Supporting Information for:

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

by:

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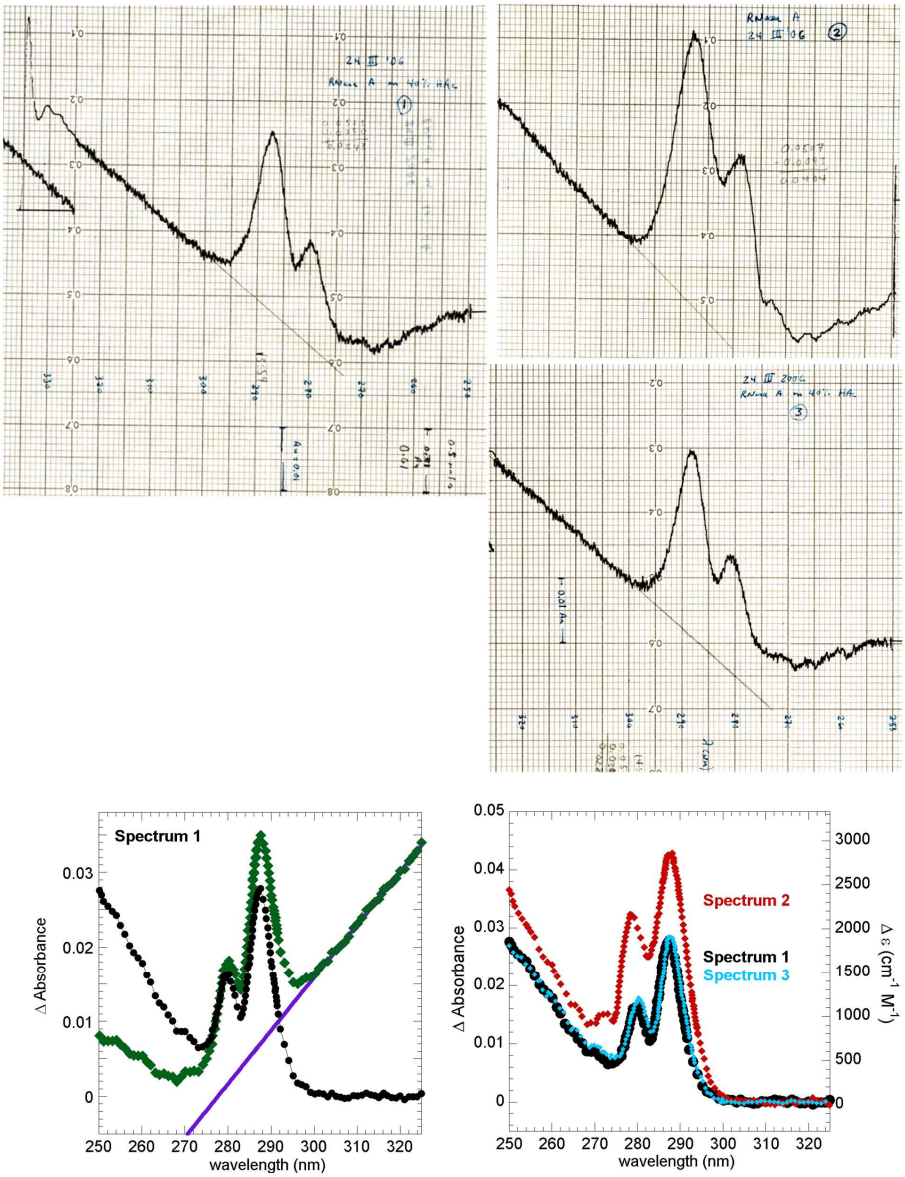
Supporting Figure 1. Ultraviolet Difference Spectroscopy of RNase A in 40% HAc



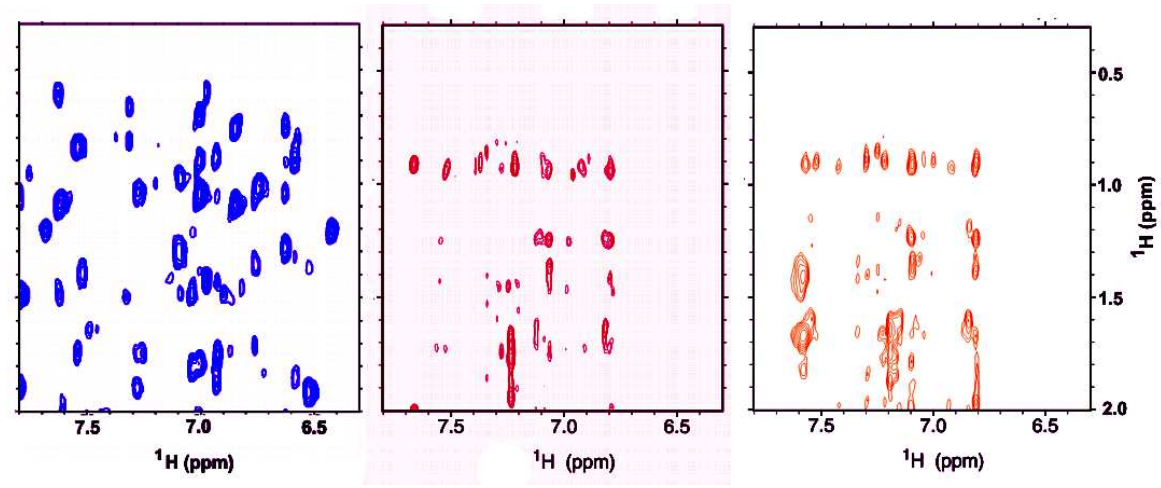
The raw ultraviolet difference spectra, shown in the **top panels** in figure above, were scanned and digitalized. Baseline correction was made by fitting a linear function to the data in the wavelength range spanning 305 to 325 nm, and this function was then subtracted from the raw data (**bottom left panel).** The three baseline corrected difference spectra are shown in the **bottom right hand panel**. The change in extinction (Δε) shown on the right y-axis, was calculated by dividing the absorbance difference (left y-axis) by the pathlength (0.4375 cm) and the protein concentration (34.2 μM).

Experimental Procedures: *Ultraviolet Difference Spectroscopy:* A Cary 210 dual beam UV spectrometer and tandem quartz cuvettes (Hellma 230-QS) were used. A solution of RNase

A (Sigma, Type XII-A, further purified as described[29]) in 40% HAc / 60% MilliQ water (H_2O) (v/v) was put in the left chamber in the sample cuvette, with water in the right chamber. In the reference cuvette, an aqueous RNase A solution was placed in the left chamber and 40% HAc / 60% water was put in the right chamber. The final protein concentration was 34.2 μM. Measurements were recorded at 22 ºC, using a scan speed of ½ nm per second.

Supporting Figure 2. Regions of the 2D ^{1}H NOESY Spectra of RNase A

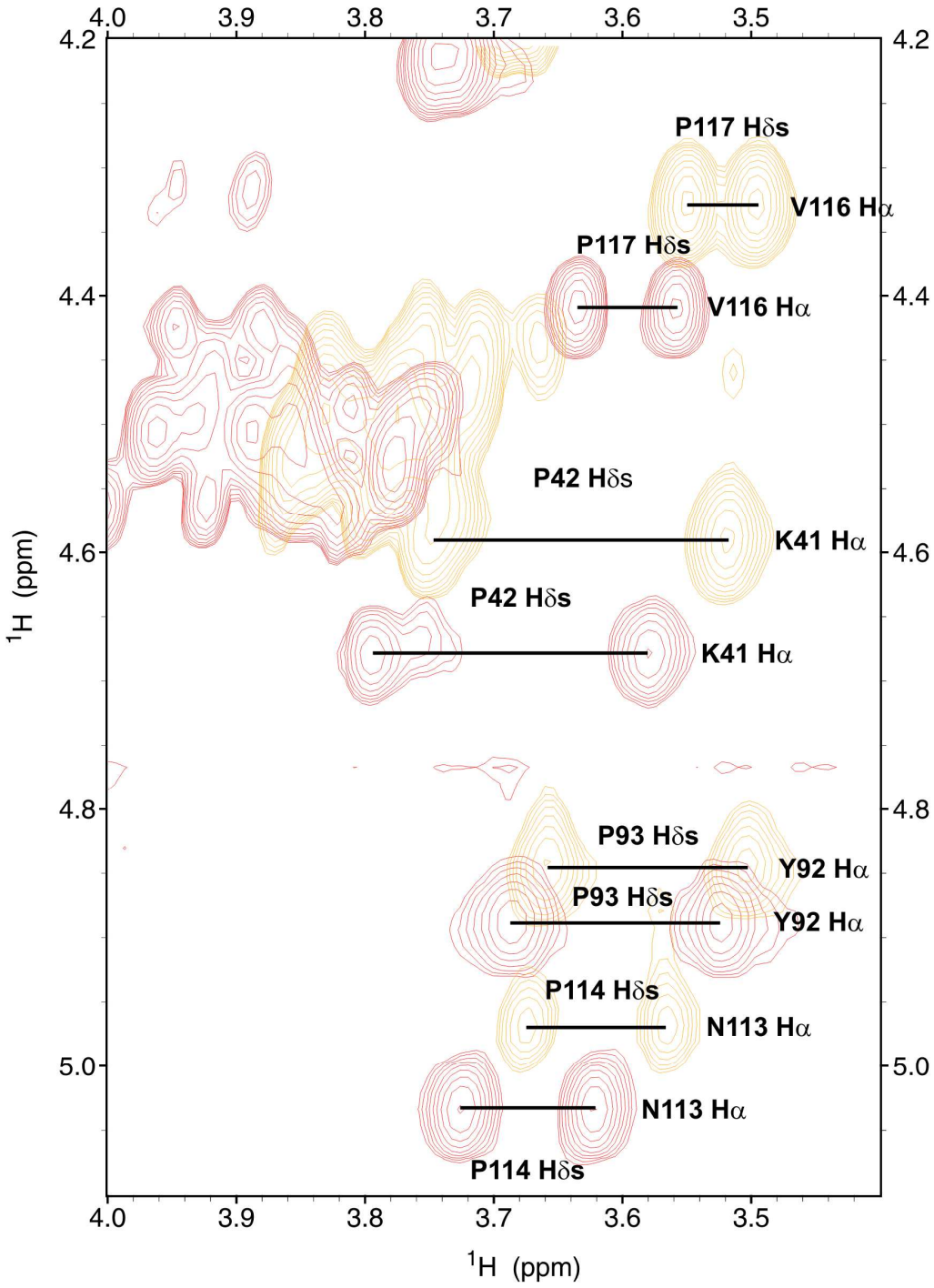
A. Aromatic to aliphatic regions of the ^{1}H 2D NOESY spectra of RNase A in aqueous solution (left panel, blue), 40% HAc (central panel, red) and 8M urea (right panel, gold).



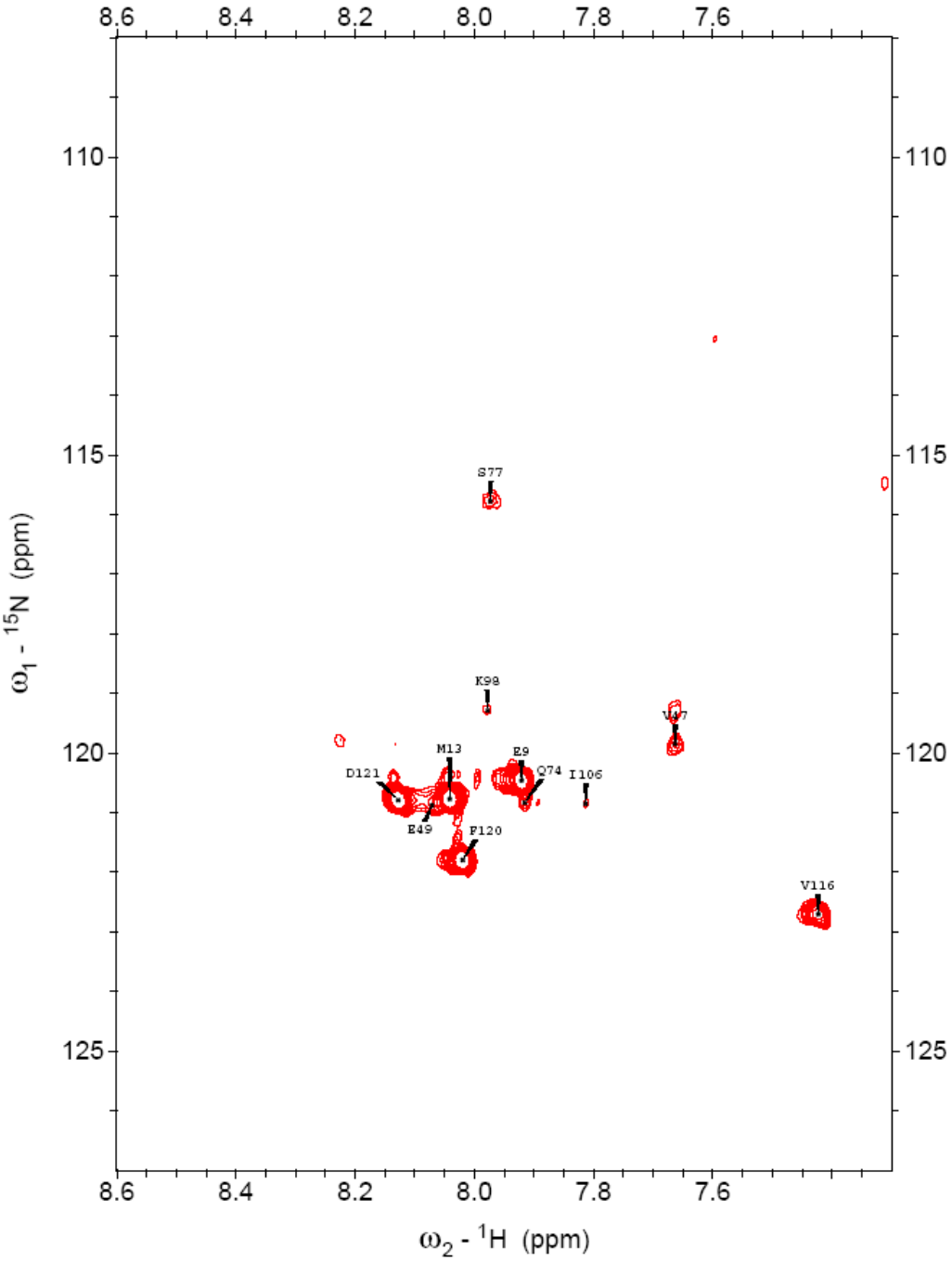
Most of the peaks appearing in this region of the NOESY spectra in 40% acetic acid or 8 M urea arise from consecutive residues such as: Val 47 – His 48, Val 54 – Gln 55, Ala 56 – Val 57, Ala 96 – Tyr 97, Lys 91 – Tyr 92, Pro 114 – Tyr 115. The Val 118 γ methyl protons resonate at some 0.07 – 0.13 ppm upfield from the other Val γ methyl protons; this could be due to a weak ring current effect from the aromatic ring of Phe 120.

Supporting Figure 2. Continued,

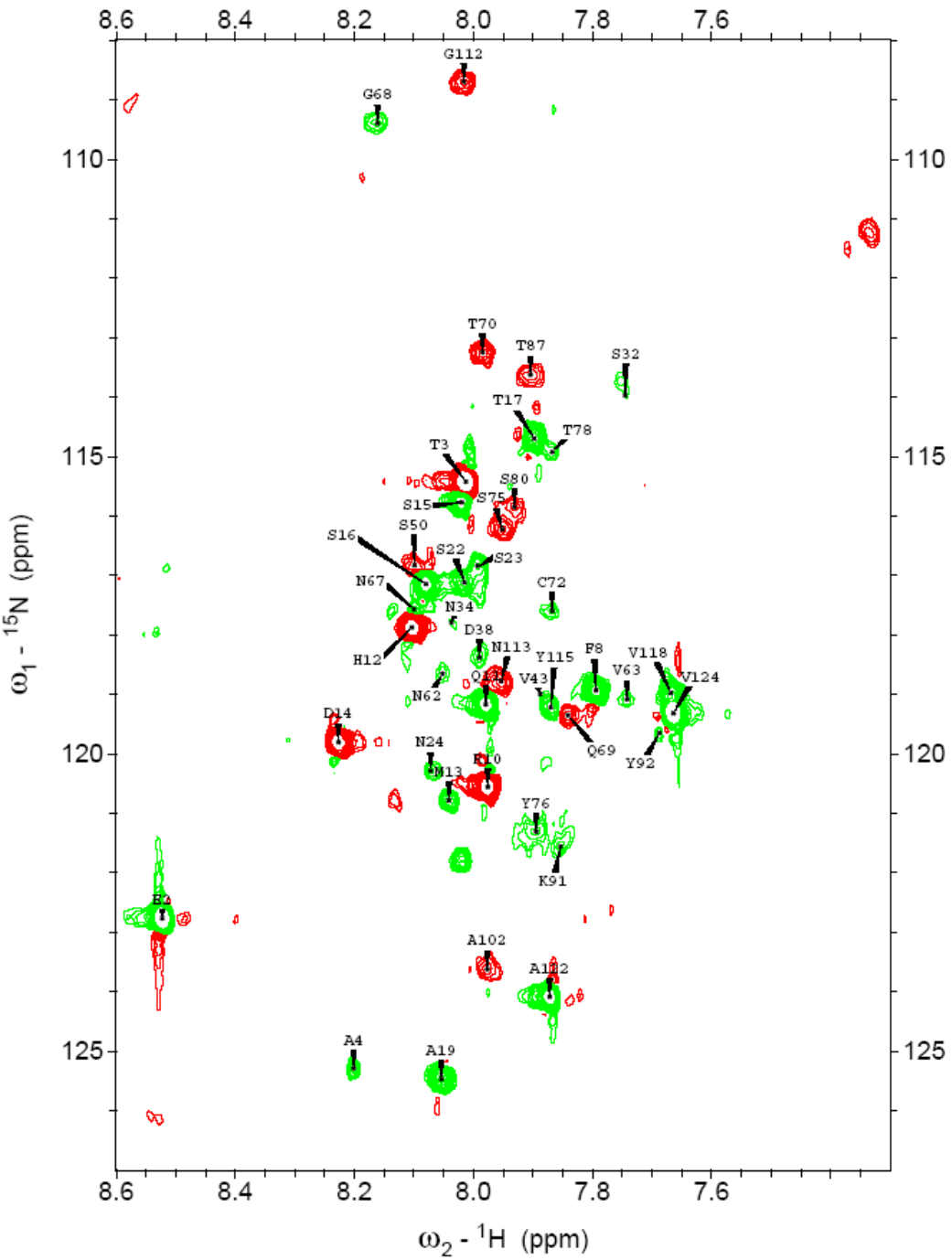
B. 2D NOESY spectra of RNase A in 40% acetic acid (red) and urea 8 M (gold). Peaks corresponding to δ protons of *trans* prolines and α protons of the respective previous residues are shown.



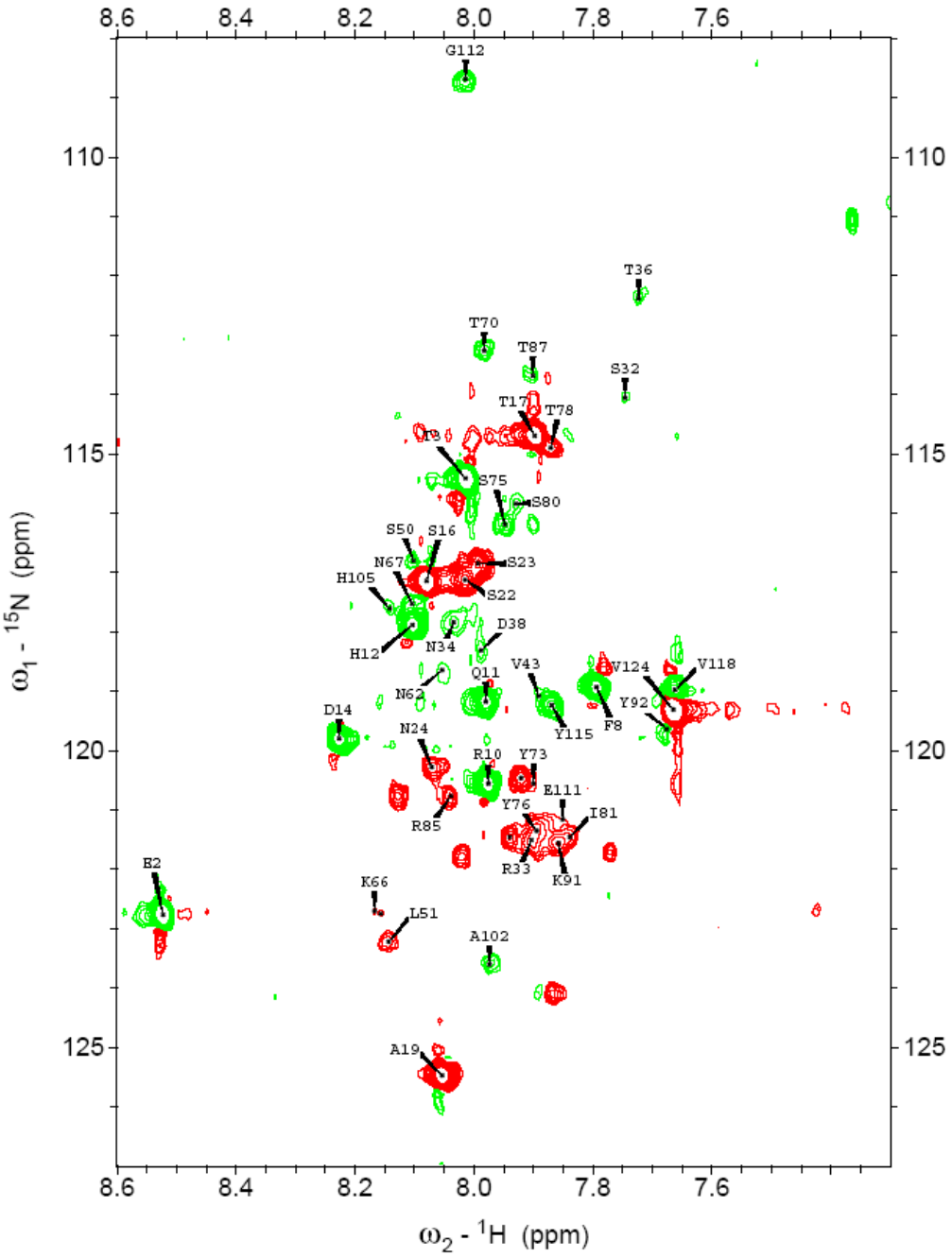
Supporting Figure 3.
Cβ- and Cγ-edited ^{1}H-^{15}N HSQC spectra of ^{13}C,^{15}N labeled RNase A in 40% HAc. In this sub-spectrum, the residues that appear are those that follow aromatic residues (Y73, Y76, Y97, Y115, H12, H48, H105, H119, F8, F46 & F120).



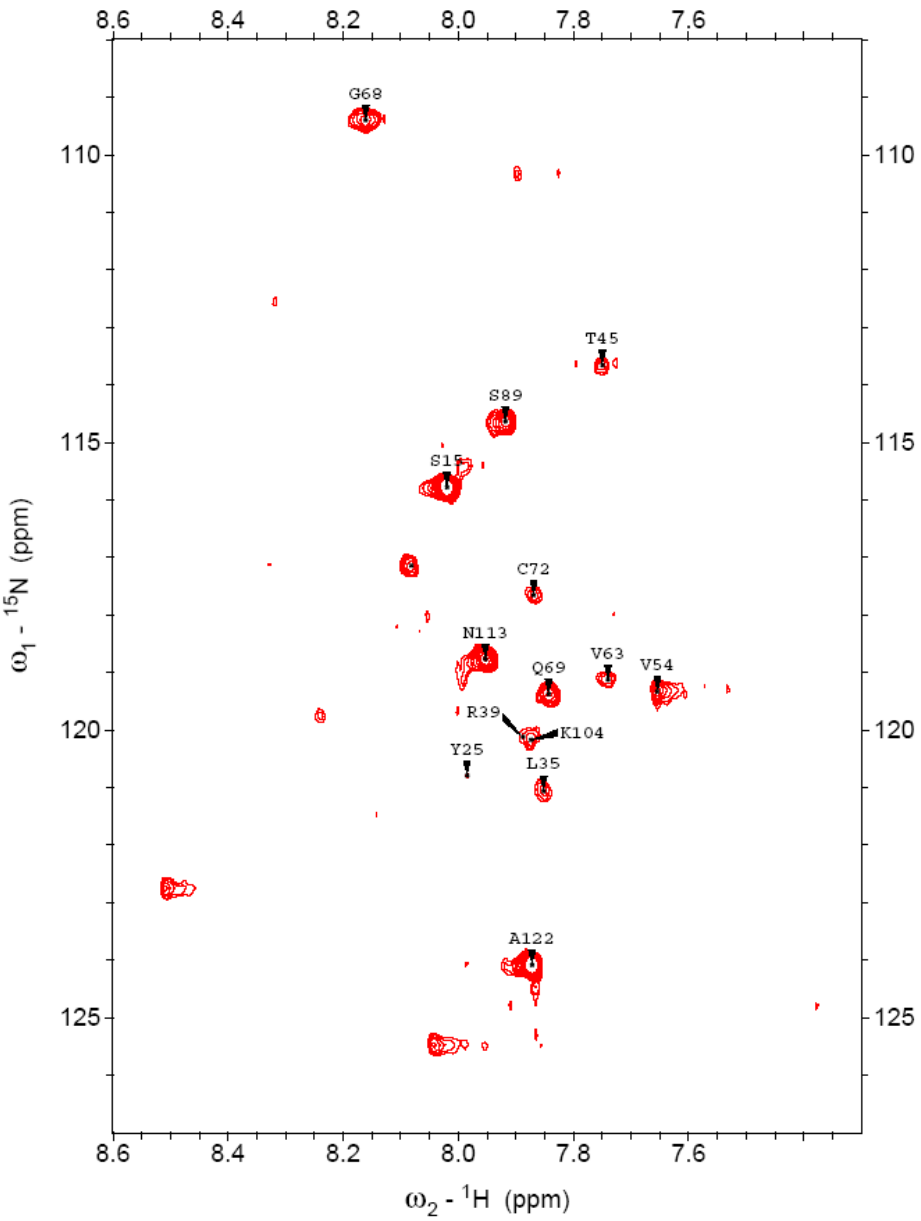
Supporting Figure 3, continued.
Cβ- and Cγ-edited ^{1}H-^{15}N HSQC spectra of ^{13}C,^{15}N labeled RNase A in 40% HAc. In this sub-spectrum, the residues that follow M, E & Q appear as negative peaks shown in red, while residues following P, K, S, T, D & N appear as positive peaks shown in green.



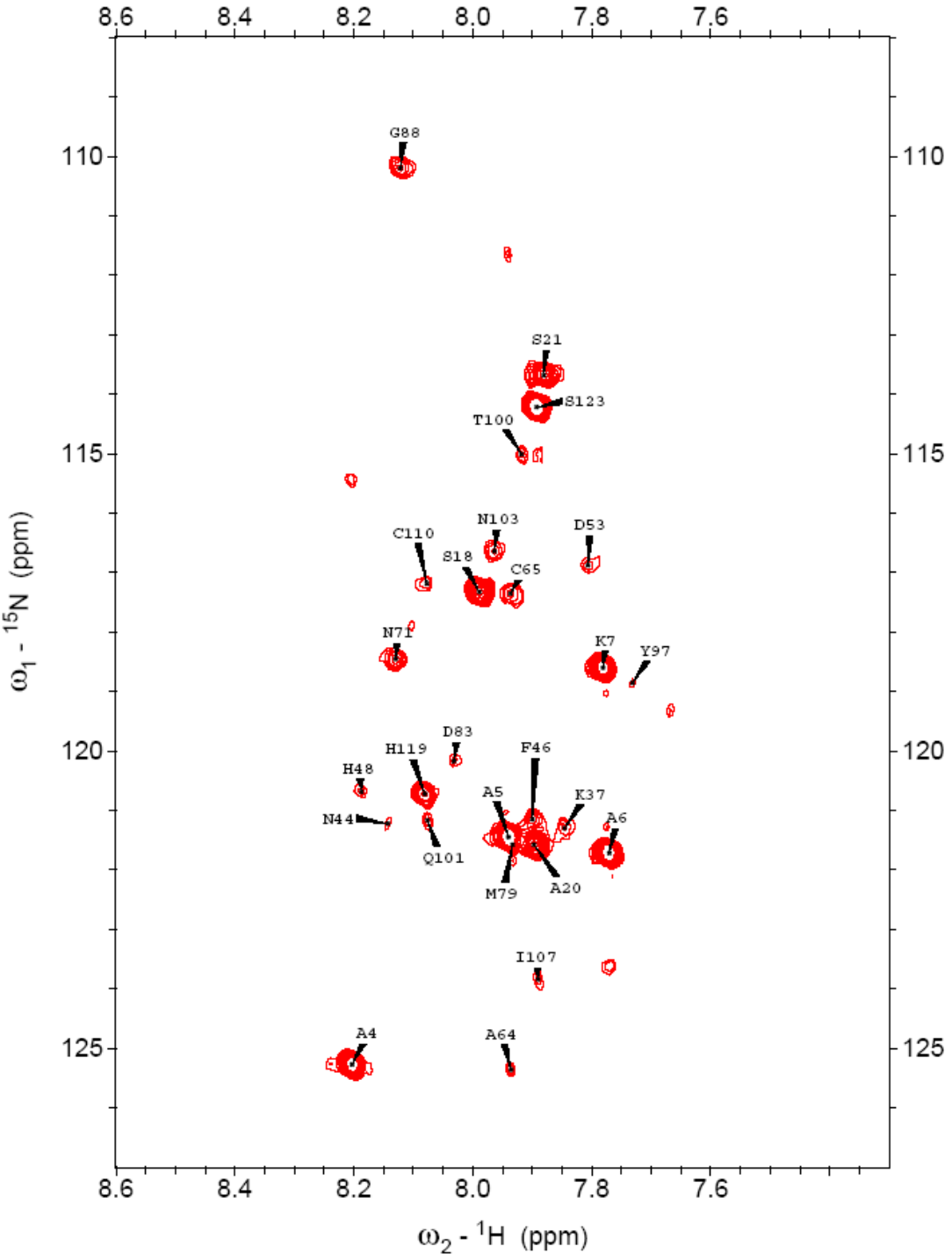
Supporting Figure 3, continued.
Cβ- and Cγ-edited ^{1}H-^{15}N HSQC spectra of ^{13}C,^{15}N labeled RNase A in 40% HAc. In this sub-spectrum, the residues following S and C appear as negative peaks (red), while residues following E, Q, L, K, M, P & R appear as positive peaks (green).



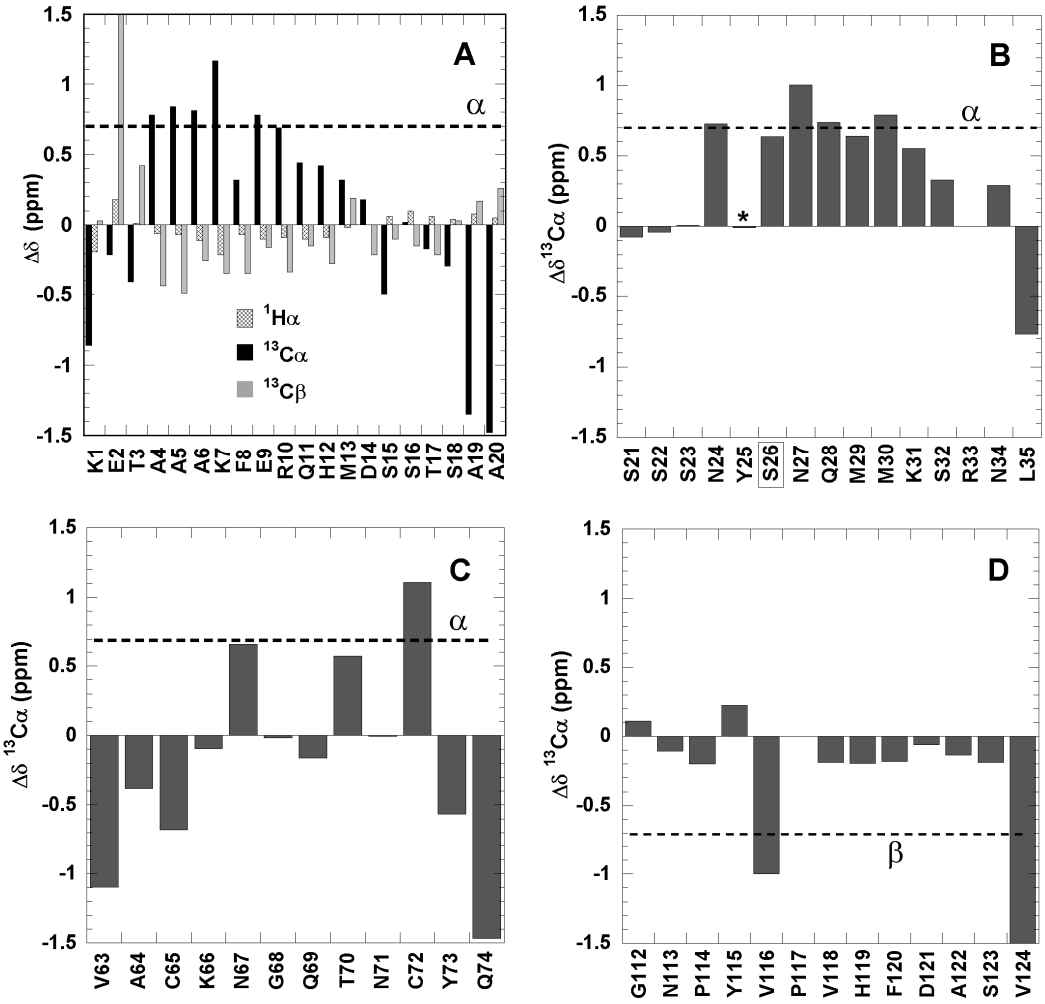
Supporting Figure 3, continued.
Cβ- and Cγ-edited ^{1}H-^{15}N HSQC spectra of ^{13}C,^{15}N labeled RNase A in 40% HAc. In this sub-spectra, residues following D, N and G appear as negative peaks (red).



Supporting Figure 3, continued.
Cβ- and Cγ-edited ^{1}H-^{15}N HSQC spectra of ^{13}C,^{15}N labeled RNase A in 40% HAc. In this sub-spectra, residues following T and A appear as negative peaks (red).



Supporting Figure 4. Secondary Structural Tendencies in RNase A peptides in 40% HAc



Detection of secondary structure in RNase A peptides by conformational chemical shift analysis. The dashed lines mark the threshold for detecting α (**panels A, B & C**) or β (**panel D**) structure. The "*" in the center, left graph marks the position of Y25, which could not be assigned. The label of S26 in this same peptide is boxed to emphasize that in the peptide Ser substitutes for Cys 26 in the native protein.

Supporting Figure 4. Continued. Secondary Structural Tendencies in RNase A peptides in 40% HAc

Experimental Procedures: S-peptide (RNase A residues 1 to 20) was obtained from Sigma (type XII-PE). Peptides corresponding to RNase A residues 21 to 35 (SSSNY**S**NQMMKSRNL), 63 to 74 (VACKNGQTNCY) and 112 to 124 (GNPYVPVHFDASV) were obtained from Caslo Labs (Lyngby, Denmark). Note that Ser 26 in the peptide (shown above in bold) is a Cys in the protein. The peptides were dissolved in water or 40% HAc and assigned using a battery of 2D NOESY, TOCSY and ^{13}C-HSQC spectra.

Results and Discussion: The helix population in the first peptide was about 23% for residues 3–9 and 11 % for residues 10–14. Residues 24-31 (RNase A numbering) in peptide II has helix content of about 23 %. Thus, the helix populations in these regions are somewhat lower in the isolated peptides than in the same segments in RNase A. Regarding residues 17–22, which have a small but significant helix population in RNase A in 40% HAc; these residues are divided between the two peptides and have no significant helix population. This suggests that the helical population of this segment in RNase A in 40% HAc is stabilized by presence of the adjacent helical segment or perhaps by long range interactions, such as the disulfide bond formed by Cys 26, which was substituted by Ser in the peptide. No significant populations of secondary structure or tendencies to adopt secondary structure were detected in the other two peptide segments.

The structural characterization of RNase A peptides reveals that the regions corresponding to helix I and II in native RNase have moderately lower helix populations in the context of two isolated peptides in 40% HAc versus the full length protein in 40% HAc. This constitutes evidence suggesting that the helical population of these parts in RNase A in 40% acetic acid is enhanced by neighboring helical segments or long range interactions, such as the Cys 26 – Cys 84 disulfide bond, which are absent in the peptides. Previous NMR and CD studies demonstrated that peptides corresponding to the first and second helices of RNase A adopt significant helical populations.[1-4] It is notable that the amount of

Supporting Figure 4. Continued. Secondary Structural Tendencies in RNase A peptides in 40% HAc

helix detected here in 40% HAc is similar to or higher than the amount detected in aqueous solution at benign pH, despite the fact that the helix stabilizing charge macrodipole[5] and the Glu 2 - ··· + Arg 10 salt bridge[6] will be absent at pH 2.0 in 40% HAc. This suggests that acetic acid, like ethanol or trifluoroethanol, might act to weakly stabilize helical structure, possibly by being less efficient compared to water molecules at forming hydrogen bonds to peptide groups. Such a helix stabilizing effect of acetic acid, together with the high helix N-capping propensities of Thr 17 and Ser 18[7] and the very favorable intrinsic helix propensities of Ala 19 and Ala 20[8] could account for the minor helix population of the Thr 17 – Ser 22 segment in RNase A in 40% acetic acid. In a previous study, the third peptide was found to be mostly unfolded in aqueous solution at pH 3.3 with only small populations of two turns formed by residues 66 – 69 and 69 – 72.[9] This minor amount of structure, detected by NOE analysis, decreased significantly when the Cys 65 – Cys 72 disulfide bond was reduced. Previous NMR studies of the fourth peptide, in water at neutral pH, showed that it is essentially extended and lacks preferred conformations save Phe 120 which may be partially structured as it shows large chemical shift changes upon adding urea or acid.[10] Both the Asn 113-Pro 114 and the Val 116-Pro 117 peptide bonds were reported to be predominately in the *trans* conformation in this peptide.

References:

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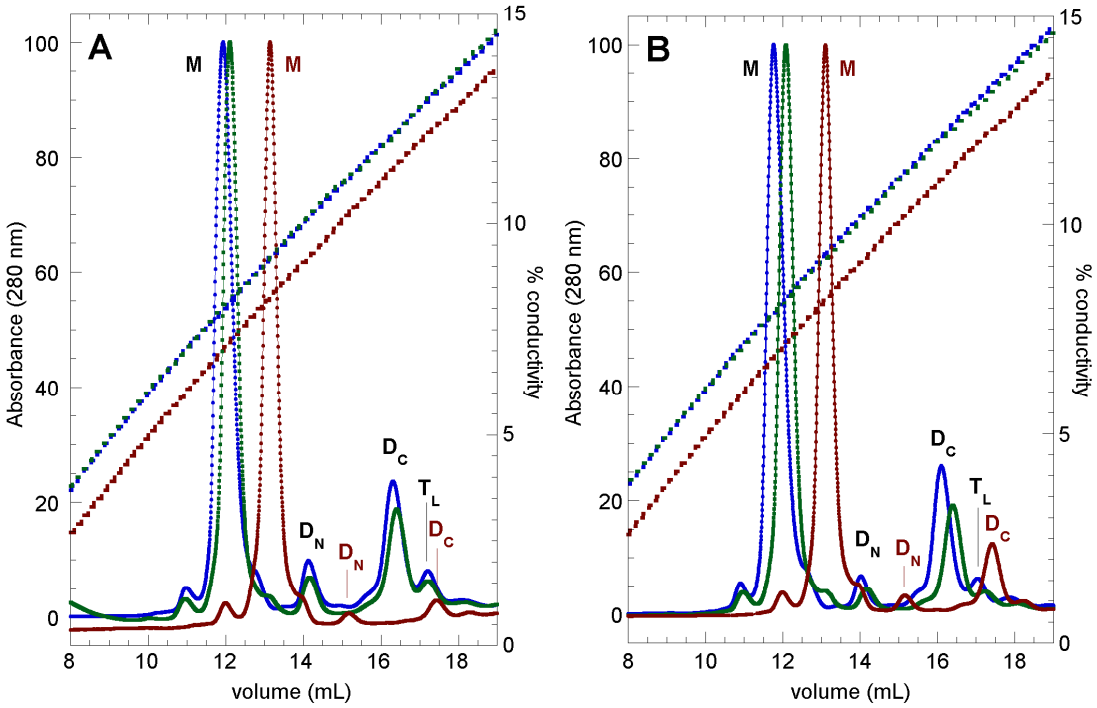
Supporting Figure 5. Representative Chromatograms of RNase A Oligomers Formed at Different Protein Concentrations During Refolding



All samples were diluted 1:10 with milliQ water just prior to injection on the FPLC column to lower the sodium phosphate concentration from 0.20 M to 0.02 M to premit the protein to bind to the column. The low concentration curve and dashed line are offset along the x-axis; this results because a larger volume of this sample was applied to the column. The oligomeric species are labeled: M, monomer; D_N, N-dimer, D_C, C-dimer; T_L, linear trimer. The dashed lines show the conductivity (right y-axis), which is proportional to the concentration of sodium phosphate used to elute the proteins. The color code for these lines matches that described below for the absorbance curves. The absorbance value of the monomeric species has been normalized to 100 to facilitate the comparison of the oligomer yields.

A. Oligomer yields after denaturation in 10 M urea followed by exchange to 0.2 M sodium phosphate buffer and refolding at high (29 mg/mL, blue curve), intermediate (4.3 mg/mL green curve) and low (0.4 mg/mL, red curve) concentrations of RNase A.

B. Oligomer yields after denaturation in 6 M GdmCl followed by exchange to 0.2 M sodium phosphate buffer and refolding at high (38 mg/mL, blue curve), intermediate (5.2 mg/mL green curve) and low (1.4 mg/mL, red curve) concentrations of RNase A.

Supporting Figure 6. Helical structure at residues 18-23 in the N-terminal swapped dimers of RNase A and human pancreatic RNase.

Top panel. Cross-eyed stereo view of the 3D domain swapped minor dimer (N-dimer, PDB **1A2W**) of RNase A. One subunit has a bent helix spanning residues 18-32 with a bend at Tyr 25. In the other subunit, residues

Supporting Figure 6. Continued. Helical structure at residues 18-23 in the N-terminal swapped dimers of RNase A and human pancreatic RNase.

18-24 are not helical. Residues 18-24 are colored gold and residues 25-32 are colored pink. The first 17 residues, which are swapped, is colored red. All other residues are colored green, except for residues 111-124, shown in blue, at the C-terminus which are swapped in the C-dimer but are not swapped in the N-dimer.

Bottom panel. Cross-eyed stereo view of the 3D domain swapped dimer of the PM8 variant of human pancreatic RNase (PDB **1H8X**). The swapped N-terminus, residues 1-17, is shown in red; residues 18–23 which adopt 3/10 α-helix, residues 24 – 34, is colored pink; and the remainder of the residues are colored green.

Supporting Table 1. RNase A Oligomer Yield

conc RNase A (mg/ml) upon redissolving after lyo. from 40% HAc	conc. RNase A upon elution after incubation in 10 M urea	conc. RNase A upon elution after incubation in 6 M GdmCl	% Monomer	% N-dimer	% C-dimer	% Linear Trimer	% cyclic trimer + higher oligomers
200[a]			63.9	3.70	25.4	4.4	2.8
103			65.4	4.5	23.4	4.1	2.7
19.80			70.5	4.05	18.9	3.6	3.2
2.46			76.3	3.85	15.6	2.9	1.4
0.50			83.1	2.75	11.9	1.8	0.55
Fit[b] b			*80.2*	*3.29*	*13.3*	*2.27*	*1.11*
m			*-7.69*	*0.41*	*5.04*	*0.93*	*0.88*
R			*0.97*	*0.71*	*0.99*	*0.94*	*0.88*
	28.55[c]		72.6	4.46	16.9	3.9	1.93
	9.29[c]		76.3	4.37	15.4	3.0	0.65
	4.3		77.3	3.80	15.0	3.1	0.81
	0.4		96.2	1.28	2.49	0	0
	Fit[b] b		*89.27*	*2.26*	*7.08*	*1.10*	*0.24*
	m		*-13.1*	*1.83*	*8.07*	*2.11*	*0.92*
	R		*0.97*	*0.96*	*0.95*	*0.97*	*0.90*
		37.85[c]	74.4	2.62	18.6	3.0	1.4
		19.25[c]	75.8	2.40	18.0	2.7	1.14
		5.2	79.5	2.0	16.1	1.9	0.5
		1.4	83.9	1.98	12.6	1.45	0.15
		Fit[b] b	*84.6*	*1.82*	*12.4*	*1.22*	*-0.04*
		m	*-6.68*	*0.46*	*4.18*	*1.12*	*0.90*
		R	*0.997*	*0.94*	*0.98*	*0.99*	*0.99*

[a] the data shown are the average values from two independent chromatograms except where noted.

[b] the data shown are the average values form three independent chromatograms.

[c] Parameters from the fit of the logarithmic equation: % species = m*log(RNase A conc.) + b to the data, with R being the correlation coefficient. To aid distinguishing these values, they are shown in *italics*.