition to conformational effects may give rise to the observed absence of enzymatic activity.

NMR Spectroscopy

The low-field region, 6.0−8.8 ppm, of the one-dimensional ^{1}H NMR spectra of the fully-reduced and AEMTS-blocked mutant, wild-type RNase A, and the fractions 2S1 and 2S2 of [C40A,C65A,C72A,C95A] RNase A is displayed in Figure 5. This region contains peaks from aromatic and histidine residues. Because the aromatic residues are present in regions that extend over the entire polypeptide chain and their chemical shift values reflect differences in conformation and solvent accessibility, comparison of this region provides a useful probe of the relative conformations of the proteins (Engler et al., 1990). The spectra of the two-disulfide-containing fractions (spectra C and D) are similar to that of the fully-reduced and blocked mutant (spectrum A) and display significantly less chemical-shift dispersion compared to the spectrum of the wild-type protein (spectrum B). This reduced chemical-shift dispersion is characteristic of an

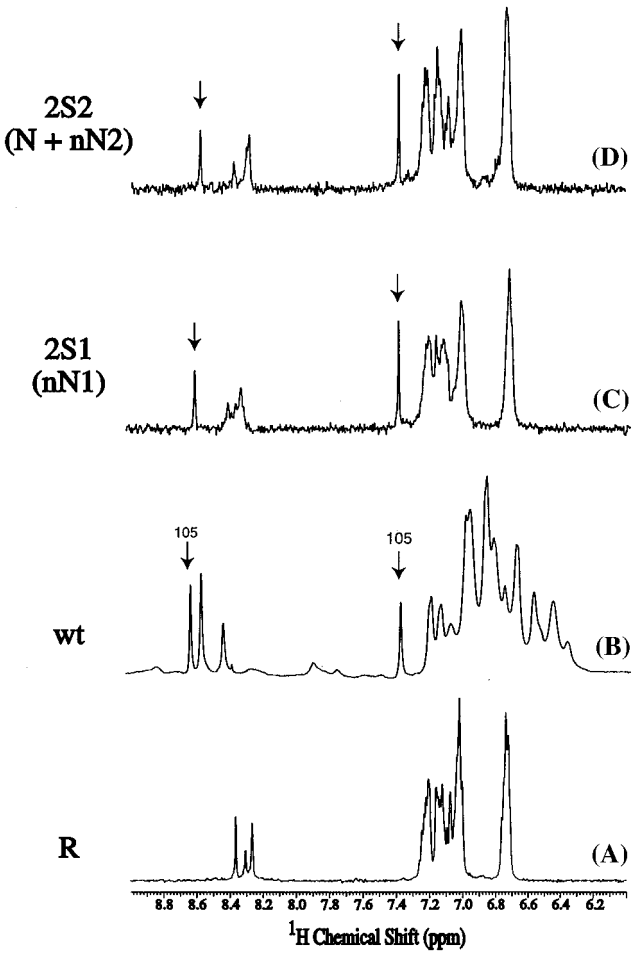

FIGURE 5: One-dimensional 500 MHZ ^{1}H NMR spectra of wild-type RNase A and [C40A,C65A,C72A,C95A] RNase A in D_2O containing 50 mM phosphate buffer at pH 6.2 and 10 °C. Labeled spectra refer to (A) the fully-reduced and AEMTS-blocked mutant, (B) wild-type RNase A, (C) fraction 2S1 consisting of two-disulfide species nN1, containing the non-native-disulfide bonds, 26−110 and 58−84, (D) fraction 2S2 containing two-disulfide species N and nN2, which contain the native (26−84 and 58−110) and non-native (26−58 and 84−110) disulfide bonds, respectively. The labeled arrows in spectrum B refer to the $C^{\epsilon 1}H$ (8.6 ppm) and the $C^{\delta 2}H$ (7.4 ppm) resonances of His 105 in the wild-type protein. The unlabeled arrows refer to peaks discussed in the text.

unfolded protein in which the proton resonances cluster around the values for a statistically-coiled peptide demonstrating that the conformations of all forms of the mutant protein are considerably disordered, as suggested by Konishi and Scheraga (1980b). Also, the spectra of the two-disulfide-containing fractions (spectra C and D) are very similar, demonstrating that the conformations of all three two-disulfide-containing species must be similar.

Two peaks in the spectra of the two-disulfide-containing proteins at 7.4 and 8.6 ppm (labeled with arrows in spectra C and D) are shifted downfield relative to the same resonances in the spectrum of the reduced and blocked mutant (spectrum A). These two resonances were shown to belong to the same histidine residue because selective inversion of the peak at 7.4 ppm resulted in a transfer of magnetization under a TOCSY spin lock to the peak at 8.6 ppm (Bax & Davis, 1985). The peak at 8.6 ppm appears at a chemical shift characteristic of $C^{\epsilon 1}H$ resonances of His in both unfolded and native forms of RNase A (Konishi & Scheraga, 1980b; Tanokura, 1983; Swadesh et al., 1984; Shimotakahara et al., 1997); therefore, the peak at 7.4 ppm must be the $C^{\delta 2}H$ resonance of the same residue. These two peaks appear at the same chemical shifts as the $C^{\epsilon 1}H$ and $C^{\delta 2}H$ resonances of His 105 in wild-type RNase A at pH 6.2 and therefore may correspond to the same residue in the spectra of the two-disulfide-containing fractions (Cohen & Schindo, 1975; Tanokura, 1983). The dispersion of chemical shifts of this set of His resonances in the spectra of the two-disulfide-containing proteins suggests that at least one His residue experiences a locally-ordered environment.

The low-field region, 6.0−8.8 ppm, of the one-dimensional ^{1}H NMR spectra of the fully-reduced and AEMTS-blocked forms of [C40S,C65S,C72S,C95S] RNase A are similar to the spectra displayed in Figure 5. Therefore, these data are not shown.

DISCUSSION

Interactions Promoting Disulfide-Bond Formation

The present study demonstrates that local interactions must direct the formation of disulfide bonds during the regeneration of [C40S,C65S,C72S,C95S] RNase A and [C40A, C65A,C72A,C95A] RNase A. Disulfide bonds contribute to the stability of a protein by reducing the conformational entropy of the unfolded state, therefore, by destabilizing this state relative to the native state. The reduction in the conformational entropy of the unfolded states of the three possible two-disulfide combinations displayed in Figure 1 was calculated using equation (1) and is listed in Table 2. Assuming that the three two-disulfide species are disordered and differ in free energy only by differences in chain entropy introduced by the formation of both disulfide bonds, the relative equilibrium populations of the three two-disulfide species were calculated and are also displayed in Table 2. These values represent the equilibrium populations expected if the polypeptide chain exists as a statistical coil, and disulfide pairing is random. For both mutant proteins, the populations of the species containing the native-disulfide pairings, 26−84 and 58−110, are greater than those predicted based on loop entropy considerations alone. Therefore, additional enthalpic interactions must be present to promote the formation of the native-disulfide bonds.

Nonrandom disulfide-bond formation was also observed for the formation of one-disulfide-containing intermediates during the oxidation of fully-reduced wild-type RNase A (Xu et al., 1996). In that study, one-disulfide-containing species were isolated and the disulfide pairing was identified. It was shown that the populations of species containing native-disulfide pairings were greater than those predicted for random disulfide-bond formation, and it was suggested that enthalpic contributions lead to the increased stability of these species. The native-disulfide bond, 65−72, was the most highly favored one, and approximately 40% of all one-disulfide-containing species contained this disulfide bond. It was suggested that local interactions direct disulfide-bond formation, resulting in a preference for these native-disulfide bonds. These local interactions, which form regions of local structure termed chain-folding initiation sites (CFIS's), are believed to direct protein-folding events by limiting the conformational space available to the polypeptide chain and have been identified in different regions of the wild-type protein (Wetlaufer, 1973; Matheson & Scheraga, 1978; Némethy & Scheraga, 1979; Baldwin, 1989; Montelione &

Scheraga, 1989; Freund et al., 1996). Preference for formation of the native-disulfide pairings observed here may result from the presence of local and medium-range interactions in the vicinity of Cys 26, 58, 84, and 110.

Evidence of Local Structure Involving a His Residue

The presence of local structure is also evident in the one-dimensional ^{1}H NMR spectra of these two-disulfide-containing mutants under conditions of solvent, pH, and temperature at which the wild-type disulfide-intact protein is fully folded. Comparison of the spectra of the oxidized forms of these mutants in Figure 5 (spectra C and D) with that of the fully-reduced and AEMTS-blocked mutant (spectrum A) demonstrates that the three-dimensional conformations of the two-disulfide-containing mutants are similar to that of the fully-reduced protein and, therefore, are predominantly disordered. However, dispersion of the $C^{\epsilon 1}H$ and $C^{\delta 2}H$ resonances of a single His residue in all two-disulfide-containing mutants suggests that this residue experiences a different local environment in the oxidized forms of the mutant relative to the fully-reduced and blocked form. Therefore, it is possible that disulfide-bond formation and the induction of some local structure in the vicinity of this residue may occur concurrently. As shown in Figure 5, the chemical shift values of these resonances are identical to the values of the $C^{\epsilon 1}H$ and $C^{\delta 2}H$ resonances of His 105 in wild-type RNase A (spectrum B). Therefore, the local structure in the two-disulfide mutants (similar to that in the wild-type protein) may result from interactions in the vicinity of His 105.

There is the possibility, however, that these differences are not due to conformational differences between the proteins. Since chemical shift values of the imidazole ring protons of His are sensitive to the electrostatic environment in the vicinity of the ring, the chemical shift values of the $C^{\epsilon 1}H$ and $C^{\delta 2}H$ resonances may simply reflect the state of a nearby Cys residue with which the His residue interacts; i.e., the His residue could interact with an uncharged half-cystine residue in the oxidized forms of the mutant and a positively-charged AEMTS-blocked residue in the fully-reduced mutant. Because none of the four His residues of the protein is preceded or followed by a Cys residue, medium-range interactions would have to be responsible for such spectra.

Regions of local structure that have been identified in unfolded forms of RNase A involve the N-terminal portion which includes His 12 (Bierzynski & Baldwin, 1982; Swadesh et al., 1984) or the C-terminal portion encompassing His 105 or His 119 (Chavez & Scheraga, 1977, 1980). By monitoring the chemical shifts of the His $C^{\epsilon 1}H$ resonances, we identified the local structure in the vicinity of His 12 in reduced *S*-sulfonated RNase A (Swadesh et al., 1984) and in the disulfide-intact guanidine-denatured protein (Bierzynski & Baldwin, 1982). Predictions, based on hydrophobic interactions in the wild-type protein, have determined that the C-terminal region including residues 105−124 is the primary region of initial structure formation or CFIS (Matheson & Scheraga, 1978). These predictions were confirmed by air- and glutathione-oxidation studies of the wild-type protein in which it was shown that the first region of the protein to fold consisted of residues 80−124 (Chavez & Scheraga, 1977, 1980). Further evidence for the existence of partially ordered structures in this region of the protein was obtained by nonradiative energy transfer studies of a peptide that corresponds to residues 105−124 of the wild-type protein (Beals et al., 1991). It was shown that these ordered structures existed at temperatures ≥20 °C and were stabilized by hydrophobic interactions. While, comparison of the NMR spectra of the two-disulfide-containing mutants displayed in Figure 5 (spectra C and D) with that of the wild-type protein (spectrum B) suggests that the isolated $C^{\epsilon 1}H$ and $C^{\delta 2}H$ resonances in spectra C and D may be assigned to His 105, assignment of these resonances to any one of the remaining His residues including His 12, 48 or 119 is also possible.

Importance for Unfolding/Folding of the Wild-Type Protein

A two-disulfide-containing species of the wild-type protein is believed to form during reduction studies of RNase A (Li et al., 1995). This work suggested that reduction of des-[65−72] with DTT^{red} at 15 °C proceeds by reduction of the 40−95 disulfide bond producing a two-disulfide species containing pairings between cysteine residues 26, 58, 84, and 110. Because no other intermediates were observed, deletion of disulfide bond 40−95 from des-[65−72], therefore, must produce a two-disulfide intermediate that contains the native-disulfide pairings 26−84 and 58−110 (Li et al., 1995). This two-disulfide intermediate is unstable and is rapidly reduced further to form the fully-reduced protein, which suggests that this species contains little native structure. The data presented here confirm this finding, demonstrating that a two-disulfide-containing species with native-disulfide bonds 26−84 and 58−110 is predominantly disordered.

Disulfide-coupled oxidation studies of wild-type RNase A also demonstrated that two-disulfide-containing folding intermediates are predominantly disordered. It was shown that all pre-transition-state intermediates are primarily disordered and undergo rapid disulfide-bond rearrangement among species with the same number of disulfide bonds (Rothwarf & Scheraga, 1993a). Formation of all three of the possible two-disulfide pairings displayed in Figure 1 demonstrates that there is not a significant difference in the stability of each of the species formed in this study. Therefore, interconversion among these species would also occur in the two-disulfide ensemble of folding intermediates of the wild-type protein.

Substitution of Cys by Ser or Ala

It should be noted that the two-disulfide species containing the native pairings, N, is the most highly populated species for [C40S,C65S,C72S,C95S] RNase A, but is the least populated species for [C40A,C65A,C72A,C95A] RNase A; for the latter, the experimentally determined populations follow the trend based on loop entropy calculations. This difference may result from the presence of different interactions involving the residues that replaced the cysteine residues, i.e., interactions involving serine versus alanine. Serine was chosen to substitute for cysteine for folding at alkaline pH because the residues differ only in replacement of a sulfur atom with an oxygen, and "Ser residues are expected to mimic best the Cys residues in the refolding protein under normal folding conditions, where the Cys thiols are ionized approximately half the time" (van Mierlo et al., 1991). Alanine, on the other hand, has been used as a substitute for cysteine residues during folding at neutral pH

because the residue "will not resist native folding events that may tend to bury free cysteines" (Staley & Kim, 1992). Because alkaline folding conditions are used in this study, viz. pH 8.1, serine may be a more appropriate substitute for cysteine.

CONCLUSION

Oxidation of reduced [C40S,C65S,C72S,C95S] RNase A and [C40A,C65A,C72A,C95A] RNase A with DTT^{ox} results in the formation of all three of the possible two-disulfide-bonded species. Disulfide pairing, however, is not completely random, and it is shown that both entropic factors and enthalpic interactions direct the formation of the two-disulfide species. One-dimensional ^1H NMR spectra demonstrate that the conformations of the three two-disulfide-containing species are similar and are predominantly disordered; however, there is evidence of local structure in the vicinity of one histidine residue. This local structure may result from the inherent hydrophobicity of a chain-folding initiation site (CFIS), from interactions produced by disulfide-bond formation or from interactions of the His residue with a nearby Cys residue.

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