Increase of RNase A *N*-terminus Polarity or *C*-terminus Apolarity Changes the Two Domains' Propensity To Swap and Form the Two Dimeric Conformers of the Protein†

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ABSTRACT: Do the polarities of the *N*-terminus or the apolarity of the *C*-terminus of bovine RNase A influence the relative yields of its two 3D domain-swapped dimeric conformers, the N-dimer and C-dimer? We have addressed this question by substituting Ala-4 or Ala-5 with serine (A4S and A5S mutants) or Ser-123 with alanine (S123A mutant) through site-directed mutagenesis. Both the polarity of the *N*-terminus and the apolarity of the *C*-terminus of RNase A were, therefore, increased. CD spectra revealed no significant differences between the secondary structures of the mutants and native RNase A. According to thermal denaturation analyses, the A4S and A5S mutants are less stable, and the S123A mutant is more stable than wild type RNase A. By subjecting the mutants under mild or drastic denaturing conditions, side-by-side with native and recombinant RNase A, to a thermally induced oligomerization procedure, the following results were obtained. (i) The *N*-terminal mutants showed a higher propensity, with respect to the native protein, to form N-dimers under mild unfolding conditions. (ii) The *C*-terminal mutant showed a higher propensity to form the C-dimer under severely unfolding conditions. These results are discussed in light of the relative stabilities of the various RNase A species under different environmental conditions, and we conclude that the hydrophilic or hydrophobic character of the RNase *N*-terminus or *C*-terminus can be an important variable governing the oligomerization of RNase A and possibly other proteins through the 3D domain-swapping mechanism.

Bovine pancreatic ribonuclease A (RNase A[1], EC 3.1.27.5) lyophilized from 40% acetic acid solutions according to the classic method of Crestfield et al. (*1*) or subjected to a thermally induced oligomerization procedure lacking the lyophilization step (*2*), forms 3D domain-swapped dimers, trimers, tetramers, and larger oligomers (*3*−*11*). Each species consists of two or more linear or cyclic conformers, forming in different quantities and showing variations in the exposure of charged residues (*3*, *4*, *10*, *11*). The structures of an *N*-terminal swapped dimer (N-dimer) (*5*), a *C*-terminal swapped dimer (C-dimer) (*6*), and a cyclic *C*-terminal swapped trimer (*7*) have been solved. For other oligomers, plausible models have been proposed (*4*, *8*−*11*). Under controlled experimental conditions, the oligomerization method of Crestfield et al. (*1*) yields the two dimeric conformers, N-dimer and C-dimer, in reproducible relative proportions of about 1:3.5−1:4. Constant ratios also characterize the yield of the larger RNase A oligomeric conformers (*8*). The oligomerization of RNase A induced by temperature, that is, by the exposure of the protein dissolved at high concentration in various solvents to temperatures varying from 23 to 70 °C (*2*), gives rise to the same 3D domain-swapped types of oligomers but in amounts and relative proportions different from those obtained by the lyophilization procedure of Crestfield et al. (*1*). Under rather mild unfolding conditions, such as heating RNase A solutions in ddH_2O, NaCl, or buffers at slightly alkaline pH to 60 °C, the relative proportions of the two dimers invert, with the N-dimer definitely prevailing over the C-dimer. However, under vigorous unfolding conditions, such as heating RNase A solutions in 40% aqueous ethanol (EtOH) or 40% aqueous 2,2,2-trifluoroethanol (TFE) (*2*) to 70 °C, the C-dimer's yield definitely prevails over that of the N-dimer. These results can be tentatively interpreted as depending, at least partly, on the different amino acid compositions of the *N*- and *C*-termini of RNase A in correlation with light or severe unfolding conditions used in the thermally induced oligomerization procedure. Indeed, the swapping portion of the *N*-terminus (residues 1−15) is prevalently hydrophilic,

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[1] Abbreviations: RNase A, ribonuclease A; N-dimer and C-dimer, *N*-terminal and *C*-terminal swapped dimers, respectively; NC-trimer and C-trimer, *NC*-terminal (linear) or *C*-terminal (cyclic) swapped trimers, respectively; N_D, N-dimer; C_D, C-dimer; NC_T, NC-trimer; C_T, C-trimer; TT, tetramer(s); ddH_2O, twice-distilled water, EtOH, ethanol; TFE, 2,2,2-trifluoroethanol; GdnHCl, guanidine hydrochloride; CD, circular dichroism.

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containing 10 out of 15 hydrophilic amino acids, that is, 67% polar residues, whereas the swapping part of the *C*-terminus (residues 116−124) is prevalently hydrophobic, with only 3 out of 9 residues, that is, 33%, that are polar amino acids. Recent experimental results indicated that sequence segments that are relatively rich in hydrophobic residues tend to induce protein aggregation and the formation of β-sheet structures (*12*). Again, the search for structure in globular proteins appears to be dominated by hydrophobic interactions (*13*), and it is known that a central hydrophobic region of α-synuclein tends to self-associate contributing to the aggregation process (*14*, *15*). Furthermore, the oligomerization of four-stranded β-sheet peptides in a tetrameric complex is a function of intermolecular hydrophobic interactions and intramolecular hydrogen bonding (*16*, *17*). Interestingly, recent results by R. Varadarajan and co-workers suggest that the stability of some ribonuclease S mutants mainly depends on packing interactions rather than on the hydrophobic driving force (*18*). It is also known that the *C*-terminal β-strand of native RNase A forms several hydrogen bonds with the protein core that are strongly protected, whereas most of the hydrogen bonds forming between the *N*-terminal α-helix (residues 3−13) and the RNase A core are weaker (*19*). Moreover, hydrogen/deuterium exchange studies show that the backbone NH groups in the *N*-terminal α-helix of RNase A unfold in the burst phase and exchange through a local unfolding event, whereas the NH groups in the *C*-terminus are remarkably resistant to severe unfolding conditions and exchange only during the global unfolding transition (*20*).

On the basis of these facts, a hypothesis that explains the results we obtained by studying the oligomerization of RNase A through 3D domain-swapping under various experimental conditions is that the event might be correlated to or depend on the mainly hydrophilic nature of the *N*-terminus on one side and the prevalently hydrophobic nature of the *C*-terminus of the protein on the other. It appears, indeed, that the RNase A *N*-terminus can unfold under relatively mild environmental conditions and swap onto the core of a second RNase A monomer, forming the resulting N-dimer, whereas, the RNase A *C*-terminus would require rather drastic environmental conditions to unfold and swap on a second RNase A monomer with the eventual formation of the resulting C-dimer. In other words, the unfolding of the RNase A *N*-terminus would require a lower energy contribution, although the unfolding of the *C*-terminus should be a more laborious process requiring a larger energy contribution. To test the hypothesis, and in the attempt to better understand the experimental conditions governing 3D domain-swapping in RNase A, we prepared three RNase A mutants by site-directed mutagenesis, with serine substituting Ala-4 or Ala-5 in two of them and an alanine substituting Ser-123 in the third. In other words, the polar character of the *N*-terminus on one side and the apolar character of the *C*-terminus on the other were enhanced. We chose these mutations in order to reduce as much as possible any perturbation of the structure of the *N*-terminus and *C*-terminus of the protein by altering charged, aromatic, catalytic, or subsite residues. Furthermore, these residues are distant from the hinge loops, and their local structure is essentially equivalent in the monomer, N-dimer, and C-dimer. The mutants were then subjected, side-by-side with native and recombinant RNase A, to some of the experimental conditions previously described within the thermally induced oligomerization procedure (*2*). The results obtained appear to support the validity of the hypothesis.

The biological significance of our study, even if performed with a model protein like ribonuclease A, is related to the increasing importance that the 3D domain-swapping mechanism has in protein aggregation. Eisenberg and co-workers, in a recent review (*21*), point out the implication of 3D domain swapping in the formation of various protein aggregates, particularly in the context of amyloid formation, prion proteins and large aggregates, and discuss the possible involvement of the mechanism in the so-called deposition diseases.

MATERIAL AND METHODS

Materials. Ribonuclease A from bovine pancreas (Type XII-A) was purchased from Sigma Chemical Co. All chemicals were of the highest possible purity. Superdex 75 HR10/30, Source 15S HR10/10, and Source HR 16/10 columns were from Amersham Biosciences. Chromatographies were performed with an Amersham Biosciences fast protein liquid chromatography system.

Preparation of RNase A Oligomers. Recombinant RNase A as well as its mutants were purified by a preliminary gel filtration step followed by ion-exchange chromatography. The main, monomeric peak of the recombinant or mutagenized protein was dialyzed against 2× distilled water (ddH_2O) and lyophilized. The enzymatic activity of the lyophilized material was ascertained by the method of Kunitz (*22*) to be identical with that of native RNase A. This material was used to prepare the oligomers. For the classic Crestfield et al. aggregation procedure (*1*), each RNase A sample was processed as described elsewhere (*3*, *4*). As for the alternative oligomerization procedure, some of the various described conditions (*2*) we have chosen are as follows. The RNase A sample was dissolved at a concentration of 200 mg/mL in (i) ddH_2O, (ii) aqueous 20% or 40% ethanol, and (iii) 40% 2,2,2-trifluoroethanol (TFE) and then heated at the proper temperature for the chosen time. At the end of the treatment, a 100-fold excess of preheated 0.1 M sodium phosphate at pH 6.7 was added and the mixture brought to 0 °C or frozen until use. In some experiments with TFE, guanidine hydrochloride (GdnHCl) was added at a final concentration of 0.2, 0.5, or 1 M. In these cases, the final dilution was performed with a 150-fold excess of 0.1 M sodium phosphate in order to properly reduce the GdnHCl concentration.

Gel Filtration. A Superdex 75 HL 26/60 (prep. grade) column, equilibrated with 0.1 M Tris-Acetate buffer at pH 8.4, containing 0.3 M NaCl was used at room temperature at a flow rate of 0.6−0.8 mL/min to purify the mixtures of the various recombinant species.

Cation-exchange Chromatography. Further purification of the recombinant species was performed using a Source 15S HR 16/10 column. Analyses of the yields of the oligomerization of the various species obtained by the thermal treatment were carried out as described previously (*3*, *4*, *10*) with a Source 15S HR10/10 column, using a 0.085−0.2 M sodium phosphate (pH 6.7) gradient at room temperature; flow rate: between 0.08 and 0.4 mL/min.

Quantification of the Oligomers Formed. Each oligomer produced was quantified by measuring the area of its peak

eluted in cation-exchange chromatography and calculating its percentage relative to the sum of the areas of all peaks eluted. The values shown in the Table and/or Figures are means of three to five determinations.

Gel Electrophoresis. Cathodic gel electrophoresis under nondenaturing conditions was performed according to Goldenberg (*23*) with slight modifications, using a β-alanine/acetic acid buffer at pH 4.0. Then 12.5% polyacrylamide gels were run at 20 mA for 100−120 min at 4 °C. Fixing and staining were performed with 12.5% trichloroacetic acid and 0.1% Coomassie brilliant blue. To analyze the yield of the various recombinant species after expression, SDS−PAGE according to Laemmli (*24*) was performed using a 10% polyacrylamide gel.

Construction of Mutants. The wild-type gene for RNase A, cloned into the expression vector pT7-7 between the *NdeI* and *HindIII* restriction enzyme sites, was kindly provided by Professor A. Di Donato (the University of Naples). Three mutants of the RNase A gene (A4S, A5S, and S123A) were obtained using the GeneEditor In Vitro Site-Directed Mutagenesis System (Promega). The mutagenesis reaction was carried out by alkaline denaturation of dsDNA containing the wild-type gene. Denaturated DNA (0.05 pmol) was added to a solution containing 0.25 pmol of selection oligonucleotide, which codes for ampicillin resistance, and 1.25 pmol of mutagenic primers. The sequences of the primers are as follows:

Mutant A4S 5′-GAAGGAAACT***T***CAGCAGCCAAG-3′
Mutant A5S 5′-GGAAACTGCA***T***CAGCCAAGTTTG-3′
Mutant S123A 5′-CTTTGATGCT***G***CAGTGTAGATCC-3′

After annealing at 75 °C for 5 min, the temperature was cooled to 37 °C, and 10× synthesis buffer (100 mM Tris-HCl at pH 7.5, containing 5 mM dNTPs, 10 mM ATP, and 20 mM DTT), T4 DNA polymerase (5−10 U), and T4 DNA ligase (1−3 U) were added. The template DNA obtained by synthesis and ligation reactions was transformed into BMH 71-18 *mut*S competent cells. Overnight cultures were used for plasmid DNA miniprep purification using the Wizard Plus SV Minipreps DNA Purification System (Promega).

A second transformation into *E. coli* JM 109 was performed to obtain a high copy number of mutants. Ten colonies of each mutant were then isolated and sequenced to confirm the presence of the desired mutation and the lack of second-site mutations.

Production and Purification of RNase A from E. coli. The production of RNase A was maximal when cultures were started from freshly transformed cells. A culture of *E. coli* strain BL 21-Gold(DE) (100 mL, $A_{600} = 0.5$ O. D.) harboring the plasmid pT7-7 containing the RNase A gene (wild-type or mutants) in a TB medium with ampicillin (50 μg/mL) was used to inoculate a larger culture (3 L divided in 6 flasks, 500 mL each) of the same medium containing ampicillin (50 μg/mL). The inoculated culture was shaken (225 rpm) at 37 °C until the early log phase ($A_{600} = 0.6$ O. D.) was reached. It was then induced to express cDNA coding for RNase A by the addition of IPTG (0.5 mM, final concentration). Shaking at 37 °C was continued in 0.1 M Tris-acetate buffer at pH 8.4 (1000 OD units/ml). Then, 50 mM phenylmethylsulfonyl fluoride (as inhibitor of serine proteases and cysteine proteases) was added (8 μL/gram of *E. coli*). Cell lysis was performed by treatment with lysozyme (0.8 mg/g of *E. coli*) and by sonication on ice (10 s, four times). After leaving the suspension at room temperature for 20 min, 4 mg of deoxycholic acid per gram of *E. coli* were added. The mixture was placed at 37 °C and stirred from time to time with a glass rod. To the very viscous lysate, 5 mM $MgCl_2$ (final concentration) and 20 μg of DNase I per gram of *E. coli* were added, and the mixture was maintained at room temperature for 30 min. After centrifugation for 30 min at 12 000 rpm and 4 °C, the pellet was washed from the Luria broth medium with 0.1 M Tris-acetate buffer at pH 8.4 (1 mL/1000 O. D. units), 2% Triton X-100, 2 M Urea, and ddH_2O. The inclusion bodies were isolated by centrifugation, re-suspended in 0.1 M Tris-acetate at pH 8.4 (containing 6 M guanidine hydrochloride, 100 mM dithiothreitol, and 10 mM EDTA) under nitrogen atmosphere. The protein solution was then dialyzed against 20 mM acetic acid. The precipitates formed during dialysis were removed by centrifugation. Renaturation of the protein was performed at room temperature, for 18 h, in 0.1 M Tris-acetate buffer at pH 8.4, containing 0.5 M L-arginine, 3 mM reduced glutathione, and 0.6 mM oxidized glutathione. The mixture was concentrated with CentriPlus 3 Millipore ultrafilters and purified with a Superdex 75 HL 26/60 (prep. grade) size exclusion column equilibrated with 0.1 M Tris-acetate buffer at pH 8.4 containing 0.3 M NaCl. The peak corresponding to RNase A was identfied by comparing its elution volume with that of native RNase A (Sigma Chem. Co., type XII A). This RNase A preparation was further purified with a second chromatographic step performed as described (*3*) using a cation-exchange Source 15S HR 16/10 column.

Circular Dichroism Spectroscopy. Far UV−CD spectra were recorded in a 0.1 cm cuvette at 25.0 °C utilizing a Jasco J-810 spectrometer equipped with a Peltier temperature control unit. The samples contained 20 μM protein and 30 mM sodium phosphate buffer at pH 6.7. Four accumulations were recorded using a bandwidth of 2 nm and a scan speed of 20 nm/min and averaged to give the final spectrum. Blank spectra acquired on buffer showed negligible CD.

Enzyme Assays. Enzymatic assays were performed with yeast RNA as the substrate according to the method described by Kunitz (*22*). The activity of the various recombinant species against yeast RNA was compared to that displayed by native RNase A used as a standard.

Analysis of RNase A Thermal Unfolding. The thermal unfolding of RNase A was followed spectrophotometrically at 287 nm (*25*, *26*) using a Beckman DU-650 spectrophotometer equipped with a thermostatically controlled water bath. The wild-type RNase A and the three mutants were dissolved in ddH_2O at a concentration of 1.0 mg/mL (*2*). At each temperature, the absorbance values of the RNase A solution were determined every 2 min, until absorbance measurements became constant. The total time was 40−50 min. Additional thermal unfolding experiments of RNase A and its variants were monitored by far UV CD at 218 or 222 nm using the JASCO J-810 instrument equipped with a programmable Peltier device for temperature control. The samples (20 μM) were heated from 20 to 85 °C at a rate of 60 °C/h, and the signal was recorded at every $^1/_2$ °C. The bandwidth was 2 nm. In one set of experiments, the samples contained 20 mM MOPS buffer; this buffer was chosen because it does not bind to or stabilize RNase A and because the pK_a of MOPS has a very small temperature dependence. In a second set of experiments, no buffer was included, and

Table 1: Ribonuclease Activity on Yeast RNA Expressed as Kunitz Units[a]

RNase A	recombinant RNase A	A4S	A5S	S123A
123 ± 11	121 ± 11	128 ± 15	127 ± 10	129 ± 12

[a] Kunitz units: ΔA per min/($A_F - A_0$) per mg enzyme per mL, where A_0 is the value of the absorbance at zero time, and A_F is the absorbance measured after 24 h.

the heating rate was increased to 80 °C/h so as to match the conditions used for the thermal unfolding experiments monitored by UV absorbance. The reversibility of unfolding was judged by measuring the ellipticity value obtained after cooling the sample back to 20 °C following thermal denaturation. On this basis, the reversibility was found to be very good; between 92% and 96% of the original signal was recovered. A six-parameter equation including terms for sloping baselines, the entropy change (ΔS), and the thermal unfolding midpoint (T_M) was fit to the thermal denaturation data using a least squares algorithm (*27*). The differences in T_M between the wild type and mutant proteins were used to calculate the free energy difference ($\Delta(\Delta G)$) of their conformational stability using the approximation $\Delta(\Delta G) \approx \Delta S * \Delta(T_M)$ given by Becktel and Schellman (*28*).

Kinetics of the Dissociation of Dimers. The dissociation kinetics of the N-dimer and C-dimer of RNase A or its mutants were measured by using 0.8 mg/mL solutions of the two dimers in 0.03 M sodium phosphate buffer at pH 6.7, maintained at 40 °C in a thermostatically controlled water bath. At the appropriate times, aliquots of 35−40 μg of each sample were diluted by adding 0.5 mL of preheated 0.1 M sodium phosphate at pH 6.7 and kept at 4 °C until use. Before use, the samples were concentrated with Centricon 3 ultrafilters, and 7−8 μg of each sample were electrophoresed under nondenaturing conditions (*23*). The determination of the amount of each species was performed using the ImageQuant program (Amersham Biosciences). The intensities of the bands were measured using the intensity of the band of a weighted amount of native bovine RNase A as a reference, which was used in this work as a standard, and electrophoresed in the first lane of the gel.

RESULTS AND DISCUSSION

First of all, we tested and compared the chromatographic, electrophoretic, and enzymatic properties of recombinant and native RNase A. The cation-exchange chromatographic pattern showed that the recombinant RNase A and mutants species were about 0.5 mL retarded in comparison with that of native RNase A (Sigma, type XII-A) from bovine pancreas (data not shown), as if its positive charge density were higher. Electrophoretic analyses under nondenaturing conditions (*23*) did not show this difference (data not shown). Enzymatic assays with native and recombinant RNase A, performed according to Kunitz (*22*), always gave quite similar results (Table 1). It can be noticed that Kunitz units/mg values are slightly higher than usual standard values (100 K.U./mg) given by Sigma for type XII-A of RNase A. This has already been observed (*29*) and can be reasonably ascribed to the higher purity of our RNase A samples because of the cation-exchange chromatography step (*3*).

We used far UV−CD spectroscopy to check whether the mutations alter the overall secondary structure of RNase A.

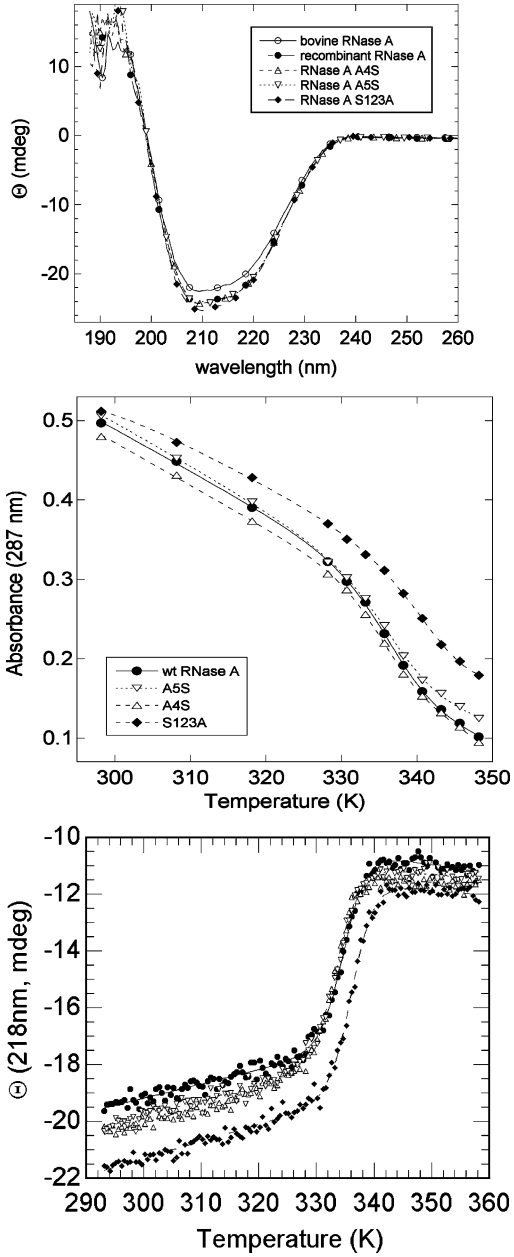

FIGURE 1: Comparison of some properties of native RNase A, wild-type recombinant RNase A, and the A4S, A5S, and S123A mutants. (A) Far UV−CD spectra of native, wild-type recombinant RNase A, and its mutants. All of the species were at 20 μM concentration, in 30 mM sodium phosphate buffer at pH 6.7. (B) Thermal unfolding of the various RNase A species dissolved in ddH_2O. Each RNase A was dissolved at a concentration of 1.5 mg/mL and progressively heated within a temperature range of 10−75 °C. At each temperature, the absorbance at 287 nm was recorded every 2 min for a total time of 40−60 min. When the absorbance remained constant for at least 10 min, its value was recorded. The data shown are representative of three experiments performed with each species. The curve is the fit of the two-state denaturation model to the data. Wild type, (—●—); A4S, (--△--); A5S, (..▽..); S123A, (--♦--). (C) Thermal denaturation of wild-type RNase A and variants dissolved in 20 mM MOPS at pH 7.0, monitored by CD as described in Materials and Methods. The curve is the fit of the two-state denaturation model to the data. Wild type, (—●—); A4S, (--△--); A5S, (..▽..); S123A, (--♦--).

The wild-type recombinant RNase A and the A4S and A5S mutants showed essentially identical CD spectra, and the S123A mutant showed a slightly enhanced minimum near 210 nm (Figure 1 A). This same minimum is somewhat less

Table 2: Thermodynamics of the Thermal Denaturation of RNase A and Its Variants

protein	ΔH kcal·mol^{-1}	ΔS cal·mol^{-1} K^{-1}	T_M K	T_M °C	ΔT_M °C	$\Delta\Delta G$ cal·mol^{-1}
In ddH_2O, monitored by UV absorbance at 287 nm						
WT	66 ± 6	195 ± 18	336.0 ± 0.7	62.8 ± 0.7		
A4S	77 ± 9	229 ± 28	334.9 ± 0.6	61.7 ± 0.6	−1.2 ± 1.0	−220 ± 190
A5S	62 ± 7	185 ± 20	336.4 ± 1.2	63.2 ± 1.2	+0.4 ± 1.4	+80 ± 270
S123A	60 ± 3	177 ± 8	341.9 ± 1.6	68.7 ± 1.6	+5.9 ± 1.7	+1140 ± 350
In 20 mM MOPS buffer at pH 7.0, monitored by circular dichroism at 218 nm[a]						
WT	112	333	334.86	61.70		
A4S	117	352	333.32	60.16	−1.54	−510
A5S	109	327	333.75	60.59	−1.11	−370
S123A	118	351	336.24	63.08	+1.38	+460
In ddH_2O, monitored by circular dichroism at 222 nm[a]						
WT trial 1	122	364	334.53	61.37		
WT trial 2	125	374	334.54	61.38		
A4S trial 1	109	326	332.85	59.69	−1.69	−620
A4S trial 2	116	348	332.77	59.61	−1.77	−650
A5S trial 1	113	339	333.12	59.96	−1.42	−520
A5S trial 2	106	317	332.98	59.82	−1.56	−580
S123A tr.1	112	335	335.80	62.64	+1.27	+ 470
S123A tr.2	112	333	335.67	62.51	+1.14	+ 420

[a] The experimental uncertainties for the experiments monitored by CD are ≈5 kcal/mol for ΔH, ≈14 cal·mol^{-1} K^{-1} for ΔS, 0.07 to 0.10°C for T_M, ≈0.14° C for ΔT_M, and 50 to 60 cal·mol^{-1} for $\Delta\Delta G$.

intense in the case of wild-type RNase A obtained from Sigma. Overall, the results strongly suggest that the mutations do not significantly alter the secondary structure of the protein.

Next, the conformational stabilities and secondary structures of the wild type and variants were determined and compared. Thermal unfolding analyses at 287 nm (*2*) of the three RNase A mutants A4S, A5S, and S123A and native RNase A, dissolved in ddH_2O, are shown in Figure 1B. When a two-state denaturation model with sloping pre and post-transition baselines was fit to these data, the T_M and ΔG values were found to be the same, within the margin of experimental uncertainties, for wild-type RNase A and the A4S and A5S variants, whereas the S123A variant showed a higher stability (Table 2). The decrease in absorbance displayed by the various species is ascribable to the increasing exposure of the tyrosine residues (*25*), mainly Tyr-25, -92, and -97 (*26*), as a function of increasing temperature. Tyr-115, located in the hinge loop (residues 112−115 (*6*)) preceding the *C*-terminus of RNase A, could also contribute to the decrease of absorbance at 287 nm, as previously reported (*2*).

Thermal unfolding transitions of RNase A and its variants were also monitored by far UV CD. Representative curves and the results of the analyses using the two-state model for all of the experiments are shown in Figure 1C and Table 2. In agreement with the results obtained by UV absorbance monitored unfolding, S123A is the only mutant that is significantly more stable than the wild-type protein. By far UV CD, however, the A4S and A5S mutants were found to be less stable than the wild-type protein. Because the precision of the CD-monitored experiments was higher (Table 2), we conclude that the A4S and A5S variants are indeed destabilized relative to wild-type RNase A. These results provide further support for the idea that the *N*-terminal α-helix is more susceptible to local unfolding, and therefore, the *N*-terminal swapping in the A4S and A5S mutants will occur more readily than in the wild type and S123A mutant. The higher stability found here for the S123A mutant is a further indication that the *C*-terminal *β*-strand is stabilized in this mutant, which suggests that higher yields of the C-dimer will be formed by this mutant under severe denaturation conditions.

Experimental Conditions Chosen to Induce RNase A Dimerization. To clarify the strategy used in this work, it may be worth recalling that in a previous investigation (*2*) the yield of the N-dimer under mild unfolding conditions (RNase A solutions in ddH_2O, sodium chloride, and buffers at slightly alkaline pH, etc.; 60 °C) was found to be higher than that of the C-dimer. On the contrary, under vigorous unfolding conditions (solutions of the protein in 40% ethanol or TFE, etc.; 60−70 °C), the amount of the C-dimer remarkably prevailed over that of the N-dimer. Moreover, the absolute quantity of RNase A dimers formed was also definitely higher.

The aim of the present work was to investigate whether changes in the polar or apolar character of the two RNase A termini could influence the 3D domain-swapping process leading to the formation of the N-dimer or the C-dimer. In particular, by site-directed mutagenesis, we increased (a) the polarity of the *N*-terminal domain by substituting the alanine residue in positions 4 or 5 of the *N*-terminus with a serine (A4S and A5S mutants) or (b) the hydrophobicity of the *C*-terminal domain of RNase A by changing the serine residue in position 123 of the *C*-terminus with an alanine (S123A mutant). Therefore, the polarity of the *N*-terminus or, alternatively, the apolarity of the *C*-terminus was increased. The dimerization pattern of these mutants was then investigated side-by-side with that of native and/or recombinant RNase A by applying some of the experimental conditions previously used and standardized with native RNase A (*2*). We chose the following conditions. Mild unfolding conditions: ddH_2O, 60 °C; intermediate unfolding conditions: 20% aqueous ethanol, 60 °C; and vigorous unfolding conditions: (a) 40% aqueous ethanol, 60 and 70 °C; (b) 40% aqueous TFE, 45 °C and 70 °C; 50% TFE, 45, 50, 55 °C; 50% TFE plus 0.5 M GdnHCl, 45 or 55 °C. The thermal treatment was applied from 0 to 120 min. It might

be also worth mentioning that the mutations we introduced into RNase A concern residues that seem not to form side-chain H bonds with other residues in native RNase A (*30*), which of course makes it easier to detect the possible effects of a change in the hydrophilic/hydrophobic character of the swapping peptides on the 3D domain-swapping mechanism.

The experimental conditions used to unfold the N- and/or *C*-terminus of RNase A are not sufficient themselves for the domain-swapped dimers to form. First, the detachment and mobilization of the swapping domains from the core of the protein (closed interface (*31*)) and the unfolding of the flexible hinge loop that links these two domains have to occur in a highly concentrated protein solution. Indeed, a highly concentrated population of protein molecules makes it easier for the open terminal domain of a monomer to find the corresponding available core domain of another monomer, reconstitute the native contacts, and form a dimer. The same reasoning can of course apply to the formation of higher oligomers. Second, after this step, the environmental conditions must change and become suitable to preserve the newly formed structure(s). Indeed, although the unfolding conditions favor the start of the oligomerization process, they can be too strong to preserve the product. The oligomerization yield may in this case decrease because of the destabilization of the newly formed structure(s) by too drastic environmental conditions. This is particularly true for the N-dimer formation under vigorous unfolding conditions. We found that the RNase A oligomers could be satisfactorily stabilized by diluting the incubation mixture at least 100 times with 0.1 M sodium phosphate buffer at pH 6.7, while slowly bringing the temperature of the sample(s) to 4 °C.

Dimerization under Mild Unfolding Conditions. The results obtained by subjecting highly concentrated aqueous solutions of the three mutants or native RNase A to a temperature of 60 °C are shown in Figure 2. Although the absolute amounts of dimers formed by the three mutants were modest, the N-swapped dimers were the prevailing forms, whereas the quantities of C-dimer formed by all species were very low (below 2%). In particular, the amounts of N-dimer formed by A4S or A5S (between 6% and 7%) were higher than the amounts of N-dimer formed by native RNase A and S123A (about 3.5% and 4.5%, respectively), and remarkably higher than the C-dimer formed by A4S and A5S as well as native RNase A (not above 1.5%). Clearly, the mild experimental conditions permitted only a low unfolding of the *C*-terminus of the various RNase A species and, therefore, a modest yield of the corresponding C-dimers. Interestingly, in the first 60 min of treatment, the S123A mutant formed no C-dimer at all (Figure 2); only later, between 60 and 120 min, could a minute quantity (below 1%) of C-dimer be found. This means that under the unfolding conditions tested, the increased hydrophobic character of the *C*-terminus in the mutant made its mobilization remarkably more difficult than that in native RNase A.

Dimerization under Intermediate Unfolding Conditions. The results obtained by heating highly concentrated solutions of the various RNase A species in 20% aqueous ethanol at 60 °C are shown in Figure 3. The relative amount of N-dimers produced was always higher than the amount of C-dimers, as is clearly reflected by the N_D/C_D ratios shown in the inset of Figure 3. However, although the amount of C-dimers formed by all RNase A species was roughly similar

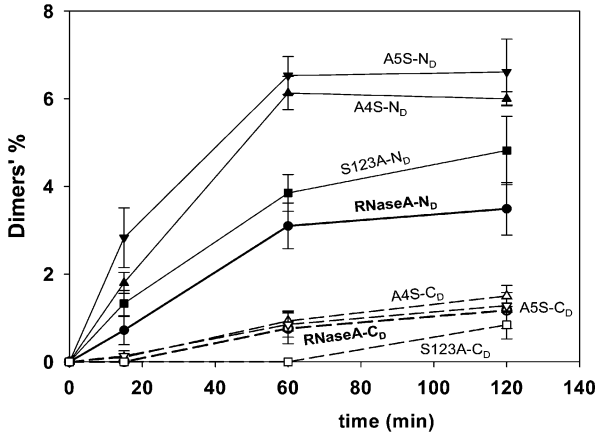

FIGURE 2: Formation of RNase A dimers after treatment in bidistilled H_2O at 60 °C. RNase A was dissolved in ddH_2O at a concentration of 200 mg/mL. Then, 2.5 μL aliquots, one for each time of incubation, contained in Eppendorf tubes were transferred to a thermostatically controlled water bath already brought to 60 °C. At the indicated times, 200 μL of preheated 0.1 M sodium phosphate buffer at pH 6.7 was added to each sample and the tube transferred to an ice-cold bath. After 5−10 min, the sample was applied on to the cation-exchange column and eluted as described (Materials and Methods). Otherwise, it was frozen until use. Each point of the curve represents the value of the area of the chromatographic peak of N_D (solid lines, filled symbols) and C_D (dashed lines, open symbols) formed by wild-type RNase A (●,○, bold lines), A4S (▲,△), A5S (▼,▽), and S123A (■,□), respectively. Each area is expressed as the percentage of the sum of the areas of the peaks of all eluted RNase A species and is the mean of four experiments.

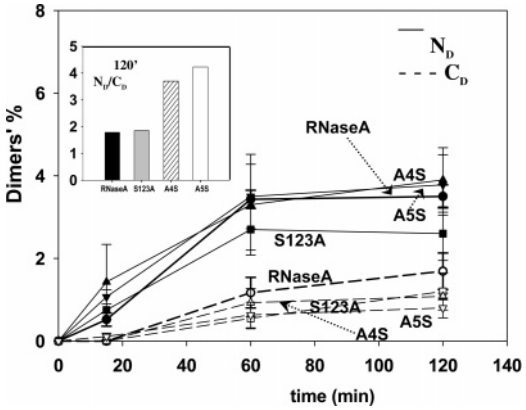

FIGURE 3: Formation of RNase A dimers after treatment in 20% aqueous EtOH at 60 °C. RNase A was dissolved in 20% aqueous ethanol at a concentration of 200 mg/mL. Then, 2.5 μL aliquots, one for each incubation time, were processed as described in Figure 2. Each point of the curves represents the value of the area of the chromatographic peak of N_D (solid lines, filled symbols) and C_D (dashed lines, open symbols) formed by wild-type RNase A (●,○, bold lines), A4S (▲,△), A5S (▼,▽), and S123A (■,□), respectively. Each area is expressed as the percentage of the sum of the areas of the peaks of all eluted RNase A species and is the mean of three experiments. In the inset, the N_D/C_D ratios measured after 120 min are shown.

to that produced under the preceding conditions (Figure 2), the quantity of N-dimers (below 4%) was actually lower. From these results, we can deduce that the intermediate unfolding conditions, although leaving almost unmodified the propensity of the *C*-terminus of the various species to unfold and, therefore, to form a C-dimer (only the S123A C-dimer slightly increases compared to the results shown in Figure 2), were probably too strong to allow an efficient survival of the N-dimers produced. One point deserves a

Table 3: N-dimers (N_D) and C-dimers (C_D) Formed by Native RNase A or Its S123A Mutant in 40% Aqueous EtOH at 60 °C[a]

time (min)	RNase A		S123A	
	% N_D	% C_D	% N_D	% C_D
5	1.0 ± 0.4	1.9 ± 0.9	2.2 ± 0.1	5.0 ± 0.4
10	1.5 ± 0.3	3.1 ± 0.5	2.7 ± 0.3	5.9 ± 0.4
15	3.5 ± 0.6	7.0 ± 0.9	2.4 ± 0.4	5.9 ± 0.3

[a] Each RNase A species was dissolved in 40% aqueous EtOH at a concentration of 200 mg/mL. Aliquots (2.5 μL), one for each time of incubation, contained in Eppendorf tubes were transferred into a thermostatically controlled water bath at 60 °C. At the indicated times, 250 μL of preheated 0.1 M sodium phosphate buffer at pH 6.7 was added to each sample and the tube transferred to an ice-cold bath. After 5−10 min, the sample was applied on to the cation-exchange column and eluted as described (Materials and Methods). Otherwise, it was frozen until use. Each value reported (with its s.d.) is the area of N_D and C_D expressed as the percentage of the sum of the areas of the peaks of all eluted RNase A species and is the mean of three experiments.

comment. The relatively low amount of the S123A N-dimer formed at 60 min incubation (slightly above 2.5%) compared to that of RNase A (about 3.5%) might indicate that the increased hydrophobic character of the *C*-terminus could in some way affect the mobilization of the *N*-terminus of the mutant, which does not occur under milder conditions (Figure 2).

Dimerization under Vigorous Unfolding Conditions. (a) Dimerization experiments in 40% ethanol at 60 °C were performed with only native RNase A and its S123A mutant, for no more than 15 min. The results are shown in Table 3. The amounts of both the N-dimer and C-dimer formed were higher for S123A than that for RNase A at 5 and 10 min of incubation but not at 15 min of incubation. At this time, RNase A actually formed more N_D and C_D than the mutant did. The experiment was in fact limited to 15 min because the amount of both dimers formed by the S123A variant decreased later, probably because of their dissociation due to the rather strong environmental conditions.

(b) Native RNase A and the S123A mutant dissolved in 40% ethanol and heated at 70 °C gave similar yields of the C-dimer (Figure 4), with a maximum at 15 min of treatment, followed by a modest decrease continuing up to 120 min and ascribable, again, to the dissociation of the dimers at high temperatures or possibly some form of irreversible denaturation. In both cases, the amount of C_D exceeded that of N_D for the first time at 15 min, the quantity of the S123A C-dimer was 16−18 times higher than that of the S123A N-dimer (see also the C_D/N_D ratios in the inset of Figure 4) and about twice that of the RNase A N_D. This result is in line with the idea that dimerization by 3D domain-swapping can be favored by the increased hydrophobic character of the RNase A *C*-terminus. Indeed, the rather strong experimental conditions chosen allowed the unfolding of the *C*-terminus of the mutant (and of native RNase A) and the formation of its C-dimer. Here again, the mutation at the *C*-terminus appears to affect either the propensity of the mutant to form N-dimer, as already proposed for the data shown in Figure 3, or its stability.

(c) The dimerization of the various RNase A species dissolved in 50% TFE and heated at 45 °C are shown in Figure 5. The absolute quantities of oligomers formed by all RNase A species were significantly higher than those found under all conditions described earlier. The amounts

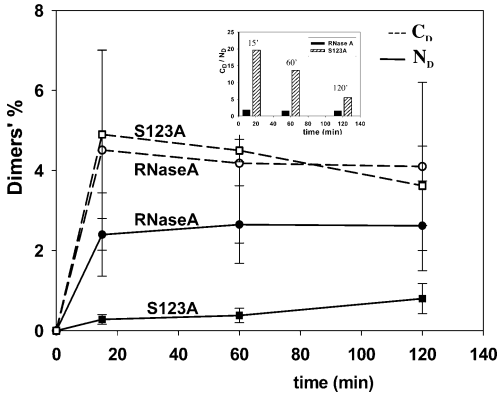

FIGURE 4: Formation of RNase A dimers after treatment in 40% aqueous EtOH at 70 °C. RNase A was dissolved in 40% aqueous ethanol at a concentration of 200 mg/mL. Then, 2.5 μL aliquots, one for each time of incubation, were processed as described in Figure 2 but at 70 °C. The species treated in this condition were only the wilde-type RNase A and the S123A. Each area is expressed as the percentage of the sum of the areas of the peaks of all eluted RNase A species and is the mean of three experiments. In the inset, the C_D/N_D ratios measured at the times indicated are compared.

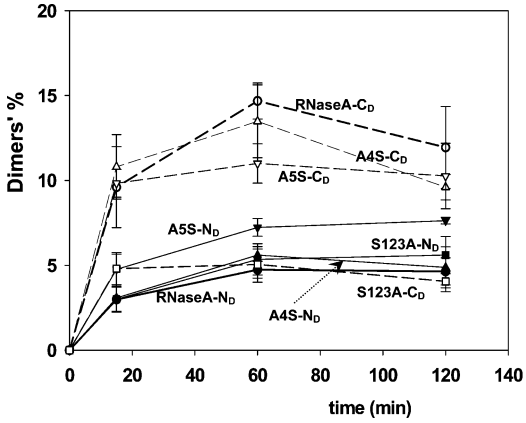

FIGURE 5: Formation of RNase A dimers after treatment in 50% aqueous TFE at 45 °C. RNase A was dissolved in 50% aqueous TFE at a concentration of 200 mg/mL. Then, 2.5 μL aliquots, one for each time of incubation, were processed at 45 °C as described in Figure 2. Each area is expressed as the percentage of the sum of the areas of the peaks of all of the eluted RNase A species and is the mean of three or four experiments.

of C-dimers formed by the native protein and the two *N*-terminal mutants remarkably increased (notably, the slightly higher stability of A5S compared to that of A4S (see Figure 1 and Table 2) could explain the lower yield of its C-dimer at 15 and 60 min), definitely surpassing their corresponding N-dimers, while the production of the S123A C-dimer still remained quite low. The increase in temperature to 50 or 55 °C did not improve these results (data not shown). Clearly, the strength of the hydrophobic interactions between the core portion of S123A and its *C*-terminus impedes their separation and thereby the swapping necessary to form a C-dimer. However, the results showed that in 50% TFE at 45 °C, the amounts of the S123A C-dimer and N-dimer became roughly equivalent.

Dimerization of Native RNase A and Its Mutants Dissolved in 50% TFE Containing Guanidine Hydrochloride (GdnHCl). Conditions that could increase the propensity of the S123A mutant to unfold and dimerize satisfactorily were obtained by dissolving it in 50% TFE containing various amounts of GdnHCl (0.2, 0.5, or 1.0 M, final concentration) at 45 °C.

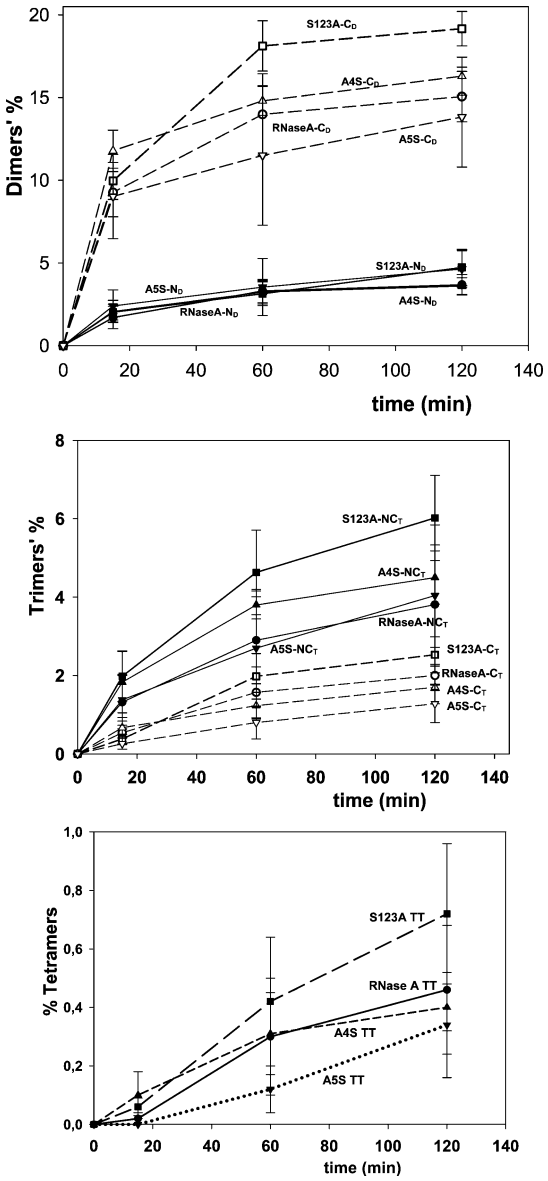

FIGURE 6: Formation of RNase A oligomers after treatment in 50% aqueous TFE containing 0.5 M GdnHCl at 45 °C; (A) dimers, (B) trimers, (C) tetramers. RNase A was dissolved in 50% aqueous TFE containing 0.5 M guanidine hydrochloride at a concentration of 200 mg/mL. Then, 2.5 μL aliquots, one for each time of incubation, were processed at 45 °C as described in Figure 2. Each area is expressed as the percentage of the sum of the areas of the peaks of all of the eluted RNase A species and is the mean of four experiments.

Identical solutions containing native RNase A and A4S or A5S were processed in parallel. The results for the mixtures containing 0.5 M GdnHCl are shown in Figure 6. Under these conditions, the tight molecular structure of the S123A mutant acquired a remarkable propensity to unfold and dimerize, and its C-dimer yield was much higher than that of the N-dimer (Figure 6A). The difference between the amount of the mutant's C-dimer and that of native RNase A was also significant; the yield of C_D was among the highest obtained thus far in oligomerization experiments performed with any RNase A species, either in this work or in previous studies (*2*). The ability of GdnHCl to enhance the yield of oligomers could be due to its action as a potent denaturant of protein structure or its capacity as a salt to screen repulsive intermolecular electrostatic interactions or both. Furthermore, recall that TFE disrupts hydrophobic interactions but strengthens hydrogen bonds, whereas GdnHCl disrupts both hydrophobic interactions and the H-bond network within the protein. The environmental conditions were probably too drastic to efficiently conserve N-dimers. Interestingly, remarkable amounts of the two trimers (*4*, *6*, *7*) also formed both from the S123A mutant and native RNase A or A4S and A5S mutants, with a net prevalence of the linear trimer NC_T, over the cyclic trimer C_T and with the S123A products always exceeding those of native RNase A (Figure 6 B). Trace amounts of tetramers (Figure 6C) were also found, with the tetramers' yield of the S123A mutant prevailing over those of the other mutants or wild-type protein, especially for the longer treatments. This could indicate that the dimer's yield does not greatly increase in longer treatments (Figure 6A) because it can evolve to higher order oligomers, especially in the case of the S123A mutant. Finally, it may be worth mentioning the following. (i) In similar experiments performed in the presence of 0.2 M GdnHCl, the amounts of the S123A C-dimer and RNase A C-dimer were equivalent (data not shown). (ii) By heating at 55 °C solutions of the S123A mutant in 50% TFE plus 0.5 M GdnHCl, an even higher absolute amount (almost 25%) of C-dimer could be obtained, although a precipitate was observed in about 50% of the experiments. (iii) In samples containing 1.0 M GdnHCl, the yield of dimers did not increase further (data not shown). (iv) Native RNase A and the three mutants formed similar amounts of the various oligomers when lyophilized from 40% acetic acid solutions (*1*, *3*) (data not shown). An easy and immediate comparison of the dimers' yields obtained after 60 min of treatment under the various unfolding conditions described in the preceding paragraphs can be made by examining Figure 7.

Conformational Stability of the Monomers of Wild-Type RNase A and Its Three Mutants. The A4S and A5S mutants show a reduced conformational stability as gauged by thermal denaturation. Ala-4 and Ala-5 are located in the first turn of the *N*-terminal helix of RNase A, and their side chains are mostly exposed, although their β-methyls contact the γ-methyls of Val 116 and Val 118. This hydrophobic contact could be disrupted by the addition of a hydroxyl group when Ala is replaced by Ser. The substitution of Ala by Ser can also be expected to destabilize this α-helix because the substitution of Ala by Ser destabilizes isolated helical peptides by 0.8 kcal/mol (*32*) and considering that there is experimental (*33*) and statistical (*34*) evidence that this kind of substitution destabilizes helices in folded proteins by the same amount as it does in peptides. However, the lower intrinsic helix stability of Ser is partly compensated (0.2 kcal/mol) by its ability to accept a hydrogen bond from amide groups in the first turn of the helix, which lack a hydrogen-bonding partner (*35*). The expected lower stability of the *N*-terminal helix due to the substitution of Ala4 or Ala5 by Ser, although not reflected as a change in the stability against thermal unfolding, could manifest itself as an increased tendency of the *N*-terminal helix to unfold locally and to separate from the protein core. This behavior could account for the faster dissociation rate of the dimers formed by these mutants.

The S123A mutant protein shows a significantly higher conformational stability compared to that of wild-type RNase A. Ser-123 is the penultimate residue of the protein and forms

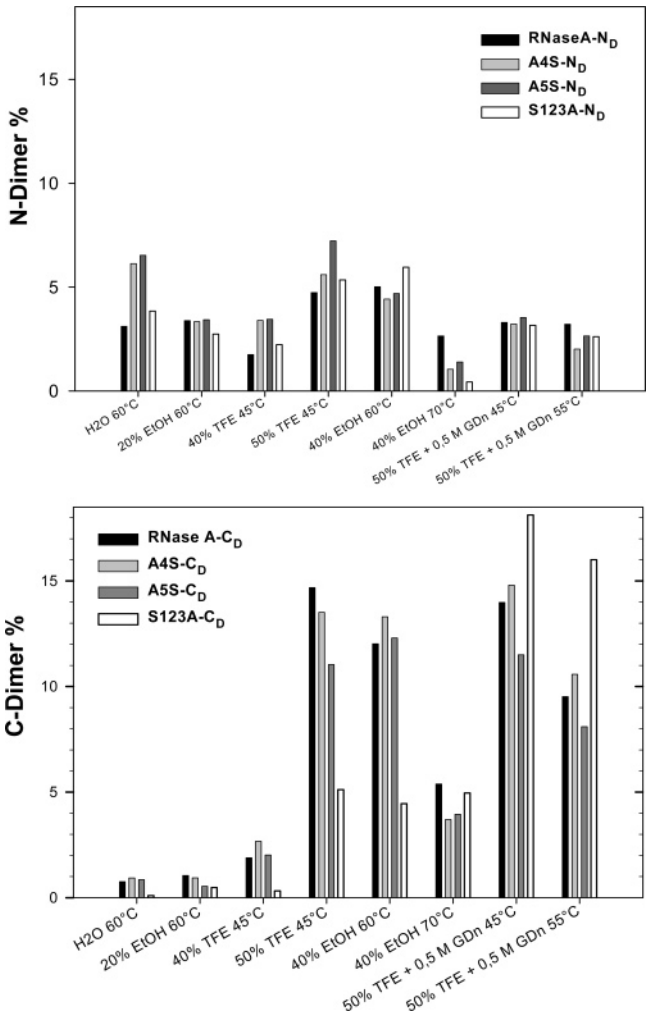

FIGURE 7: Comparison of the yield of dimers formed by RNase A and the three mutants after 60 min of treatment under the different conditions previously described. (A) N_D; (B) C_D. Each bar represents the area expressed as the percentage of the sum of the areas of the peaks of all eluted RNase A species. The error bars are omitted here. GDn means GdnHCl.

part of the *C*-terminal β-sheet. Computational (*36*, *37*) and experimental studies (*38*) differ in the values they obtain for the intrinsic β-sheet propensities of the 20 amino acids, although, in general, the difference for substituting Ser for Ala was calculated or found to be small. Ser-123 could be destabilizing relative to Ala because its hydroxyl group is poorly solvated and/or lacks hydrogen-bonding partners in the folded protein. This explanation is plausible, considering that this hydroxyl group is mostly buried, and its side chain lies near some hydrophobic side chains (Ile-81, Ile-106, Phe-120, Val-124). The increased stability afforded by the Ala-123 substitution is consistent with the lower yield of C-dimers by this variant under mildly destabilizing conditions. However, once formed, the C-dimer of the S-123A variant reconstitutes the native contacts at the closed interface and can be expected to be more stable, which is perfectly in line with the high yield of C-dimer obtained under severely destabilizing conditions.

As discussed above, the observed yield of oligomers crucially depends on the strength of the denaturing conditions: when too mild, too little of the open intermediate forms, and when too severe, the stability of the dimer is compromised. This is particularly true when treatments are performed in the same medium but at increasing temperatures (see Figure 7 B). The results obtained here demonstrate that a mutation can shift the balance between these two factors; in the cases of A4S and A5S, the mutation promotes local unfolding, increasing the dimer's yield under mildly denaturing conditions, and in the case of S123A, the substitution increases the stability and, therefore, the relative yield of the C-dimer under severely unfolding conditions.

Stability against the Dissociation of the Two Dimers Formed by Wild-Type RNase A and Its Three Mutants. It has been reported that the N-dimer (N_D) is more stable against dissociation than the C-dimer (C_D) (*39*, *40*) mainly because of the larger open interface (*31*) present in the N_D with respect to that of the C_D and not the closed interface because of the presence of the latter in both the native monomer and the oligomers (*31*). However, bearing in mind that the stability against the dissociation of the RNase A oligomers generally depends on several factors (*8*) and decreases as the size of the oligomers increases (*5*, *8*), conditions favoring an enhanced survival of the oligomers were found to be a low temperature, low protein concentration (below 0.1−0.2 mg/mL) at pH close to 6.7, sodium phosphate buffer higher than 0.05 M (as indeed reported many years ago (*1*, *41*), a sodium phosphate buffer at pH 6.7 favors the survival of the dimers). In our hands, under the conditions described, the C-dimer is more stable than the N-dimer, contrary to what was reported elsewhere (*39*, *40*), but the discrepancy can be ascribed to one of the variables mentioned above, particularly the protein concentration (*8*). Here, we determined the dissociation rate of the N_D and the C_D formed by native RNase A and its three mutants. The conditions chosen were an oligomer concentration of 0.8 mg/mL, 0.03 M sodium phosphate buffer at pH 6.7, and an incubation temperature of 40 °C. The results obtained are shown in Figure 8. The half-lives ($t_{1/2}$) for the N_D and C_D of each species were calculated and reported in Table 3. It is clear that the dimeric species formed by the S123A mutant are the most stable, whereas the dimers of A4S and A5S dissociate even more readily than the N_D formed by native RNase A. Taking into account that the mutations introduced are located in the closed interface and do not involve the open interfaces of the dimers and that in these conditions the C_D is more stable than the N_D for the wild type and the S123A mutant, we can propose that the closed interface contributes to the different stabilities of the two dimeric species. From the stability values determined for the monomers and dimers, the whole qualitative energetic profile of the formation/dissociation process of these species can be constructed (Figure 9), where it can be noticed that the mutations introduced affect the C_D's stability more than that of the N_D.

Considering that the $t_{1/2}$ values are directly (and quantitatively) related to the barrier height between the dimers and the transition state for dissociation, it is striking that the N-dimer and C-dimer have different $t_{1/2}$ values in the case of the wild-type and the S123A mutant, but the values for $t_{1/2}$ are essentially the same (within experimental uncertainty) for the A4S and A5S mutants. This suggests that the mechanism of dissociation of the N-dimer and C-dimer is different in the case of the wild type and the S123A mutant and would very likely occur, as shown in Figure 9, through N-open and C-open transition states, respectively.

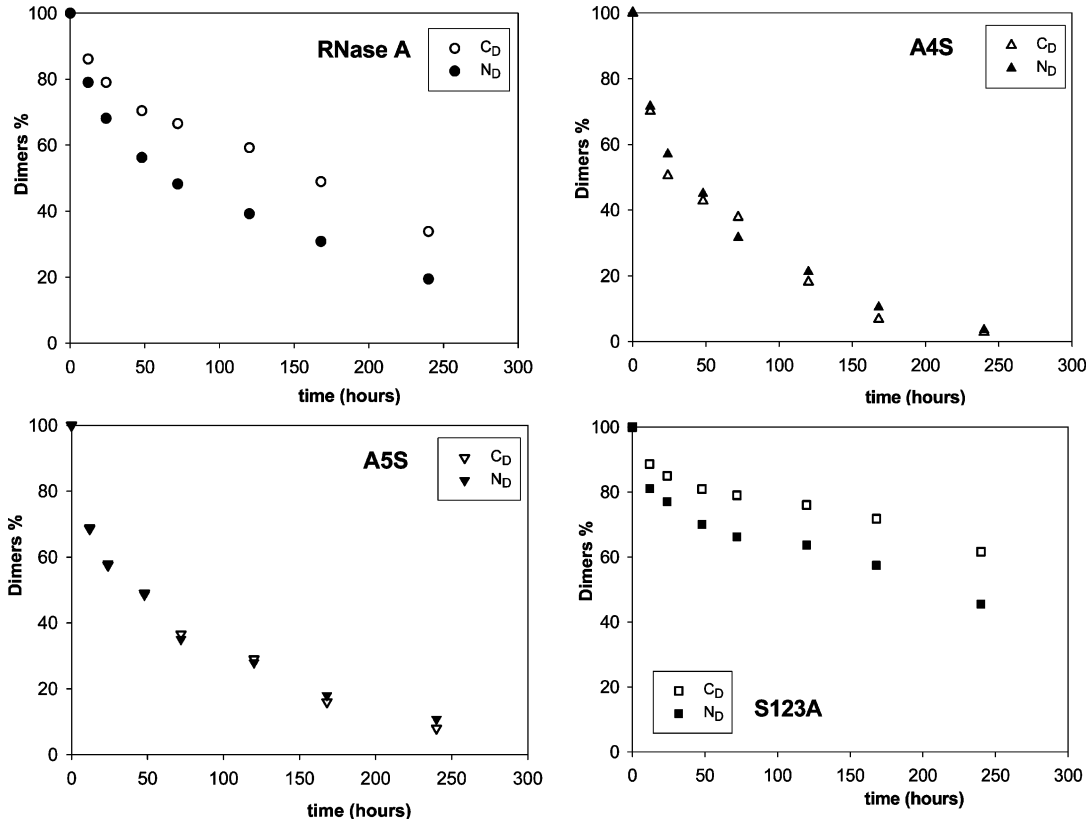

FIGURE 8: Time-course dissociation of N_D and C_D formed by (A) RNase A, (B) A4S, (C) A5S, and (D) S123A. The dimeric samples were incubated in 0.03 M sodium phosphate buffer at pH 6.7 at 40 °C. At various times, the samples (50 μg, 0.8 mg/mL each) were diluted 10-fold with 0.1 M sodium phosphate at pH 6.7 and kept at 4 °C until cathodic PAGE analysis performed under nondenaturing conditions (*22*) to measure the percent of dimer survival. The points represent the percent of dimers that survived, measured using an Image J-Quant program.

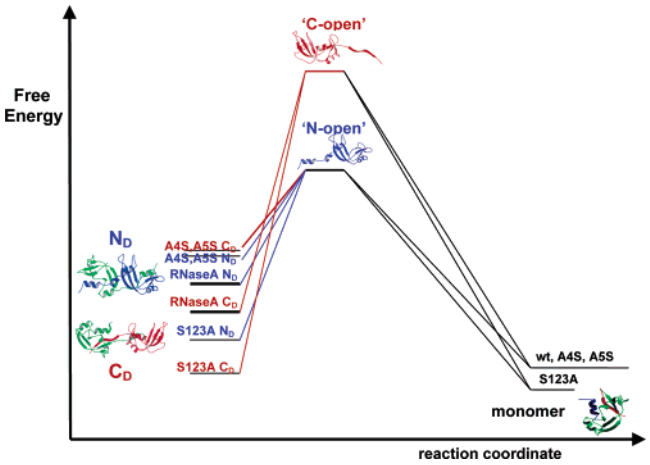

FIGURE 9: Energetic profiles of the dissociation of the N- and C-dimers formed by the various RNase A species. Blue lines: N-dimers; red lines C-dimers.

However, the essentially similar $t_{1/2}$ values observed for the N-dimer and C-dimer of the A4S and A5S mutants suggest that their mechanism of dissociation is the same and that the transition state is the same. Because the *N*-terminal helix is weakened in these mutants, it could be separating from the protein core more readily in both the N-dimer and the C-dimer of these mutants. Therefore, we propose that the transition state of both the N-dimer and the C-dimer of A4S and A5S is the N-open form.

Contributions of the N- and C-Termini to the Formation of the C-Dimer and the N-Dimer, respectively. From the $t_{1/2}$ values (Table 3), it is clear that the mutations introduced in the *N*-terminus (A4S and A5S) not only reduce the stability of the N_Ds but also affect the stability of the corresponding C_Ds, whose reduction is proportionally even higher (compared to the stability of native RNase A). However, the mutation introduced at the *C*-terminus (S123A) dramatically increases the stability of not only the C_D but also the corresponding N-dimer. On the basis of these facts and on other observations reported in this article (see discussion of results shown in Figures 3 and 4), we could suggest that the mutations introduced in the *N*-terminus of the protein influence not only the extent of the swapping of the *N*-terminus but also the production of the oligomers forming through a *C*-terminal domain-swapping mechanism and that conversely the S123A mutant affects not only the aggregation via C-swapping but also the yield of the species formed via N-swapping. The reciprocal control in the aggregation propensity of the two domains involved in RNase A oligomerization through 3D domain swapping also emerged in the study of the 3D domain-swapped oligomers formed by two other ribonuclease species, RNase B (*42*) and RNase S (*43*). It is worth recalling that for RNase S the interaction between the *N*-terminal helix and the protein core is also weakened, in this case by proteolysis. Recently, we concluded that in the dissociation of the RNase S dimer the release of the S-peptide (*N*-terminal helix) was the rate-limiting step and that after its loss the interactions between the swapped *C*-terminal β strand with the other monomer are so weakened that dissociation occurs quite rapidly. A similar mechanism could account for the experimental results

Table 4: $t_{1/2}$ Values[a] of the Dissociation of N-dimers and C-dimers of Native RNase A or Its Mutants

	RNase A	A4S	A5S	S123A
N_D	78 h	43 h	49 h	215 h
C_D	143 h	40 h	50 h	376 h

[a] Values calculated from the linear regression of ($\ln(100\ D_t/D_0)$), where the values of D_t represent the percent of *D* measured at time *t* (and reported in the curves displayed in Figure 8), and D_0 is the value of *D* measured at zero dissociation time (100%).

regarding the dissocation of the A4S and A5S mutants of RNase A. This line of reasoning leads to a remarkable and novel conclusion, namely, that the dissociation mechanism or quaternary unfolding pathway of an oligomeric protein can be altered by mutation. Even for monomeric proteins, reports of changes in the unfolding pathway are infrequent; for example, Udgaonkar and co-workers found that the unfolding pathway of barstar could be altered by changes in temperature at pH, ionic strength, and the presence or absence of chemical denaturants (*44*).

Comparison with the Effects of Mutation on the 3D Domain-Swapped Oligomerization of Other Proteins. Mutational studies on a number of proteins (*45*) including RNase A (*46*) have shown that the shortening or lengthening of the hinge loop can greatly alter their capacity to form 3D domain-swapped oligomers. Point mutations in the hinge loop can also produce large changes; alterations of 9 orders of magnitude for the K_D of dimer dissociation were reported for Pro/Ala variants of suc1 (*47*). Outside the hinge loop, the mutagenesis of a residue at the domain interface present in the oligomer but not the monomer of human pancreatic RNase was shown to promote its dimerization (*48*).

In contrast, the mutation of residues within the swap domain itself should have much smaller effects because these residues have the same conformation and form the same stabilizing interactions at the closed interface in both the monomer and the oligomer (*31*). Bearing this in mind, the substantial effects of the A4S, A5S, and S123A mutants (all of these residues being located at the closed interface and quite distant from the hinge loops) reported here are particularly remarkable. This line of reasoning further emphasizes the importance of hydrophilicity and hydrophobicity on the kinetic and thermodynamic competition between domain opening in the monomer versus the oligomer.

CONCLUSIONS

The hydrophilic or hydrophobic character of the *N*- and *C*-terminal ends of RNase A is an important variable governing the oligomerization of ribonuclease A by 3D domain swapping. The hypothesis that protein oligomerization in general and RNase A oligomerization in particular, occurring through the 3D domain-swapping mechanism, could be influenced by the hydrophilic or hydrophobic character of their swapping domains and, therefore, by changes occurring in that character has been strengthened by the experiments reported in this work. Two types of ribonuclease A mutants were used: two *N*-terminal mutants A4S and A5S, endowed with a higher polarity in their *N*-termini compared to native that of RNase A, and one *C*-terminal mutant S123A, endowed with a higher hydrophobicity in its *C*-terminus compared to that of the native enzyme. These RNase A variants, at high concentrations, were subjected to oligomerization by a thermal procedure that uses mild or vigorous unfolding conditions without a lyophilization step. We obtained the following results. (i) The stabilities of the RNase A variants against thermal denaturation showed the following rank order: A4S $\approx$ A5S $<$ wild type RNase A $<$ S123A. The *N*-terminal mutants, with increased polarity in their *N*-termini, showed a higher propensity with respect to native RNase A to form N-dimers under relatively mild unfolding conditions. The small differences concerning the formation and dissociation of the A4S and A5S dimers could be considered to be insignificant. (ii) The *C*-terminal mutant, with its *C*-terminus more hydrophobic than that of native RNase A, showed a higher propensity to form the C-dimer under drastic unfolding conditions. Both results should be interpreted with some caution. In the first case, the yield of the mutants' N-dimer could be modest because of its low stability, which decreases the survival of the dimer if the environmental conditions become too strong. In the second case, the tight molecular structure of the S123A mutant, because of the increased hydrophobicity of its *C*-terminus, needs rather drastic environmental conditions to unfold. Under strong unfolding conditions, remarkable amounts of the two types of trimers as well as traces of tetramers also form. In conclusion, the 3D domain-swapping mechanism leading to RNase A oligomerization appears to be governed by the hydrophilic or hydrophobic character of the two RNase A termini involved in the process. Therefore, polarity or apolarity of the swapping domains of RNase A involved in the formation of oligomers, and possibly of other proteins oligomerizing by 3D domain-swapping, constitute important variables controlling the event. (iii) The C-dimer formed by the RNase A S123A mutant is, under the conditions described in this work, the most stable species against dissociation, and the C-dimers formed by the A4S and A5S mutants are even less stable than the N-dimer formed by native RNase A. (iv) One swapping domain of RNase A appears to influence the propensity of the other to swap, with the eventual formation of a dimer or more generally an oligomer.

NOTE ADDED IN PROOF

While this manuscript was under review, a thorough study of the pressure-induced unfolding and refolding of Ribonuclease A and variants was published (*49*), which supports the importance of hydrophobic interactions and van der Waals packing of *C*-terminal residues to the stability and folding of this enzyme.

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