

## NMR Spectroscopy Reveals that RNase A is Chiefly Denatured in 40% Acetic Acid: Implications for Oligomer Formation by 3D Domain Swapping

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**Abstract:** Protein self-recognition is essential in many biochemical processes and its study is of fundamental interest to understand the molecular mechanism of amyloid formation. Ribonuclease A (RNase A) is a monomeric protein that may form several oligomers by 3D domain swapping of its N-terminal  $\alpha$ -helix, C-terminal  $\beta$ -strand, or both. RNase A oligomerization is induced by 40% acetic acid, which has been assumed to mildly unfold the protein by detaching the terminal segments and consequently facilitating intersubunit swapping, once the acetic acid is removed by lyophilization and the protein is redissolved in a benign buffer. Using UV difference, near UV circular dichroism, folding kinetics, and multidimensional heteronuclear NMR spectroscopy, the conformation of RNase A in 40% acetic acid and in 8 M urea has been characterized. These studies demonstrate that RNase A is chiefly unfolded in 40% acetic acid; it partially retains the native helices, whereas the  $\beta$ -sheet is fully denatured and all X-Pro peptide bonds are predominantly in the *trans* conformation. Refolding occurs via an intermediate,  $I_N$ , with non-native X-Pro peptide bonds.  $I_N$  is known to be populated during RNase A refolding following denaturation in concentrated solutions of urea or guanidinium chloride, and we find that urea- or GdmCl-denatured RNase A can oligomerize during refolding. By revealing the importance of a chiefly denatured state and a refolding intermediate with non-native X-Pro peptide bonds, these findings revise the model for RNase A oligomerization via 3D domain swapping and have general implications for amyloid formation.

### Introduction

Bovine pancreatic ribonuclease A (RNase A) is a small and extremely well-known enzyme.<sup>1</sup> Native RNase A in aqueous solution is a folded, monomeric protein with three  $\alpha$ -helices (helix I, residues 3–13; helix II, 24–34; and helix III, 50–60) and six  $\beta$ -strands (residues 43–49, 61–63, 72–74, 79–87, 96–111, and 116–124).<sup>2</sup> It has four prolines. The peptide bonds of prolines 93 and 114 adopt the *cis* conformation in the folded monomer, whereas prolines 42 and 117 are *trans*.<sup>2,3</sup> The structure is stabilized by four disulfide bonds linking cysteines 26 to 84, 40 to 95, 58 to 110, and 65 to 72.<sup>4</sup> RNase A possesses six Tyr, at positions 25, 73, 76, 92, 97 and 115; three Phe, at positions 8, 46, and 120; and no Trp.<sup>5</sup>

RNase A can form a series of 3D domain swapped oligomers by exchanging the N-terminal  $\alpha$ -helix or the C-terminal  $\beta$ -strand

or both.<sup>6–10</sup> These oligomers have additional biological and enzymatic activities that the monomeric protein lacks.<sup>11–13</sup> The formation of these oligomers may be related to amyloidogenesis, as the novel structure in the RNase A major dimer consists of a two-stranded  $\beta$ -sheet that resembles amyloid structure.<sup>7</sup> Moreover, the structures of some oligomers have provided convincing evidence that 3D domain swapping is a mechanism for amyloid formation.<sup>14–16</sup> Amyloid forms via 3D domain swapping when monomers swap a homologous domain or

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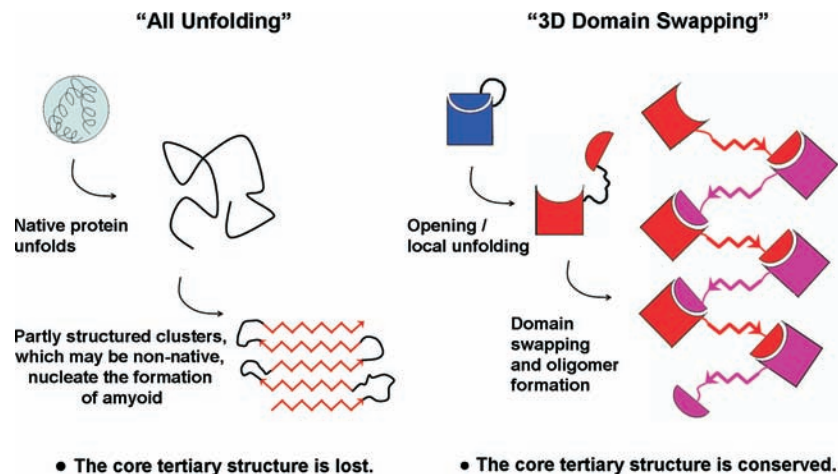
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**Figure 1.** Two possible mechanisms for protein oligomerization and amyloid formation.

secondary structural element which had detached from its subunit by a mild local unfolding or opening reaction (Figure 1).<sup>17</sup> An alternative mechanism, for which strong experimental evidence is also available,<sup>18</sup> postulates that the native protein structure unfolds more or less completely prior to amyloid formation (Figure 1).<sup>19</sup>

RNase A oligomerization is commonly induced by incubating the protein in 40–50% aqueous acetic acid for 1 h, then the acid is removed by lyophilization, and finally the protein is redissolved in benign conditions such as neutral pH phosphate buffer.<sup>20</sup> RNase A is generally thought to undergo oligomerization via a mild, local unfolding and subsequent 3D domain swapping reactions in the 40% acetic acid solution (Figure 1). Nevertheless, we have reported that RNase A and RNase S oligomerization, provoked by an alternative procedure involving the heating of highly concentrated RNase solutions in 40% aqueous ethanol,<sup>21</sup> is most efficient at temperatures where these proteins are completely unfolded as gauged by far-UV circular dichroism.<sup>22</sup>

These observations prompted us to characterize more completely the conformation of RNase A under conditions that are most commonly used to promote its oligomerization, namely, 40% acetic acid. The goal here is to determine whether RNase A has a locally opened, native-like conformation or is completely unfolded in 40% acetic acid and to use these data to better understand how this protein oligomerizes. First, the overall degree and rate of unfolding induced by 40% acetic acid were measured using solvent perturbation UV difference spectroscopy, near-UV CD spectroscopy, and 1D <sup>1</sup>H NMR spectroscopy. Next, the chemical shifts of the backbone and <sup>13</sup>Cβ nuclei in <sup>13</sup>C,<sup>15</sup>N-labeled RNase A were determined to reveal the extent of unfolding on the level of individual atoms on the basis of their conformational chemical shifts ( $\Delta\delta$ ). As a comparative benchmark, the chemical shifts of these nuclei in conditions

where RNase is essentially completely unfolded, i.e., 8 M urea at pH 2.5,<sup>23</sup> were also determined. Some previous NMR studies of protein denatured states at low pH have revealed structured clusters of aromatic groups or disulfide bonds or both.<sup>24–28</sup> To test the possible contribution of long-range interactions to RNase A structure formation in 40% acetic acid, four peptides corresponding to different segments of RNase A were studied by NMR spectroscopy. The ability of two commonly used protein denaturants, urea and guanidinium chloride (GdmCl), to induce an unfolded state suitable for RNase A oligomerization during refolding was characterized. These results and a study of the dependence of the oligomer yield on the RNase A concentration during refolding implicate a native-like refolding intermediate with non-native X-Pro peptide bonds in RNase A oligomerization. Finally, a model for the RNase oligomerization process based on these findings is presented.

## Experimental Section

**Near-Ultraviolet Circular Dichroism (near-UV CD).** A JASCO J810 instrument equipped with a Peltier temperature control module was used to record spectra at 25.0 °C in the near UV. Four accumulations spanning a wavelength range of 250–350 nm were recorded at 0.5 nm steps for each spectrum. The bandwidth was set to 1.5 nm and the scan speed was 20 nm per min. Reference spectra recorded on 10 mM sodium phosphate (pH 7), 40% acetic acid (from Pancreac)/60% water (v/v), and 8 M urea pH 2.5 (from Merck) were subtracted from the spectra of protein solutions. All spectra were recorded in a 5 mm path length cuvette. The concentration of RNase A (Sigma, type XII-A, further purified as previously described<sup>29</sup>) was 2.8 mg in 0.5 mL, or 410  $\mu$ M.

**Titration of RNase A with Acetic Acid.** Solutions of RNase A in Milli-Q water or already containing some acetic acid were titrated

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by adding small amounts of glacial acetic acid to the protein sample. The sample was incubated for a few minutes at 25.0 °C, which had been found to be sufficient to reach equilibrium, and then the CD signal at 290 nm was averaged for 120 s. The signal was corrected for dilution and the two-state model for the native  $\rightleftharpoons$  denatured equilibrium<sup>30</sup> was utilized to analyze the data to obtain the *m*-value and the free energy of conformational stability,  $\Delta G$ .

**Unfolding and Refolding Kinetics of RNase A.** The unfolding rate of RNase A in 40% HAc was measured at 22.0 °C by near-UV CD at 290 nm. A 0.30 mL solution of Milli-Q water containing 2.8 mg of RNase A was preincubated at 22.0 °C in the cuvette within the spectrometer and at zero time, 0.20 mL of deuterated acetic acid [acetic-*d*<sub>3</sub> acid-*d*<sub>1</sub> (99.5% atom D), Aldrich] was added, and the CD signal at 290 nm was monitored for 500 s. This experiment was done twice.

Aliquots of 2.8 mg of RNase A that had been unfolded in 40% HAc at room temperature for a time that is sufficient to allow the isomerization of the X-Pro peptide bonds to reach cis  $\rightleftharpoons$  trans equilibrium were lyophilized overnight. At zero time, the samples were dissolved in an aqueous solution of 2.50 M GdmCl, 50 mM MES, pH 6.2, which had been preincubated in the cuvette at 10 °C, and the refolding kinetics was monitored for 1 h at 290 nm. Under these conditions RNase A molecules with non-native X-Pro peptide conformations refold slowly.<sup>31</sup> To test for the presence of the native-like intermediate, I<sub>N</sub>, in refolding, 40% HAc treated and lyophilized RNase A (2.8 mg) was redissolved in 0.10 mL of 0.20 M sodium phosphate pH 6.7 at 0 °C, and after 15 s, this solution was added to the CD cuvette containing 0.40 mL of a solution of GdmCl at 10.0 °C. The final buffer composition was 3.00 M GdmCl (from Sigma), 0.04 M sodium phosphate, 0.04 M MES, pH 6.3. This experiment was repeated once. A negative control experiment, in which the 15 s refolding step was skipped, and a positive control experiment substituting 0.20 M sodium phosphate for 0.80 M Na<sub>2</sub>SO<sub>4</sub>, which is known to populate a native-like intermediate,<sup>32</sup> in the 15 s step were performed.

For all these experiments, the observed kinetic rates were determined by fitting a single- or double-exponential equations to the data using Kaleidagraph 3.6 (Synergy Software).

**Nuclear Magnetic Resonance.** Recombinant bovine pancreatic ribonuclease A uniformly labeled with <sup>13</sup>C and <sup>15</sup>N was prepared as previously described<sup>32</sup> using labeled Martek9 media from Spectra Stable Isotopes. NMR spectra of RNase A and its peptides were recorded at 25 °C in (i) 90% Milli-Q H<sub>2</sub>O containing 200 mM sodium phosphate, pH 6.7, 10% D<sub>2</sub>O (abbreviated below as “water”), (ii) 40% deuterated acetic acid, 50% H<sub>2</sub>O, 10% D<sub>2</sub>O (abbreviated as “40% HAc”), or (iii) 8 M urea at pH 2.5 with 90% H<sub>2</sub>O, 10% D<sub>2</sub>O (abbreviated as “8 M urea”). The pH of the 8 M urea samples was adjusted to pH 2.5 with HCl to approach the pH of the 40% HAc samples, which is about 2.0. The samples also contained 50 μM sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), whose most upfield resonance was used as the internal chemical shift reference. Carbon-13 and nitrogen-15 were referenced indirectly to the <sup>1</sup>H signal of DSS using ratios of 0.251 449 530 and 0.101 329 118, respectively.<sup>33</sup> All spectra were collected on a Bruker 800 MHz AV NMR spectrometer equipped with a triple resonance (<sup>1</sup>H, <sup>13</sup>C, <sup>15</sup>N) cryoprobe and Z-gradients. 2D NOESY, <sup>15</sup>N-HSQC, and <sup>13</sup>C-HSQC, and 3D HNCOC, HNCA, HNCACB, and CBCACONH spectra were recorded on an RNase A sample uniformly labeled in <sup>15</sup>N and <sup>13</sup>C dissolved in 40% HAc or 8 M urea. Additional spectra, namely, HNHA, HACAHN, and HNCO, were acquired on <sup>13</sup>C,<sup>15</sup>N RNase A dissolved in 40% HAc. Peak

assignments were made by following standard procedures. The assignments were corroborated using spectra recorded using C $\beta$ -edited and C $\gamma$ -edited <sup>1</sup>H-<sup>15</sup>N HSQC experiments employing a series of novel pulse sequences that select the signals according to the side chain structure.<sup>34</sup> Peptides, dissolved in water or 40% HAc, were assigned using a battery of 2D NOESY, TOCSY, and <sup>13</sup>C-HSQC spectra.

**Secondary Structure Characterization.** Secondary structure was identified by comparing the experimentally determined chemical shift values with standard chemical shift values for short, unstructured peptides in 8 M urea, pH 2,<sup>35</sup> for our spectra recorded in 40% HAc or 8 M urea. At neutral pH, the values of Wishart et al.<sup>36</sup> were used. In our hands, the chemical shift of valine C $\beta$  from Schwarzingler et al.<sup>35</sup> gave anomalous results, so it was replaced with the value from Wishart et al.<sup>36</sup> In all cases, sequence-dependent corrections were introduced.<sup>35</sup> The fractional populations of helical structures were estimated using the following values of  $\Delta\delta$  for 100%  $\alpha$ -helix: <sup>13</sup>C $\alpha$  = 3.09 ppm<sup>37</sup> and <sup>13</sup>C' = 2.21 ppm.<sup>38</sup>

**Percent Yield of RNase A Oligomers as a Function of Their Concentration.** RNase A (15.46 mg) was dissolved in 40% HAc (concentration: 19.8 mg/mL  $\rightleftharpoons$  1.41 mM) and incubated for 70 min at room temperature. The sample was then divided into six 125 μL aliquots (containing 2.48 mg of RNase A each) and then lyophilized overnight (16 h). The aliquots were dissolved in different volumes of 200 mM sodium phosphate buffer, pH 6.7. The resulting protein concentrations of the solutions ranged from 35 μM to 14 mM. These aliquots were incubated 35–60 min on ice before analysis using cation exchange chromatography<sup>21</sup> using a Mono S column attached to an Amersham Biosciences AKTA FPLC system. Elution was performed using a 10–170 mM sodium phosphate gradient at pH 6.7 and monitored by absorbance at 280 nm.

**Test for Oligomer Formation in 10 M Urea or 6 M GdmCl.** The ability of two commonly used protein denaturants, namely, 10 M urea and 6 M GdmCl, to induce RNase A oligomer formation was tested. RNase A (200 mg/mL) was dissolved in 10 M urea or 6 M GdmCl and incubated for 1 h at 22 °C to allow unfolding and Pro isomerization. Next, the denaturant was rapidly removed by gel filtration in a column pre-equilibrated in 0.2 M sodium phosphate. Half milliliter fractions were collected and incubated on ice. The quantity of oligomers formed in different fractions with a range of RNase A concentrations was determined by cation exchange chromatography. Just before injection, the aliquots were diluted 1:10 with Milli-Q water; this dilutes the 0.2 M sodium phosphate present after gel filtration and enables RNase A to bind to the Mono S column.

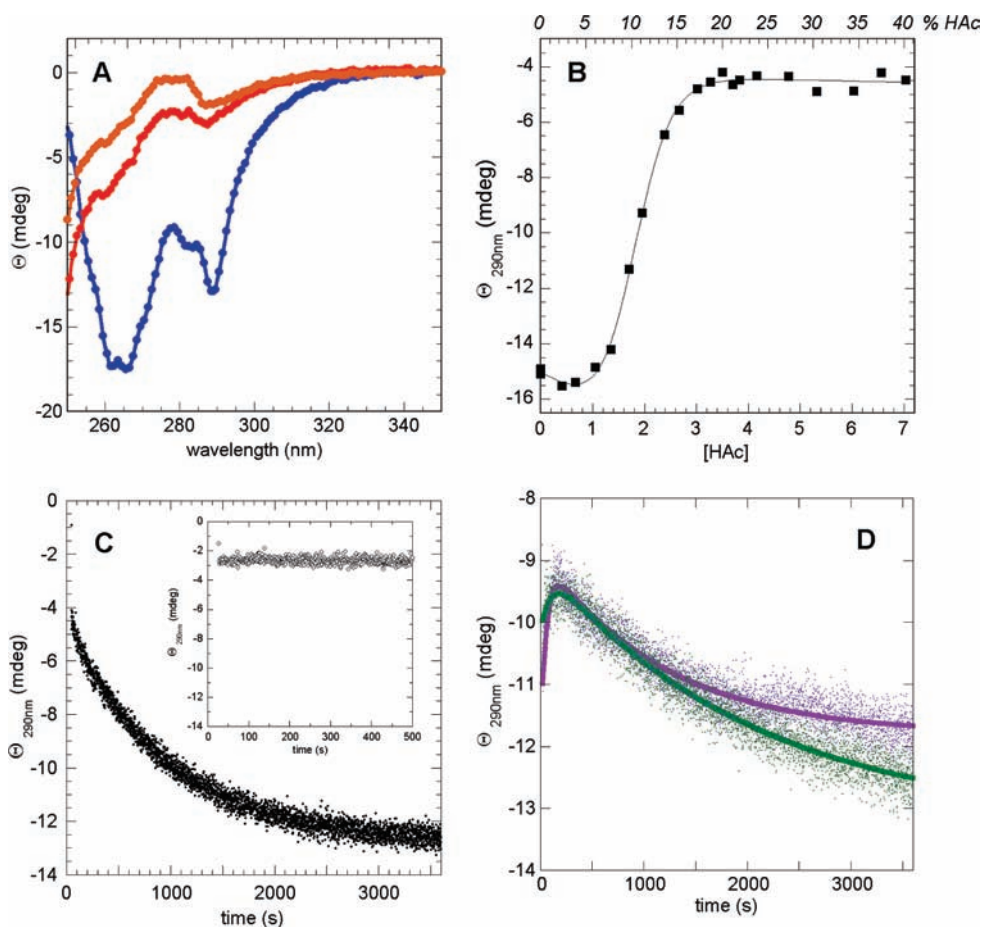
## Results

**Ultraviolet Difference and Circular Dichroism Spectroscopies.** The presence of 40% HAc induces large changes in the UV difference spectrum of RNase A; the mean  $\Delta\epsilon_{287\text{ nm}}$  is  $2200 \pm 600\text{ cm}^{-1}\text{ M}^{-1}$  (Supporting Information Figure 1). This value is much larger than the spectral changes observed for agents that perturb the solvation of the protein surface without causing denaturation<sup>39</sup> and is more than half of the values of  $\Delta\epsilon_{287\text{ nm}}$  of  $2800\text{ cm}^{-1}\text{ M}^{-1}$  for the refolding of RNase A from concentrated GdmCl<sup>31</sup> and of  $3100\text{ cm}^{-1}\text{ M}^{-1}$  reported previously for the GdmCl-induced denaturation of RNase A.<sup>40</sup>

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**Figure 2.** Conformation and folding of RNase A by near-UV CD. (A) Near-UV CD spectra of RNase A in 50 mM MES buffer pH 6.2 (blue), 40% HAc (red), or 8 M urea (gold). The spectrum of RNase A in 0.20 M sodium phosphate buffer at pH 6.7 is very similar to that in 50 mM MES buffer (data not shown). (B) Titration of RNase A (0.5 mM concentration) with acetic acid followed by near-UV CD at 290 nm. The curve represents the fit of a two-state unfolding model to the data. (C) Refolding kinetics of RNase A in 2.5 M GdmCl, 50 mM MES pH 6.2, which previously had been unfolded in 40% HAc and lyophilized. The unfolding kinetics of RNase A upon addition of acetic acid is shown in the inset. (D) Two-step assay for detecting  $I_N$ ; 40% HAc treated and lyophilized RNase A was refolded for 15 s in 0.20 M sodium phosphate pH 6.7 (green points) or 0.80 M sodium sulfate, 50 mM MES pH 6.2 (purple points) before adding to a 3 M (final concentration) of GdmCl. The curves (green for sodium phosphate and purple for sodium sulfate) show the fit of a two-exponential equation to the data.

In 10 mM sodium phosphate (pH 6.7) or 50 mM MES (pH 6.2), the near-UV CD spectra of RNase A shows a minimum in ellipticity in the near-UV (Figure 2A), which is chiefly due to the fixed conformations of Tyr25, Tyr92, and Tyr97 in the folded protein.<sup>41</sup> The amplitude of this minimum is greatly reduced in 40% HAc or 8 M urea, indicating that the Tyr side chains are no longer rigid. We also attempted to measure far-UV CD spectra of RNase A in 40% HAc; these efforts were thwarted by the high absorbance of acetic acid in the far ultraviolet.

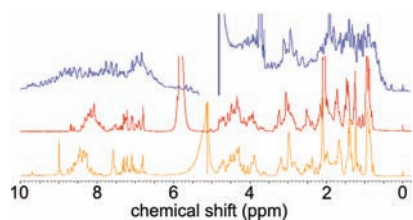
Next, RNase A was titrated with HAc and the changes in the near-UV CD signal at 290 nm were monitored (Figure 2B). The signal remains relatively constant below 1 M (7%) HAc. Above this value, the signal characteristic of native structure drops sharply until HAc concentrations are greater than 3.4 M (20%) HAc, where another plateau is reached. These data are well-fit by a two-state model for a native  $\rightleftharpoons$  denatured equilibrium. The analysis of the data with this model yields values of  $3.0 \pm 0.3$  kcal mol<sup>-1</sup> for  $\Delta G$  and  $1700 \pm 140$  cal mol<sup>-1</sup> M<sup>-1</sup> for the  $m$ -value. Values of  $\Delta G = 1.1$  kcal mol<sup>-1</sup> and  $m$ -value =  $2050$  cal mol<sup>-1</sup> M<sup>-1</sup> were reported for the urea-

induced unfolding of RNase A at pH 2.0, 25 °C.<sup>23</sup> Since the  $m$ -value is a quantitative measure of the amount of buried surface that is exposed during unfolding,<sup>42</sup> the magnitude of the  $m$ -value by HAc titration is evidence that RNase A has undergone a conformational change that is greater than a local opening event and is more consistent with a global unfolding reaction.

Kinetic experiments show that RNase A unfolding in 40% HAc is complete within the 30 s experimental deadtime (Figure 2C, inset). Thus, X-Pro bond isomerization could begin within seconds once RNase A is dissolved in 40% HAc. When RNase A, which had been incubated in 40% HAc and subsequently lyophilized, was redissolved in 2.50 M GdmCl, pH 6.2 at 10.0 °C, the majority of the molecules refold in a slow phase with  $k_{\text{obs}} = 1.05 \times 10^{-3}$  s<sup>-1</sup> (Figure 2C). This behavior is characteristic of the refolding of unfolded RNase A molecules possessing equilibrium *cis* and *trans* populations of X-Pro peptide bonds.<sup>31</sup> Finally, a two-step assay was performed. RNase A, previously treated with 40% HAc and lyophilized, was briefly refolded in 0.20 M sodium phosphate buffer or 0.80 M sodium sulfate before addition of GdmCl to a final concentration of 3

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**Figure 3.** 1D  $^1\text{H}$  spectra of RNase A in phosphate buffer 0.2 M, pH 6.7 (blue); in 40% acetic acid (red); and in 8 M urea (gold). RNase in phosphate buffer is well-folded. The decrease in peak dispersion of RNase in acetic acid and in urea indicates that protein is unfolded in those conditions.

M. Both a fast unfolding phase ( $k_{\text{obs}} = 0.015 \text{ s}^{-1}$ ) and a slow refolding phase ( $k_{\text{obs}} = 5 \times 10^{-4} \text{ s}^{-1}$ ) are observed (Figure 2D). This fast unfolding phase is a hallmark of the intermediate  $\text{I}_\text{N}$ ,<sup>31</sup> which has native-like structure but non-native X-Pro peptide bonds.<sup>43</sup> In these conditions,  $\text{I}_\text{N}$  unfolds because its GdmCl unfolding midpoint of less than 1 M.<sup>31</sup> Fully native RNase A has a GdmCl unfolding midpoint of 3.4 M and is stable in 3 M GdmCl, pH 6, 10 °C.<sup>31</sup> The slow refolding phase observed here is due to a rate-limiting step of X-Pro bond isomerization in the denatured polypeptide chains. No fast unfolding phase was seen in the negative control experiment lacking the 15 s incubation in sodium phosphate (data not shown).

**1D  $^1\text{H}$  and 2D  $^1\text{H}$  NOESY NMR Spectroscopy.** The presence of folded structure in RNase A was surveyed by 1D  $^1\text{H}$  NMR (Figure 3). In aqueous solution, the resonances of RNase A are widely separated. This high dispersion is a hallmark of its folded structure. In contrast, the 1D  $^1\text{H}$  spectra in 40% HAc or 8 M urea reveal much less dispersion. This strongly suggests that most nuclei have lost the unique environment they had in the folded protein and that the protein is now denatured.

The 2D NOESY spectrum of RNase A in aqueous solution contains a myriad of NOE crosspeaks arising from tertiary structure contacts. Most of the crosspeaks arising from the proximity of aromatic and aliphatic protons in the protein core are missing in the NOESY spectra recorded in 40% HAc or 8 M urea (Supporting Information Figure 2A), which reflects a breakdown of tertiary structure. Interestingly, all four X-Pro peptide bonds are chiefly *trans* in both 40% HAc or 8 M urea, as detected by X $\alpha$ H-Pro $\delta$ H NOEs and the Pro  $^{13}\text{C}\beta$   $\delta$  values (Table 1 and Supporting Information Figure 2B), whereas Tyr92-Pro93 and Asn113-Pro114 peptide bonds are *cis* in native monomeric RNase A.

**Characterization of RNase A in 40% Acetic Acid via Multidimensional Heteronuclear NMR.** The  $^1\text{H}$ – $^{15}\text{N}$  HSQC spectra of RNase A in 40% HAc and 8 M urea are shown in Figure 4. Despite the severe overlap in the  $^1\text{H}$  dimension, good dispersion is observed in the  $^{15}\text{N}$  dimension, as is typical for denatured proteins. On the basis of these spectra and the 3D spectra, the assignment of 97% of the  $^{15}\text{N}$ ,  $^1\text{H}_\text{N}$ ,  $^{13}\text{C}\alpha$ , and  $^{13}\text{C}\beta$  and 92% of the  $^{13}\text{C}'$  and  $^1\text{H}\alpha$  nuclei in 40% HAc, as well as 99% of the  $^{13}\text{C}\alpha$ , 96% of the  $^{13}\text{C}\beta$ , and 94% of the  $^1\text{H}_\text{N}$  and  $^{15}\text{N}$  resonances in 8 M urea, pH 2 could be obtained. These chemical shift values have been deposited in the BMRB database (file number 16503). The assignments in 40% HAc were corroborated using C $\beta$ -edited and C $\gamma$ -edited  $^1\text{H}$ – $^{15}\text{N}$  HSQC experiments (Supporting Information Figure 3).

Previous studies of chemical shifts in native proteins have shown that  $\Delta\delta$  can be used to identify segments of secondary

**Table 1.**  $^{13}\text{C}\beta$  Chemical Shifts of Pro Residues

|                                | $\delta$ $^{13}\text{C}\beta$ (ppm) |                |
|--------------------------------|-------------------------------------|----------------|
|                                | 40% HAc                             | 8 M urea, pH 2 |
| Pro42                          | 31.77                               | 32.22          |
| Pro93                          | 31.80                               | 32.32          |
| Pro114                         | 31.78                               | 32.06          |
| Pro117                         | 31.72                               | 32.24          |
| <hr/>                          |                                     |                |
| <i>trans</i> -Pro <sup>a</sup> |                                     | 32.1           |
| <i>cis</i> -Pro <sup>a</sup>   |                                     | 34.5           |

<sup>a</sup> values of Richarz and Wüthrich<sup>67</sup> for X-Pro in the *cis* or *trans* conformation employing the correction of 1.5 ppm of Wishart et al.<sup>38</sup> applied to take into account the difference in the chemical shift reference values of dioxane<sup>67</sup> and DSS (this work). The small systematic difference in the  $^{13}\text{C}\beta$  chemical shifts measured here in 40% HAc versus 8 M urea pH 2.5 is almost certainly due to solvent effects on the position of the DSS reference.

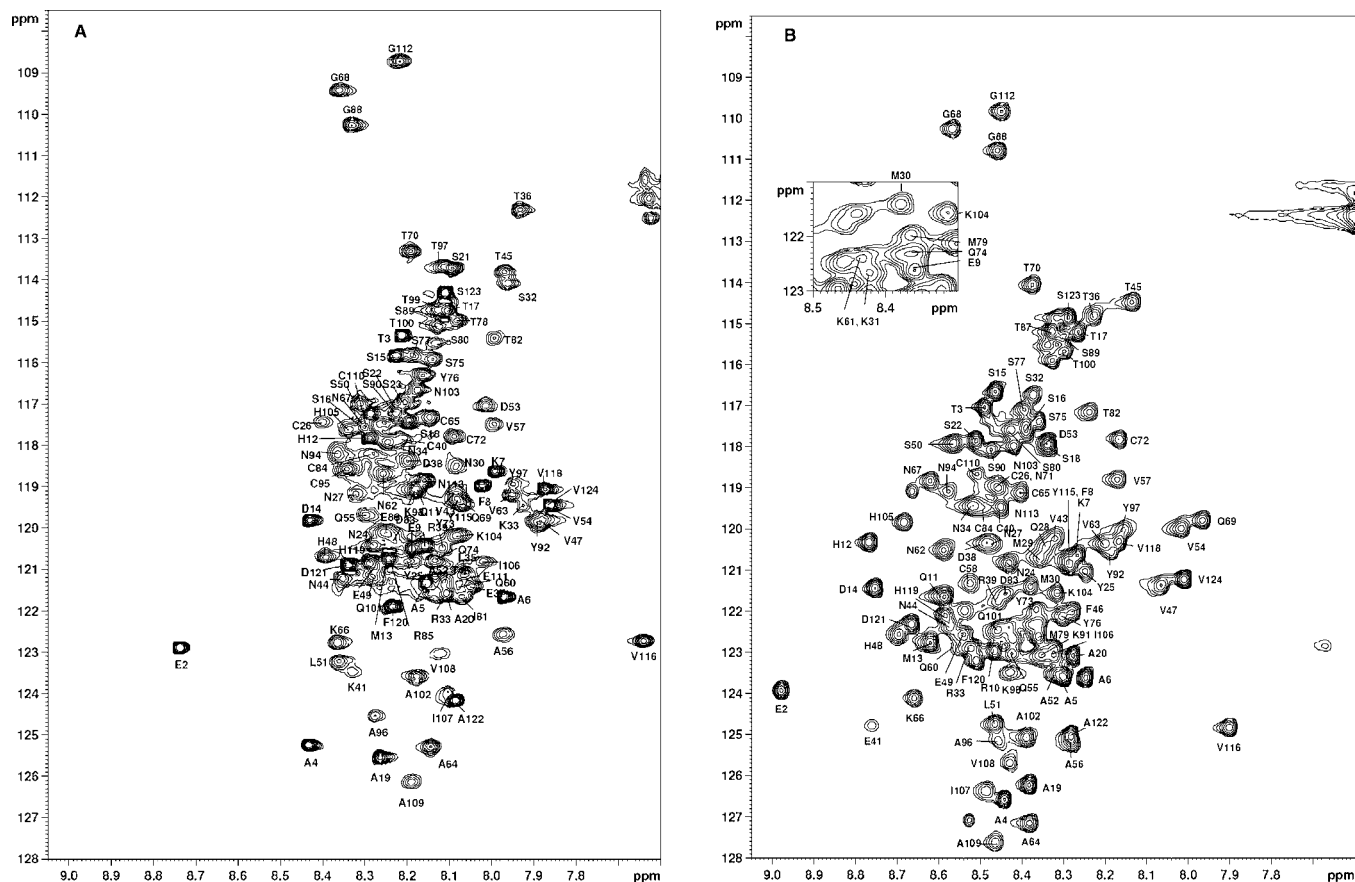
structure in folded proteins and to detect and estimate the population of secondary structure elements in partly folded peptides or proteins. A summary of reported average conformational shifts for 100%  $\alpha$ -helix and  $\beta$ -sheet structure is reported in Table 2. The conformational shifts of the  $^{13}\text{C}$  nuclei ( $^{13}\text{C}\alpha$ ,  $^{13}\text{C}\beta$ , and  $^{13}\text{C}'$ ) are particularly useful tools for detecting secondary structure, as they are highly sensitive to the backbone structure and are scarcely influenced by other factors, such as aromatic ring current effects, as is the  $^1\text{H}\alpha$  nucleus.

The  $^{13}\text{C}$  conformational chemical shifts of RNase A in 40% HAc are shown in Figure 5. Three segments of helical structure can be clearly detected. The first segment approximately spans residues 3–12. Its helix population is about 26%, based on the average  $\Delta\delta$  values of the  $^{13}\text{C}\alpha$  and  $^{13}\text{C}'$  nuclei. This first segment corresponds to the position of the first  $\alpha$ -helix in native RNase A. The  $^{13}\text{C}\beta$  nuclei are not used to estimate the helical population because this nucleus is not as good at discriminating helix from random coil as the  $^{13}\text{C}\alpha$  and  $^{13}\text{C}'$ .<sup>38,44</sup> The second helical segment includes residues 17–33. The estimated helical population is highest in the center of this segment (residues 23–25 and 29–30, 55% helix) and lower at its beginning (residues 17–22, 19% helix) and end (residues 31–33, 17% helix). Most of this second helical segment (residues 24–33) matches the position of the second helix in folded RNase A. However, the first six residues (17–22) adopt a loop in the native monomeric protein.<sup>2</sup> The third helical segment detected in RNase A in 40% acetic acid is composed of residues 50–60. The helix population is about 45% in the first six or seven residues and only 14% in the last four or five residues. This third helical segment is in the same place as the third helix in native RNase A. The lower helical population for the last four or five residues in this segment in 40% HAc may be related to the observation that the C-terminus of this helix is irregular in aqueous solution under benign conditions.<sup>2</sup> Besides these three helical regions, there is one segment, composed of residues 115–124, with a weak tendency toward adopting  $\beta$ -strand structure; only the  $\Delta\delta^{13}\text{C}'$  values reach the threshold for detecting  $\beta$  structure. The C-terminus of RNase A is rich in  $\beta$ -branched and bulky residues, which tend to favor extended backbone structures, such as  $\beta$ -strands.

In 8 M urea, pH 2.5, the conformational chemical shifts of RNase A are small and no segments of partially populated secondary structural elements are identified (Figure 5). These

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**Figure 4.** (A)  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of  $^{13}\text{C}$ ,  $^{15}\text{N}$  RNase A in 40% HAc. (B)  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra of  $^{13}\text{C}$ ,  $^{15}\text{N}$  RNase A 8 M urea, pH 2.5.

**Table 2.** Estimating Secondary Structure Content from  $\Delta\delta$  Values

| nuclei                | $\Delta\delta$ (ppm)                    |   |
|-----------------------|---|---|
|                       | 100% $\alpha$ -helix                    | 100% $\beta$ -sheet (ppm)               |
| $^{13}\text{C}\alpha$ | <b>+3.09<sup>a</sup></b>                | -1.48 <sup>a</sup> , -1.6 <sup>b</sup>  |
| $^{13}\text{C}\beta$  | -0.38 <sup>a</sup> , +1.05 <sup>c</sup> | +2.16 <sup>a</sup> , +1.7 <sup>b</sup>  |
| $^{13}\text{C}'$      | <b>+2.21<sup>d</sup></b>                | -2.21 <sup>d</sup>                      |
| $^1\text{H}\alpha$    | -0.39 <sup>e</sup> , -0.14 <sup>f</sup> | +0.37 <sup>e</sup> , +0.45 <sup>f</sup> |

<sup>a</sup> From Spera and Bax;<sup>37</sup> values shown in **bold** are used in the text to estimate the helix content. <sup>b</sup> From Santiveri et al.<sup>68</sup> (for estimating  $\beta$ -strand populations in peptides). <sup>c</sup> Average from Wang and Jardetzky.<sup>44</sup> <sup>d</sup> Calculated from Wishart and Sykes.<sup>38</sup> <sup>e</sup> From Wishart et al.<sup>69</sup> <sup>f</sup> From Osapay and Case<sup>70</sup> (based on theoretical calculations).

results are consistent with previous biophysical studies that found RNase A to behave like a completely unfolded, disulfide bond cross-linked random coil in these conditions.<sup>23,45</sup>

**Characterization of RNase A Peptides in 40% Acetic Acid.** To obtain additional data regarding the possible participation of long-range interactions in stabilizing the helical structure that is partly populated in 40% HAc, four peptides corresponding to helix I (residues 1–20, also known as the S-peptide), helix II (residues 21–35), an internal loop closed by the C65–C72 disulfide bond (residues 63–74), and the last loop and  $\beta$ -strand (residues 112–124) of RNase A have been structurally characterized by NMR spectroscopy in 40% HAc. Partial helix structure was detected in the first two peptides in positions corresponding to the position of helix I and helix II in the complete RNase A molecule, but not in the other two peptides

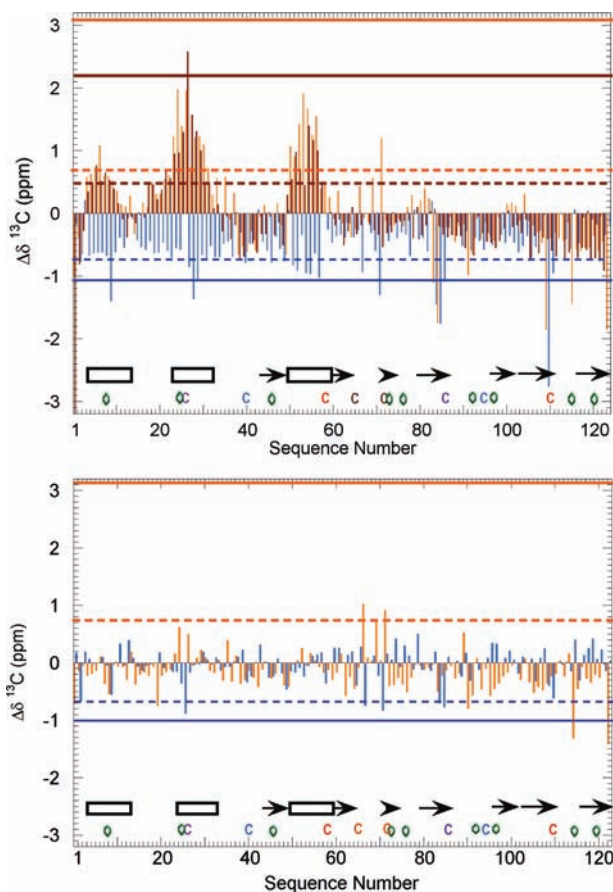
(Supporting Information Figure 4). These results provide evidence that long-range interactions generally play minor roles in stabilizing the helical structure present in RNase A in 40% HAc. Some possible minor contributions are discussed in the text accompanying Supporting Information Figure 4.

**Dependence of the Oligomer Yield on the RNase A Concentration.** The results reported here indicate that RNase A is mainly unfolded and shows no signs of oligomerization in 40% HAc. Therefore, oligomerization must occur in the subsequent steps, either lyophilization or the redissolution of the freeze-dried protein in neutral pH buffer. An additional experiment was performed to gain insight into the refolding and oligomerization process. After incubation in 40% acetic acid and lyophilization, aliquots of RNase A were dissolved in varying volumes of sodium phosphate buffer. The percent oligomer yield, quantified by cation exchange chromatography, was found to increase with the logarithm of the RNase A concentration (Figure 6A,B, Supporting Information Table 1). The elution profile from cation exchange chromatography of RNase A oligomers observed here after treatment with 40% acetic acid and lyophilization are similar to previously reported results.<sup>8,10,21,29</sup> A control sample of lyophilized RNase A, which had not been treated with 40% acetic acid, showed a small amount of N-terminal swapped dimer and a tiny quantity of C-terminal swapped dimer; this is consistent with previously reported results.<sup>21</sup>

**RNase A Oligomer Formation following Unfolding by Urea or GdmCl.** To test if the thoroughly unfolded protein conformations ensembles provoked by commonly used denaturants are also able to form oligomers during refolding, samples

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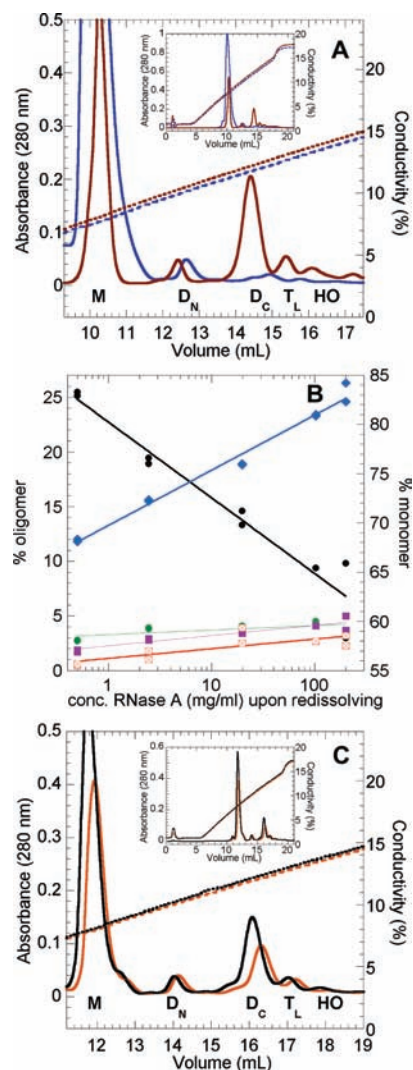


**Figure 5.** Conformational chemical shifts of RNase A in 40% acetic acid (top panel) and 8 M urea pH 2 (bottom panel).  $^{13}\text{C}\alpha$  is shown in orange;  $^{13}\text{C}\beta$ , blue; and  $^{13}\text{C}\gamma$ , maroon. The solid lines represent the  $\Delta\delta$  values expected for 100%  $\alpha$ -helix for  $^{13}\text{C}\alpha$  (orange),  $^{13}\text{C}\beta$  (blue), and  $^{13}\text{C}\gamma$  (maroon). The dashed lines represent the  $\Delta\delta$  limits for detecting  $\alpha$ -helix in the  $^{13}\text{C}\alpha$  (orange),  $^{13}\text{C}\beta$  (blue), and  $^{13}\text{C}\gamma$  (maroon) nuclei. Green hexagons mark the position of aromatic residues. The letters “C” show the position of Cys residues; Cys with the same color form a disulfide bond.

of RNase A were denatured in 10 M urea or 6 M GdmCl. Following a gel filtration step to remove the denaturant and transfer the protein to 0.2 M sodium phosphate buffer, analysis by cation exchange chromatography revealed the formation of RNase A oligomers (Figure 6C). As was observed for oligomerization occurring after denaturation by 40% acetic acid, the yield of oligomers after urea or GdmCl denaturation was found to depend on the logarithm of the protein concentration during refolding (Supporting Information Table 1 and Figure 5).

## Discussion

The main goal of this work has been to elucidate the conformation of RNase A in 40% acetic acid and to use these findings to improve our comprehension of how RNase A oligomerization occurs. A first set of experiments using UV difference and CD spectroscopies and 1D  $^1\text{H}$  NMR spectroscopy, indicates that most or all of the protein groups that are buried in native RNase A quickly become solvent exposed in 40% acetic acid. Moreover, the 2D NOESY NMR spectrum shows a loss of long-range contacts between aromatic and aliphatic groups, which are indicative of tertiary structure. The peptide bonds preceding all four prolines of RNase A in 40% HAc are predominately in the *trans* conformation as occurs in RNase A denatured in 8 M urea pH 2.5. Thus, observation of



**Figure 6.** Yield of RNase A oligomers: effect of concentration and denaturant. (A) Cation exchange chromatogram of RNase A oligomers formed after incubation in 40% HAc, lyophilization, and redissolving in 0.2 M sodium phosphate buffer (solid red curve) or dissolving RNase A without HAc treatment (solid blue curve). The conductivity (broken lines, right y-axis) is proportional to the sodium phosphate gradient used to elute the protein. The oligomeric species are labeled as follows: M, monomer;  $\text{D}_\text{N}$ , N-dimer;  $\text{D}_\text{C}$ , C-dimer;  $\text{T}_\text{L}$ , linear trimer; HO, cyclic trimer and higher oligomers. The inset shows the complete chromatograms. (B) Effect of RNase concentration after lyophilization on oligomer yield. Percentage of monomer is shown in black, C-dimer in blue, N-dimer in green, linear trimer in violet, and other oligomers in red. (C) Cation exchange chromatograms of RNase A oligomers formed after denaturation in 10 M urea (solid red curve) or 6 M GdmCl (solid black curve). Broken lines show the conductivity, and oligomeric species are labeled as in panel A. The complete chromatograms are shown in the inset.

the *trans* conformation of Tyr92-Pro93 and Asn113-Pro114 in 40% HAc is consistent with a loss of native structure of RNase A. It is important to point out, however, that the *trans* conformation at Pro114 could predispose the protein for oligomer formation via C-terminal swapping, since the major RNase dimer,<sup>7</sup> major trimer,<sup>8</sup> and probably all tetramers<sup>46</sup> contain at least one subunit with a *trans* Asn113-Pro114 peptide bond. The loop formed by residues 33–41 creates stabilizing interactions with both Tyr92 and residues 10–13 at the N-terminus. Therefore, a Tyr92-Pro93 *trans* peptide bond may

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disrupt these interactions and thus indirectly favor the detachment and swapping of the N-terminus.

Our in-depth study of RNase A structure from conformational chemical shifts reveals that in 40% acetic acid the protein loses essentially all the  $\beta$ -sheet structure but has partial helical structure at positions that correspond approximately to the locations of the three helices in native RNase A, except for the segment composed of residues 17–22. These residues, which adopt a minor population of  $\alpha$ -helix in 40% acetic acid, are in a loop, which is flexible,<sup>47</sup> in native monomeric RNase A. It is interesting that one subunit of the 3D domain N-terminal swapped dimer of RNase A has a bent  $\alpha$ -helix spanning residues 18–32,<sup>6</sup> and a similar segment composed of residues 18–23 adopts a  $3_{10}$  helix in the domain-swapped dimer of the PM8 variant of human pancreatic RNase<sup>48</sup> (Supporting Information Figure 6). Therefore, it is possible to suggest that the presence of nascent helical structure in the 17–22 residue segment in 40% acetic acid may predispose the polypeptide chain for the formation of the N-dimer, and future experiments will test this proposal. However, this nascent helical structure does not seem to be essential for N-dimer formation, since it is absent in urea or GdmCl denatured RNase A and substantial amounts of N-dimer form upon transfer of RNase A from these denaturant solutions to a benign buffer. Studies of the conformations of apo-myoglobin<sup>49</sup> and the C-terminal domain of ribosomal L9 protein<sup>50,51</sup> at low pH in HCl have revealed partially populated  $\alpha$ -helices that extend beyond their limits in the native protein. In contrast to molten globules states forming at low pH, which retain native-like secondary structure, conserve the global tertiary structure, and bury their poorly packed hydrophobic groups,<sup>52</sup> RNase A in 40% HAc loses its  $\beta$ -sheet structure, its hydrophobic core is solvent exposed, and only a fraction of its helical structure is retained. In conclusion, RNase A in 40% HAc adopts a chiefly unfolded state and is not a mildly unfolded “open intermediate” (Figure 1).

A comparative study of the conformation of RNase A in 8 M urea at pH 2 revealed an essentially complete loss of all secondary structural elements. Some investigations of protein conformations in 8 M urea have identified partial structure, typically clusters of aromatic rings or disulfide groups packed on Trp indole groups.<sup>24–26,53</sup> The lack of residual or non-native structure in RNase A in 8 M urea at pH 2 may be attributed to its relative poorness of aromatic groups and in particular its lack of Trp residues.

#### A New Model for RNase A Oligomerization in 40% HAc.

The data reported here have implications for the formation of RNase A oligomers, since the 40% acetic acid treatment does not lead to a locally unfolded “open” intermediate but a chiefly unfolded state. Recalling that RNase oligomerization is induced by (i) incubation in 40% HAc, (ii) lyophilization, and (iii)

redissolving the protein in a benign buffer, we propose that oligomerization is not likely to occur during lyophilization, since the motion of the protein chains will be minimized by the cold temperature ( $-50\text{ }^{\circ}\text{C}$ ). Moreover, the loss of solvent molecules during freeze-drying tends to disrupt rather than promote protein structure,<sup>54</sup> so it is improbable that RNase A will refold during lyophilization as the acetic acid molecules are removed. Future studies based on infrared or solid-state NMR spectroscopies of lyophilized RNase A samples are planned to test this point directly.

If lyophilization were to tangle RNase A chains and thereby predispose them for oligomer formation during lyophilization, one would expect the percent yield of oligomer to be independent of the final RNase A concentration after dissolving in neutral pH buffer. Instead, the oligomer yield is proportional to the logarithm of the final RNase A concentration. This is the behavior expected by kinetic collision theory for RNase A molecules colliding and combining in bimolecular reactions to form oligomers once after being dissolved in buffer<sup>55</sup> and is evidence that RNase A molecules refold and oligomerize upon dissolving in the neutral pH buffer.

The refolding of RNase A molecules from concentrated solutions of denaturants such as guanidinium chloride or urea has been thoroughly studied.<sup>31,40,41,43,56–60</sup> When RNase A is denatured briefly at low temperatures, the *cis* peptide bonds of Tyr92-Pro93 and Asn113-Pro114 conserve this conformation and the protein molecules are able to refold quickly if returned to solution conditions that favor the native state. However, if the RNase A molecules are left in denaturing conditions for longer periods of time, the peptide bonds of Tyr92-Pro93 and Asn113-Pro114 slowly isomerize to an equilibrium mixture of *trans* to *cis* conformations, with a ratio of approximately 4:1 *trans*:*cis*. The native state of RNase A is strongly destabilized by the presence of a *trans* peptide bond between residues 92 and 93 or 113 and 114 or both.<sup>61</sup> Indeed, when denatured RNase A molecules with an equilibrium mixture of *trans* and *cis* X-Pro peptide bonds are transferred to solution conditions where the native state is marginally stable, the RNase A molecules with *trans* peptide bonds at Tyr92-Pro93 or Asn113-Pro114 or both refold slowly and the refolding rate is limited by *trans*  $\rightarrow$  *cis* isomerization of these bonds. In contrast, when denatured RNase A molecules with an equilibrium mixture of *trans* and *cis* X-Pro peptide bonds are transferred to conditions that strongly favor the native state, RNase A molecules can rapidly refold to an enzymatically active, native-like intermediate called “ $I_N$ ”, which is distinguished from the fully native state in that it has a lower conformational stability and possesses *trans* peptide bonds at Tyr92-Pro93 or Asn113-Pro114 or both.

These previous investigations, and results reported here in Figure 2D, show that the  $I_N$  intermediate forms when HAc

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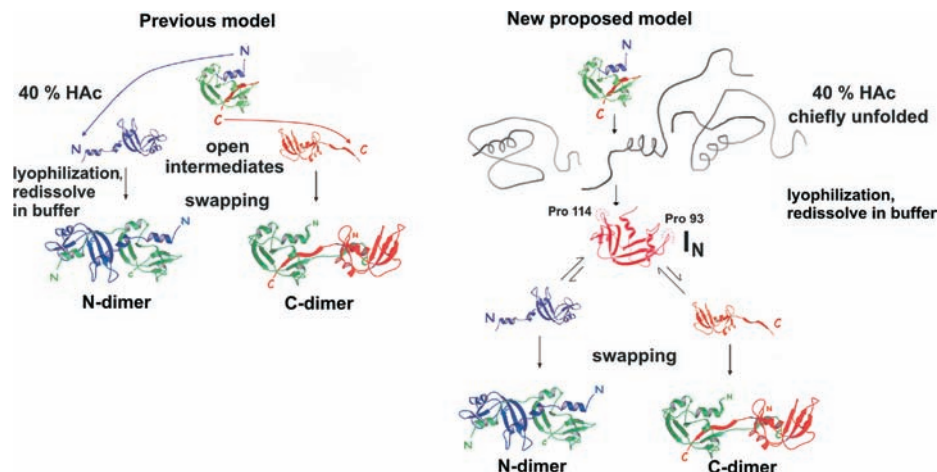
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**Figure 7.** Models for RNase A oligomerization in 40% acetic acid. The previous model (left panel) assumed that 40% HAC induced a mild local unfolding or opening of the N- or C-terminal segments. After the acetic acid is removed by lyophilization and the proteins are redissolved in a benign buffer, the terminal segments of the “open intermediates” would swap to form oligomers. The new proposed model (right panel) reflects our findings that RNase A is principally unfolded in 40% HAC, with only some fractional populations of helix. All X-proline peptide bonds equilibrate predominantly to the *trans* conformation. After removing the acetic acid by freeze-drying and placing the protein in a benign buffer, some of the protein molecules rapidly fold to a native-like intermediate  $I_N$ , which has non-native *trans* peptide bonds at positions Tyr92-Pro93 or Asn113-Pro114 or both. The lower conformational stability of this intermediate and the perturbation of the *trans* Asn113-Pro114 peptide bond on the native monomeric conformation favor the formation of the open intermediates, which then would collide and swap their terminal segments to form oligomers. This figure has been adapted in part from ref 8. Note that the disulfide bonds remain intact in RNase A in 40% acetic acid.

denatured and lyophilized RNase A molecules with *trans* peptide bonds at Tyr92-Pro93 or Asn113-Pro114 or both are redissolved in the 0.20 M sodium phosphate buffer typically used to form oligomers. We propose that  $I_N$  is the key intermediate responsible for RNase A oligomerization. The lower stability of  $I_N$  would favor the detachment of the N-terminal segment (helix I and the following loop) from the rest of the protein, thus promoting the formation of the N-dimer and higher order oligomers containing a swapped N-terminus. As mentioned previously, the nascent helical structure in the 17–22 residue segment forming in 40% HAC, together with the indirect effect of the *cis* 92–93 peptide bond on the anchoring of helix I, may also predispose RNase A for oligomers with N-terminal swapping. With regard to the C-terminal swapping, although the presence of a *trans* peptide bond at positions 113–114, present in most molecules of  $I_N$ , severely disrupts the native loop conformation in monomeric RNase A,<sup>59</sup> we expect it to strongly favor the formation of the C-dimer. The Asn113-Pro114 peptide bond is *trans* in the C-dimer and this conformer is essential for the formation and stability of a novel two-stranded  $\beta$ -sheet in the “hinge loop” segments (residues Glu111-Val116) that link the C-dimer subunits.<sup>7</sup> The proposed mechanism for HAC-induced RNase A oligomerization is summarized in Figure 7.

The isomerization of X-Pro peptide bonds has also been proposed to play a crucial role in the formation of 3D domain swapped oligomers in other proteins.<sup>62,63</sup> The formation of RNase A C-swapped dimers and higher order oligomers, including quasi-amyloid fibrils, can be also favored by point mutations or insertions that strengthen the stability of the hinge loop  $\beta$ -sheet structure.<sup>15,64,65</sup> These and other factors affecting the formation of 3D domain swapped oligomers by mammalian

pancreatic type ribonucleases have been reviewed recently.<sup>66</sup> Previously, two models for protein oligomerization and amyloid formation that contrast mild unfolding and domain swapping versus extensive or complete unfolding have been developed by the laboratories of Eisenberg and Dobson. On the basis of our present results with RNase A, which has long served as a paradigm for protein oligomerization via 3D domain swapping, we propose that other proteins oligomerizing by 3D domain swapping may actually become thoroughly unfolded at some point during this process.

## Conclusions

The conformation of RNase A in 40% HAC is found to be mostly but not completely unfolded. All X-Pro bonds are predominantly in the *trans* conformation and the hydrophobic core and the  $\beta$ -sheet structure unfold completely. However, three  $\alpha$ -helices are partly populated in RNase A in 40% HAC in approximately the same positions as the three native helices. No helical structure is found in 8 M urea at pH 2.5. We also find that RNase A can oligomerize after thorough unfolding in concentrated solutions of urea or GdmCl, followed by a gel filtration step, which exchanges the denaturant for a refolding buffer. The yield of RNase A oligomers depends on the logarithm of RNase A concentration during refolding. These results, and a consideration of the RNase A refolding literature, lead us to propose that RNase A oligomerization does not occur in 40% HAC solution, as was previously assumed. Instead, RNase A molecules refold to a native-like intermediate whose lower

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conformational stability and content of *trans* X-Pro peptide bonds promotes the formation of 3D domain swapped oligomers. These findings are pertinent for understanding how amyloid forms, since other 3D domain swapped oligomers might also undergo unfolding events of greater magnitude than those needed to detach the swap domain.

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**Supporting Information Available:** Graphs showing (i) UV difference spectra of RNase A in 40% acetic acid, (ii) regions of NOESY spectra of RNase A in 40% HAc and 8 M urea, (iii) C $\beta$ - and C $\gamma$ -edited  $^1\text{H}$ - $^{15}\text{N}$  HSQC spectra, (iv) the conformational chemical shifts of RNase A peptides, (v) representative chromatograms of RNase A oligomers forming after urea and GdmCl denaturation, and (vi) structures of the RNase A N-dimer and RNase 1 PM8 dimer and a table summarizing RNase A oligomer yield following denaturation in 40% HAc, 10 M urea, or 6 M GdmCl. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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