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Thermal Aggregation of Ribonuclease A

A CONTRIBUTION TO THE UNDERSTANDING OF THE ROLE OF 3D DOMAIN SWAPPING IN PROTEIN AGGREGATION*

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By lyophilizing RNase A from 40% acetic acid solutions, two dimeric aggregates, the "minor" and "major" dimers (named here N-dimer and C-dimer, respectively), form by 3D domain swapping at a ratio of 1:4. Trimeric and tetrameric aggregates are also obtained. The two dimers and the higher oligomers also form without a lyophilization step. By keeping RNase A dissolved at a high concentration (generally 200 mg/ml) in various media at temperatures ranging from 23 to 70 °C for times varying from a few minutes to 2 h, various oligomers, in particular the two dimeric conformers, formed in quite different amounts, often inverting their relative quantities depending on the more or less severe unfolding conditions. When unfolding mainly concerned the N terminus of the protein, richer in hydrophilic residues, the N-dimer, formed by 3D domain swapping of the N-terminal α-helix of each monomer, prevailed over the C-dimer. Under more vigorous denaturing conditions, where also the C terminus of RNase A, richer in hydrophobic amino acids, unfolded, the C-dimer, formed by 3D domain swapping of the C-terminal β-strand, prevailed over the other, possibly because of the induction to aggregation promoted by the hydrophobic residues present in the C termini of the two monomers.

Protein aggregation is presently an event of remarkable interest. Severe human neurodegenerative diseases are in fact characterized by the presence of aggregated forms of proteins (β-amyloid in Alzheimer's disease, α-synuclein (a primary component of Lewy bodies) in Parkinson's disease, a definitely modified form of the prion protein in various transmissible encephalopathies, etc.), and protein aggregation also characterizes the so-called polyglutamine expansion diseases (Huntington's disease, Kennedy's disease, etc.) (1).

Bovine ribonuclease A is a structurally versatile protein, appropriate to study the manner in which a protein aggregates. By lyophilization from 40–50% acetic acid solutions, the protein aggregates in the form of several types of oligomers, ranging from dimers (2) to pentamers and possibly higher aggregates, each species existing in at least two conformational isomers produced in invariable different amounts (3, 4). The two dimers of RNase A have been well characterized and shown to be 3D domain-swapped oligomers (5, 6), with the domain swapping involving either the N or C terminus or both of each subunit. For one of the trimers (the quantitatively major one), a very plausible linear structure has been proposed (6), whereas the crystal structure of the other shows a circular trimer, similar to a propeller, formed by the swapping of the C-terminal β-strand of each monomer (7). Models for the putative structures of the two tetrameric conformers have also been proposed (4, 8).

Interestingly, RNase A also aggregates under relatively mild conditions. Park and Raines (9) reported that, at 37 and 65 °C and pH 6.5, it forms domain-swapped dimers, whereas many years ago it was shown that high substrate concentrations can induce the dimerization of RNase A (10). It has been suggested that the 3D domain-swapping mechanism forming RNase A dimers or higher order aggregates may have implications for the formation of amyloid-like fibrils (5, 6, 11), and strong support for this idea is given by the recently reported dimerization through a 3D domain-swapping mechanism of the amyloidogenic protein cystatin C (12) and the human prion protein (13).

We have studied here the oligomerization of RNase A occurring under various conditions that favor the unfolding and mobility of the N and/or C terminus of the protein, and therefore the domain swapping event, without a lyophilization step. Our aim was to contribute to the understanding of the 3D domain-swapping mechanism in protein aggregation. The aggregates, mainly the two dimeric conformers, but also higher order oligomers, formed in varying relative, often inverted proportions depending on the experimental conditions.

EXPERIMENTAL PROCEDURES

Materials—Ribonuclease A from bovine pancreas (Type XII-A) was from Sigma. All chemicals were of the highest purity available. Superdex 75 HR10/30 and Source 15S HR10/10 and HR16/10 columns were from Amersham Biosciences. Chromatographic experiments were performed with an Amersham Biosciences fast protein liquid chromatography system.

Preparation of RNase A Aggregates—RNase A was dissolved in various media, and aggregation was performed as described below, always without a lyophilization step: (*a*) 20 or 40% ethanol in water and an RNase A concentration of 200 mg/ml; (*b*) 20 or 40% ethanol in 0.08 M sodium phosphate buffer (pH 6.7) and an RNase A concentration of 200 mg/ml; (*c*) 40% 2,2,2-trifluoroethanol (TFE)[1] in water and an RNase A concentration of 200 mg/ml; (*d*) 40% acetic acid (pH ~1) and an RNase A concentration of 66.7 mg/ml; (*e*) 5, 10, or 30% aqueous acetone and an RNase A concentration of 200 mg/ml; (*f*) 40% aqueous acetone and an RNase A concentration of 170 mg/ml; (*g*) 0.05 M glycine HCl buffer (pH 3) and an RNase A concentration of 200 mg/ml; (*h*) double-distilled water (pH 6.0) and an RNase A concentration of 200 mg/ml; (*i*) 0.02 M NaCl (pH 7) and an RNase A concentration of 200 mg/ml; (*j*) 0.0125 M $Na_2B_4O_7$ and 0.0152 M HCl buffer (pH 8.5) and an RNase A concentration of 200 mg/ml; (*k*) 0.05 M KCl/H_3BO_3 and 0.0208 M NaOH buffer (pH

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Dedicated to the memory of Eraldo Antonini, eminent biochemist, on the 20th anniversary of his premature death.

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[1] The abbreviation used is: TFE, 2,2,2-trifluoroethanol.

9) and an RNase A concentration of 200 mg/ml; and (*l*) 0.05 M KCl/H_3BO_3 and 0.0437 M NaOH buffer (pH 10) and an RNase A concentration of 200 mg/ml. Aliquots (2 μl) of each RNase A solution were maintained for appropriate times, ranging from minutes to hours, at the chosen temperature. At the end of each incubation time, 200 μl of 0.2 M sodium phosphate buffer (pH 6.7), preheated at the same temperature (sometimes at room temperature), were added, and the sample was brought to 0 °C. Aliquots of the RNase A solutions indicated above were also kept at 23 °C for the same times chosen for the experiments at 60 °C. At the end of each incubation time, 200 μl of 0.2 M sodium phosphate buffer (pH 6.7) were added.

Gel Filtration—After the treatment described above, each sample was frozen until used or applied to a Superdex 75 HR10/30 column equilibrated with 0.2 M sodium phosphate buffer (pH 6.7) at 23 °C. Elution was performed at 23 °C with the same buffer at a flow rate of 0.01–0.2 ml/min. Every aggregated species emerging from the column was identified by checking its elution position and comparing it with that of the well characterized oligomers obtained by the usual lyophilization procedure (3, 4), which were always used as standards.

Ion-exchange Chromatography—Analysis and separation of the various RNase A aggregates were performed under conditions identical to those described elsewhere (3, 4). Their identification was performed by comparison with the elution pattern of standard aggregates (see above).

Quantification of the Aggregates Formed—Each aggregate produced under the various experimental conditions was quantified by measuring the area of its peak appearing on the gel-filtration or ion-exchange chromatogram and calculating its percentage relative to the sum of the areas of the peaks of all RNase A species eluted, monomeric RNase A included. The values shown in the table and/or figures are the means ± S.D. of several determinations.

Gel Electrophoresis—Cathodic gel electrophoresis under nondenaturing conditions was performed according to Goldenberg (14) with slight modifications, using β-alanine/acetic acid buffer (pH 4). 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. Identification of the aggregates was performed by comparison of their R_F values with those of the aggregates obtained by the lyophilization procedure, already well characterized and used here as standards.

Analysis of the Thermal Unfolding of RNase A—The thermal unfolding of RNase A was followed spectrophotometrically at 287 nm using a Beckman DU-650 spectrophotometer equipped with a thermostatically controlled water bath. The protein was dissolved in the various media at a concentration of ~1.5 mg/ml. At each temperature, the absorbance values of the RNase A solution were determined every 2 min, for a total time of 60 min, until measurements became constant for at least 10 min.

RESULTS AND DISCUSSION

Lyophilization of RNase A from 40% acetic acid solutions produces two dimeric conformers at a rather invariable ratio of ~1:4, one called in fact the "minor" dimer and the other the "major" dimer (3, 4). The minor dimer forms by 3D domain swapping of the N-terminal α-helix (residues 1–15) of the RNase A monomer (5), and the major dimer forms by 3D domain swapping of the C-terminal β-strand (residues 116–124) of each RNase A subunit (6). Here we have simply named them the N-dimer and C-dimer, respectively, on the basis of the mechanism of their formation (5, 6).

By the lyophilization procedure, higher oligomers of RNase A also form: two trimers in constant, quite invariable amounts (ratio 1.5:1), formerly called the major and minor trimers (3, 4, 6), as well as two tetramers, a minor and a major one, forming at a ratio of 1:1.6 (4). However, dimers and larger aggregates of RNase A also form under conditions that allow the unfolding of the N and/or C terminus of the protein, but without including a lyophilization step. Moreover, the relative proportions of the two dimers can change and even be inverted depending on the experimental conditions. The experiments were performed by maintaining native monomeric RNase A, dissolved at a high concentration (usually 200 mg/ml) in the various fluids described under "Experimental Procedures," at temperatures ranging from 23 to 70 °C for times ranging from a few minutes to 2 h. The treatment was ended by diluting the incubation mixture 1:100 with 0.2 M sodium phosphate buffer (pH 6.7) and transferring it to an ice bath. The aggregates formed were separated by gel filtration. When necessary, each gel-filtered oligomeric species or the incubated mixtures as such were analyzed by ion-exchange chromatography. In both cases, identification of the various oligomers formed was carried out as described under "Experimental Procedures," *i.e.* by using the well characterized dimers, trimers, or tetramers (3, 4), recently prepared, as standards. A control of the nature of the various oligomers was also performed, when necessary, by gel electrophoresis under nondenaturing conditions (see "Experimental Procedures"). In all cases, the relative amounts of the two dimeric conformers and of the higher order oligomers were calculated as described under "Experimental Procedures."

Aggregation of RNase A Dissolved in Various Media and Incubated at 60 or 23 °C—As shown in Fig. 1 (*A* and *B*, *blue lines*) and Table I, a solution of RNase A (200 mg/ml) in 40% aqueous or buffered ethanol heated at 60 °C for 30 min produced, similar to the lyophilization procedure, the C-dimer in a higher amount and the N-dimer in a lower amount, the ratio of the former to the latter being ~2:1 (lower than the 4:1 ratio usually found with the lyophilization procedure). Similar results were obtained when RNase A dissolved in 40% aqueous TFE was maintained at 60 °C for 30 min (C-dimer/N-dimer ratio of 2:1). Under identical conditions, significant amounts of trimers or tetramers also formed. Because these oligomers were collected by gel filtration and not by ion-exchange chromatography, no complete separation of the two conformers of each species was obtained. The trimers and tetramers indicated here (Table I) are therefore mixtures of the two trimeric and tetrameric conformers (3), respectively.

By incubating (60 °C, 30 min) RNase A dissolved in either 20% aqueous or buffered ethanol, as well as in many other fluids (Fig. 1, *A* and *B*, *red lines*; and Table I), we found instead that the relative proportions of the two dimers inverted, the N-dimer often prevailing over the C-dimer. Unexpectedly, oligomerization of the protein also occurred when incubation was performed at room temperature (23 °C). Under these conditions, the prevalence of the N-dimer over the C-dimer was observed in all cases, including RNase A solutions in 40% ethanol or TFE, although the aggregates often formed in quite modest amounts (Fig. 1, *A* and *B*, *green lines*; and Table I).

It is also worth mentioning that incubation of a 10-fold less concentrated RNase A solution (20 mg/ml) in 40% ethanol or TFE at 60 °C gave only traces of dimers. This points out the importance of protein concentration in the aggregation process.

Oligomerization of RNase A Dissolved in 40% Aqueous Ethanol and Heated at 45, 50, or 60 °C—To analyze the results described in more detail, we studied the formation of RNase A oligomers by incubating solutions of the protein in 40% aqueous ethanol for up to 2 h at 45, 50, or 60 °C. By analyzing aliquots withdrawn from the incubation mixture at the times indicated, we obtained the results presented in Fig. 2. Fig. 2*A* shows RNase A aggregation at 45 °C. At this temperature, the reaction did not reach equilibrium even after 2 h of incubation. The modest amounts of oligomers formed regularly increased with time; and the N-dimer was definitely prevalent over the C-dimer. Small amounts of trimers also appeared after 70 min of incubation. A notable point is that, at zero time, *i.e.* during the short time necessary to prepare the incubation mixture at room temperature (23 °C) and to transfer an aliquot of it to an ice bath, RNase A appeared to aggregate quite efficiently, with the N-dimer and C-dimer reaching amounts of 3.4 and 1.2%, respectively. However, after some minutes at 45 °C, the amount of both aggregates decreased, as if they were dissociated. It is worth pointing out that, from these and other results,

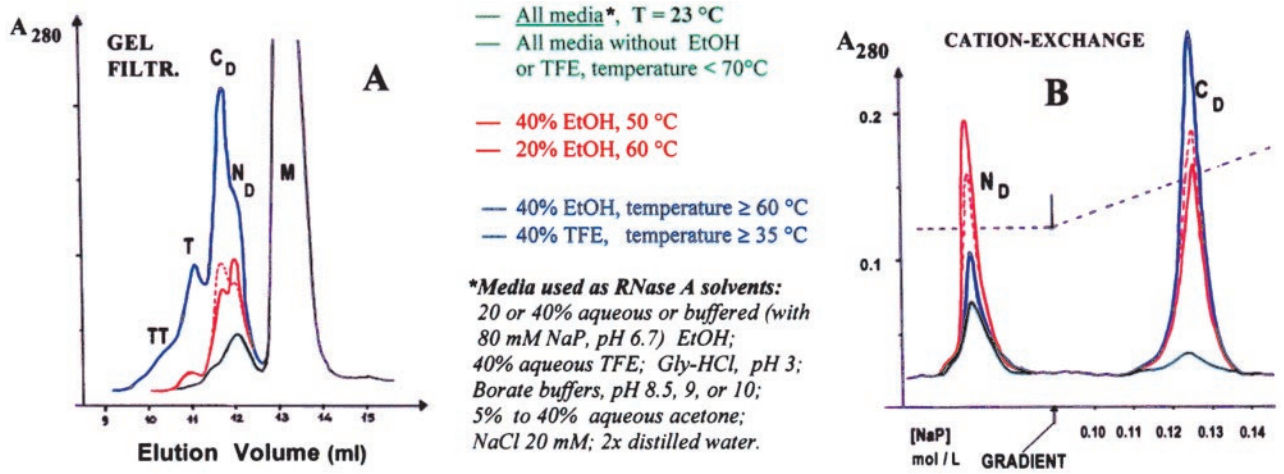

FIG. 1. **Patterns of RNase A aggregates obtained by thermal treatment under different environmental conditions.** The RNase A oligomers were obtained as follows. Eppendorf tubes containing 2 μl (0.4 mg) of each solution were put in a thermostatically controlled bath at one of the temperatures indicated for 30 min (*dashed red line* refers to 60-min kinetics). Then, 200 μl of 0.2 M sodium phosphate buffer (pH 6.7), preheated at the same temperature used in the incubations, were added. Each sample was transferred to an ice-cold bath. After 5 min at 0 °C, the sample was applied to a gel-filtration Superdex 75 HR10/30 column (*A*) or an ion-exchange Source 15S HR10/30 column (*B*) (3). As a control, aliquots of each RNase A solution were brought to 0 °C immediately after preparation, kept for 10 min at 0 °C, and then chromatographed at 23 °C. No significant differences were observed compared with the data obtained by incubating the same solutions at 23 °C. In *A* and *B*, identification of the various oligomers formed was performed by comparison of their elution properties with those of the already well characterized dimers, trimers, or tetramers, recently prepared by the lyophilization procedure (3) and used as standards in this work. *M*, monomer; *T*, trimer; *TT*, tetramer; C_D, C-dimer; N_D, N-dimer.

TABLE I

Dimeric, trimeric, and tetrameric aggregates formed by RNase A dissolved in various media and kept for 30 min at 60 or 23 °C

The RNase A concentration in each fluid was 200 mg/ml, except in 40% acetone, for which the RNase A concentration was 170 mg/ml. Eppendorf tubes containing 2 μl of each solution were put in a thermostatically controlled water bath at 23 or 60 °C for 30 min. Then, 200 μl of 0.2 M sodium phosphate buffer (pH 6.7) at 23 °C or preheated at 60 °C were added to the samples in the 23 and 60 °C water baths, respectively. Each sample was transferred to an ice-cold bath. After 5 min at 0 °C, the sample was applied to a gel-filtration or ion-exchange column or frozen (−20 °C) until used. Elution was performed, and the amounts of aggregates formed were calculated as described under "Experimental Procedures." As controls, aliquots of each RNase A solution were brought to 0 °C immediately after preparation, kept for 10 min at 0 °C, and chromatographed at 23 °C. No significant differences were observed compared with the data obtained by incubating the same solutions at 23 °C and then chromatographing. Values are means ± S.D. N_D and C_D, N-dimer and C-dimer, respectively; T, trimer (mixture of the two conformers). TT, tetramer (mixture of the two conformers); ddH_2O, double-distilled H_2O.

Solution of RNase A in:	N_D at 60 °C	C_D at 60 °C	T at 60 °C	TT at 60 °C	N_D at 23 °C	C_D at 23 °C	No. of experiments	
							60 °C	23 °C
	%	%	%	%	%	%		
40% aqueous ethanol	8.40 ± 1.59	16.52 ± 2.89	6.90 ± 1.77	3.28 ± 1.31	2.21 ± 0.67	0.82 ± 0.19	9	7
40% buffered ethanol[a]	8.48 ± 1.33	17.11 ± 1.49	5.62 ± 0.89	1.90 ± 1.26	0.56 ± 0.40	0.31 ± 0.10	5	4
40% aqueous TFE	8.72 ± 1.53	17.93 ± 1.04	6.21 ± 1.48	1.70 ± 0.58	1.73 ± 0.35	0.54 ± 0.18	5	5
20% aqueous ethanol	7.40 ± 2.44	6.24 ± 3.37	2.40 ± 1.58	0.79 ± 0.56	2.26 ± 0.48	0.65 ± 0.22	7	8
20% buffered ethanol[a]	5.50 ± 0.70	2.74 ± 0.59	0.60 ± 0.38	0	1.60 ± 0.31	0.52 ± 0.12	3	3
Gly-HCl buffer(pH 3)	2.41 ± 0.14	0.80 ± 0.49	0	0	1.89 ± 0.69	0.53 ± 0.26	4	5
dd H_2O	2.61 ± 1.02	1.50 ± 0.51	0	0	2.20 ± 0.68	0.63 ± 0.24	6	6
0.02 M NaCl(pH 7)	2.21 ± 0.50	0.90 ± 0.31	0	0	1.88 ± 0.61	0.57 ± 0.16	5	5
Borate buffer								
pH 8.5	3.41 ± 0.79	0.80 ± 0.31	0	0	1.22 ± 0.20	0.08 ± 0.03	6	4
pH 9.0	3.53 ± 0.39	0.82 ± 0.34	0	0	0.71 ± 0.10	0.05 ± 0.03	9	5
pH 10	4.19 ± 1.68	0.93 ± 0.47	0	0	0.84 ± 0.43	0.05 ± 0.02	10	5
30% acetone	4.73 ± 1.10	2.08 ± 1.06	1.10 ± 0.14	0.29 ± 0.07	3.23 ± 0.28	1.32 ± 0.26	5	4
40% acetone	5.01 ± 1.95	2.29 ± 1.31	1.06 ± 0.38	0.31 ± 0.11	2.40 ± 0.29	1.10 ± 0.50	4	3

[a] With 0.08 M sodium phosphate buffer (pH 6.7).

it appears that RNase A has a remarkable propensity to oligomerize (see also Ref. 9), and this propensity might even increase after some treatment. This could be the reason why commercial preparations of RNase A are sometimes found to contain considerable amounts of aggregated material (15).

At 50 °C (Fig. 2*B*), oligomerization was more conspicuous, reaching equilibrium after 60 min of incubation. Although the experimental data appear to be highly variable between ~5 and 30 min, an inversion of the relative proportions of the N-dimer and C-dimer seemed to occur after 35 min.

At 60 °C (Fig. 2*C*), definitely larger amounts of all oligomeric species were produced, and the quantity of the C-dimer was always remarkably higher than that of the N-dimer. At 60 min of incubation, the relative amounts were 9.7% for the N-dimer and 16.4% for the C-dimer. Their ratio (1:1.7) is far from the 1:4 ratio usually obtained with the lyophilization procedure. The RNase A trimer, already formed at 45 °C (Fig. 2*A*), became more abundant at 50 °C (Fig. 2*B*) and definitely increased at 60 °C (Fig. 2*C*). After 1 h of incubation, it was present at 9.7%, identical to the N-dimer. At the same time, the quantity of tetramers formed, negligible at 50 °C (Fig. 2*B*), was ~5% at 60 °C (Fig. 2*C*). No significant increase in the amount of the two dimers and the higher order aggregates occurred after 60 min of incubation.

Oligomerization of RNase A Dissolved in 40% TFE and Heated at 60 °C—A similar pattern of oligomerization was

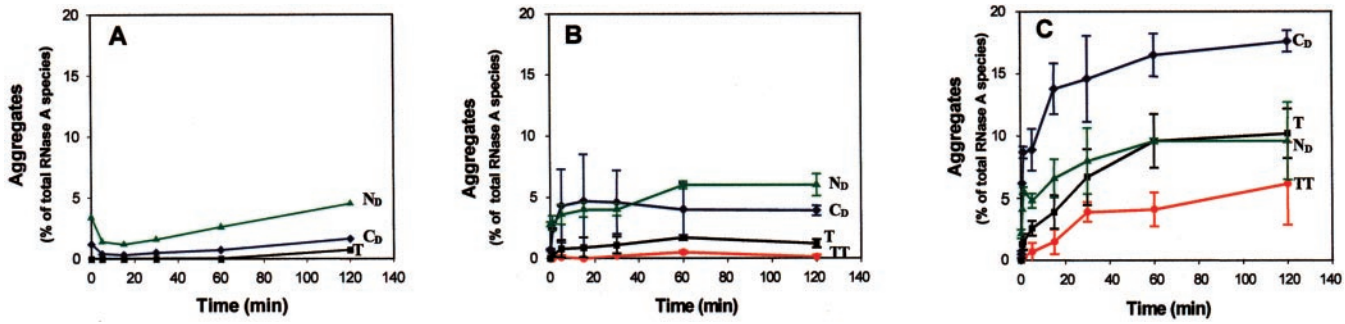

FIG. 2. **Formation of aggregated dimers, trimers, and tetramers by RNase A dissolved in 40% aqueous ethanol and incubated at 45, 50, or 60 °C.** RNase A was dissolved in 40% ethanol at a concentration of 200 mg/ml. For each time of incubation, Eppendorf tubes containing 2-μl aliquots were transferred to a thermostatically controlled water bath already brought to 45 °C (*A*), 50 °C (*B*), or 60 °C (*C*). At the times indicated, 200 μl of preheated 0.2 M sodium phosphate buffer (pH 6.7) were added to each sample, and the tube was transferred to an ice-cold bath. After 5 min, the sample was applied to a gel-filtration column and eluted as described under "Experimental Procedures." Otherwise, it was frozen until used. Each point of the curves represents the value of the area of the chromatographic peak of the N-dimer (N_D; *green*), C-dimer (C_D; *blue*), trimer (*T*; *black*), or tetramer (*TT*; *red*). Each area is expressed as the percentage of the sum of the areas of the peaks of all eluted RNase A species. Each point is the mean of two to four experiments for *A–C*, respectively.

found upon incubation at 60 °C of RNase A dissolved in 40% TFE (Fig. 3). After 60 min, the formation of the C-dimer (~17% at 60 min) was remarkably favored over that of the N-dimer (7.6%), as well as over that of the trimeric and tetrameric species (7.8, and 2.7%, respectively), the amounts of which were lower than the amounts found in 40% ethanol at the same temperature (Fig. 2*C*).

Oligomerization of RNase A Dissolved in 20% Aqueous Ethanol and Heated at 60 °C—The pattern of the oligomerization of RNase A under these conditions (Fig. 4), which was rather similar to the pattern observed at 50 °C in 40% ethanol (Fig. 2*B*), reveals that these two conditions are qualitatively equivalent and indeed critical (see also Table I) for the aggregation process. They appear to be a transition point between the experimental conditions favoring the prevalence of one or the other of the two dimers. In conclusion, under these conditions, the quantities of the two dimers fluctuated around an average value of ~50% of each conformer (see also Fig. 1, *A* and *B*, *solid* and *dashed red lines*). Because of its significance, the analysis of RNase A aggregation in 20% ethanol at 60 °C was repeated, but the two conformers were separated by ion-exchange chromatography. The results of four different experiments (data not shown) are in very good agreement with those obtained by gel filtration.

Analysis of the Thermal Unfolding of RNase A Dissolved in Various Media—The transition profiles for the thermal unfolding of RNase A dissolved in many of the fluids used in the experiments described were determined spectrophotometrically at 287 nm and are shown in Fig. 5. The decrease in absorbance (16, 17) is ascribable to the increased exposure of tyrosine residues as a function of the increase in temperature. Recent studies using Tyr-to-Phe mutants have assigned the absorbance change upon unfolding largely to three buried tyrosines at positions 25, 92, and 97 (18). Therefore, the steep changes in absorbance detected (Fig. 5) should reasonably be essentially ascribed to the global unfolding of the whole protein. However, Tyr^{115}, located in the hinge loop of the RNase A C terminus (residues 112–115) (6), might also be important in the absorbance changes that occur in the unfolding events leading to C-dimer formation. The temperatures at which the absorbance inflections occurred (Fig. 5) varied under different conditions: when RNase A was dissolved in 40% aqueous TFE or ethanol, the absorbance lowering took place at lower temperatures, whereas under milder conditions (borate buffer at pH 8.5 or 10, double-distilled water, etc.), the absorbance changes shifted toward higher temperatures. Therefore, the correlation between RNase A unfolding and the prevalence of

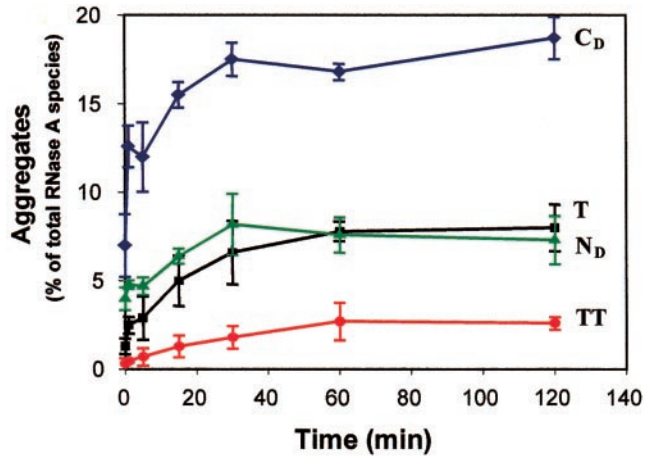

FIG. 3. **Formation of aggregated dimers, trimers, and tetramers by RNase A dissolved in 40% aqueous TFE.** RNase A was dissolved in 40% TFE at a concentration of 200 mg/ml. For each incubation time, Eppendorf tubes containing 2-μl aliquots were transferred to a thermostatically controlled water bath already brought to 60 °C. At the times indicated, each sample was processed as described in the legend to Fig. 1. Each point of the curves represents the value of the area of the chromatographic peak of the N-dimer (N_D; *green*), C-dimer (C_D; *blue*), trimer (*T*; *black*), or tetramer (*TT*; *red*). Each area is expressed as the percentage of the sum of the areas of the peaks of all eluted RNase A species. Each point of the curves is the mean of three experiments.

one or the other dimeric conformer is clear: under conditions in which the absorbance lowering occurs definitely below 60 °C, the C-dimer, formed by the C-terminal end-swapping mechanism (6), is produced in higher amounts. This means that unfolding occurs at both the N and C termini of the protein. Under milder conditions in which the absorbance lowering occurs above 60 °C, the N-dimer is more abundant, indicating the prevalent unfolding (and swapping) of the N terminus of RNase A. Under intermediate conditions (20% ethanol at 60 °C and 40% ethanol at 50 °C) in which absorbance changes in the protein occur just below or close to 60 °C, the amounts of the two dimeric conformers become similar, possibly indicating that the unfolding events occurring at the N and C termini of RNase A are equivalent, as well as the energy requirements for the two aggregation events occurring via 3D domain swapping.

The steep absorbance inflection related to the unfolding of RNase A in 40% TFE occurred at ~32 °C (Fig. 5). To further analyze the correlation between the thermal unfolding of the protein and the yield of one or the other dimeric conformer, the

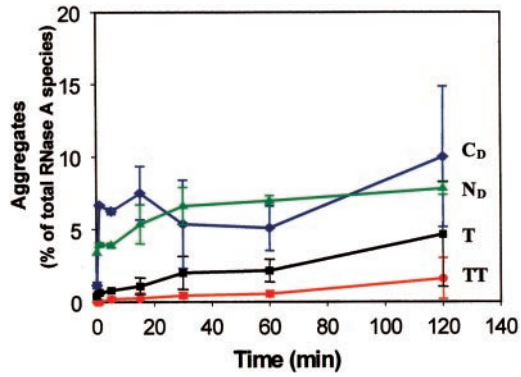

FIG. 4. **Formation of aggregated dimers, trimers, and tetramers by RNase A dissolved in 20% aqueous ethanol.** RNase A was dissolved in 20% ethanol at a concentration of 200 mg/ml. For each time of incubation, 2-μl aliquots were dispensed in Eppendorf tubes and transferred to a thermostatically controlled water bath already brought to 60 °C. At the times indicated, each sample was processed as described in the legend to Fig. 1. Each point of the curves represents the value of the area of the chromatographic peak of the N-dimer (N_D; *green*), C-dimer (C_D; *blue*), trimer (*T*; *black*), or tetramer (*TT*; *red*). Each area is expressed as the percentage of the sum of the areas of the peaks of all eluted RNase A species. Each point is the mean of four experiments.

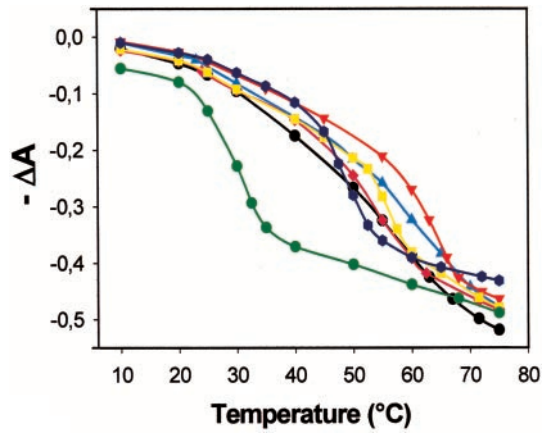

FIG. 5. **Thermal unfolding of RNase A dissolved in various media.** In each fluid (H_2O (*red*), borate buffer at pH 8.5 (*cyan*) or 10 (*black*), glycine HCl buffer at pH 3 (*pink*), 20% (*yellow*) or 40% (*blue*) ethanol, or 40% TFE (*green*)), RNase A was dissolved at a concentration of 1.5 mg/ml 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 and used for drawing the curve. The absorbance in H_2O, extrapolated to 0 °C, was used as the A_0 value.

oligomerization process was studied at a concentration of 200 mg/ml RNase A in 40% aqueous TFE heated at lower temperatures (30–45 °C) for 120 min. The results obtained are shown in Table II. Although the amount of the N-dimer was higher at 30 °C than that of the C-dimer at any time of incubation, an inversion of their relative proportions possibly occurred between 30 and 32 °C. At 32 °C, as well as at 35 °C and even more at 45 °C, the amount of the C-dimer became definitely higher than that of the N-dimer. Moreover, by comparing the results obtained upon the aggregation of RNase A under all conditions used (Figs. 1–4 and Tables I–IV) with the relative absorbance inflections detected (Fig. 5), the correlation mentioned above appears to be confirmed.

Oligomerization of RNase A in 40% Acetic Acid or in 5–40% Acetone—We also analyzed the oligomerization of RNase A in 40% acetic acid solutions (protein concentration of 66.7 mg/ml) heated at 60 °C for 120 min (data not shown). In aliquots withdrawn from time to time from the incubation mixture, both dimers were found in very modest quantities (1–2%), which, however, could partly be also ascribable to the definitely lower protein concentration used in these experiments. The amount of the C-dimer was twice that of the N-dimer over the entire course of the experiment. Moreover, in the solution prepared at 23 °C to perform the experiment, the C-dimer was found, once again, in amounts (4–5%) definitely higher than those measured later during the 2-h incubation at 60 °C. In other words, it appears, as already mentioned, as if exposure of the RNase A solution to too drastic conditions could result in the dissociation of the oligomer(s) formed at room temperature.

We also studied the oligomerization pattern of RNase A dissolved in 5–40% acetone and heated at 60 °C for up to 120 min (Table III). The relative proportions of the two dimers were similar to those forming under mild unfolding conditions (all media at 23 °C; borate buffer at pH 8.5, 9, or 10; water; NaCl; etc.). Under all conditions, the N-dimer formed at a higher amount than the C-dimer. Unfortunately, the thermal transition curve of RNase A in acetone could not be determined because of the known strong absorbance of acetone in UV light.

Oligomerization of RNase A at 70 °C—To try to understand the results obtained in the presence of acetone, the temperature of incubation of RNase A dissolved in several different concentrations of acetone was increased from 60 to 70 °C. Under these conditions, an inversion of the proportions of the two dimers took place, with the prevalence now of the C-dimer over the N-dimer (Table IV). This result can be explained as due to a more severe unfolding of RNase A obtained at 70 °C. The next step was to heat (70 °C, 60 min) RNase A dissolved in double-distilled water or borate or glycine HCl buffer. When these RNase A solutions were incubated at 23 and 60 °C (Fig. 1, *A* and *B*, *green lines*; and Table I), the amounts of the N-dimer were always higher than those of the C-dimer. Now, at 70 °C (Table IV), the relative proportions of the two dimeric conformers inverted in all cases, with the quantity of the C-dimer prevailing over that of the N-dimer. This result could be explained with the data shown in Fig. 5: the absorbance inflection of the RNase A thermal unfolding profile occurred below 70 °C in all media used. The almost identical pattern of RNase A aggregation occurring in aqueous solvents in the presence or absence of various concentrations of acetone could be explained on the basis of the solvation process. Acetone, ethanol, and TFE are strong denaturing agents, but acetone, unlike ethanol or TFE, is not a hydrogen donor. Therefore, in the presence of various concentrations of acetone, the protein domains exposed in the first sphere of solvation are preferentially solvated by water (hydrogen donor) and not by acetone. In contrast, solvation by ethanol and TFE (if present in aqueous media) is possible because they are hydrogen donors like water, but less structured than water, entropically favoring ethanol and TFE in their solvating action.

We completed the analysis at 70 °C by a 60-min incubation at this temperature of RNase A dissolved in 40% ethanol or TFE, *i.e.* under vigorous unfolding conditions, or in 20% ethanol, which at 60 °C might be taken as a point of transition between mild and strong unfolding conditions. The amount of the C-dimer always prevailed over that of the N-dimer (Table IV). The absolute amounts of the two dimers found at 70 °C were lower than those measured at 60 °C, which again suggests that the oligomers can be destabilized if exposed to too drastic treatments.

RNase A Oligomers Larger than Dimers—Trimers and tetramers also formed, as already shown, under various experimental conditions: at 50 °C in 40% ethanol, at 60 °C in 20% ethanol, as well as at 60 °C in 40% ethanol or TFE and also at

TABLE II

Formation of dimeric aggregates by RNase A dissolved in 40% TFE as a function of time and temperature

Eppendorf tubes containing 2 μl of a solution of RNase A in 40% TFE (concentration of 200 mg/ml) prepared at 23 °C were transferred to a thermostatically controlled water bath already brought to the temperatures chosen for the incubation. At the times indicated, each sample was processed as described in the legend to Table I. The values are means of duplicated experiments. N_D and C_D, N-dimer and C-dimer, respectively.

T	5 min		15 min		30 min		60 min		120 min	
	N_D	C_D	N_D	C_D	N_D	C_D	N_D	C_D	N_D	C_D
°C	%		%		%		%		%	
30	0.48	0.14	0.68	0.27	0.33	0.12	0.59	0.17	0.77	0.19
32	0.28	0.33	1.00	1.70	0.84	1.66	0.70	1.60	1.28	1.84
35	1.76	2.39	1.36	1.49	2.12	6.55	1.99	3.98	1.56	3.15
45	1.86	5.80	2.28	8.33	3.01	9.49	2.97	9.43	3.12	10.07

TABLE III

Oligomers formed by RNase A dissolved in aqueous acetone as a function of different acetone concentrations and time of incubation at 60 °C

2 μl of 200 mg/ml RNase A dissolved in 5, 10, or 30% aqueous acetone or 170 mg/ml RNase A dissolved in 40% acetone were dispensed in Eppendorf tubes for each time of incubation and immediately transferred to a thermostatically controlled water bath already brought to 60 °C. At the times indicated, each sample was processed as described in the legend to Table I. The values are means of duplicated experiments.N_D and C_D, N-dimer and C-dimer, respectively.

Acetone	15 min		30 min		60 min		120 min	
	N_D	C_D	N_D	C_D	N_D	C_D	N_D	C_D
%	%		%		%		%	
5	1.25	0.13	2.06	0.47	2.85	0.59	2.60	0.42
10	1.39	0.33	2.10	0.39	2.20	0.42	3.39	0.98
30	1.28	0.42	1.95	0.64	3.41	0.85	3.88	1.26
40	2.38	0.51	2.25	0.72	4.19	1.25	2.78	1.01

TABLE IV

Oligomers formed by RNase A dissolved in various media and incubated at 70 °C for 60 min

Eppendorf tubes containing 2 μl of RNase A (200 mg/ml; 170 mg/ml for 40% acetone) dissolved in the various media were put in a thermostatically controlled water bath preheated at 70 °C. After 60 min, the samples were processed as described in the legend to Table I. The values are means of duplicated experiments. N_D and C_D, N-dimer and C-dimer, respectively; T_1 and T_2, major and minor trimers, respectively; ddH_2O, double-distilled H_2O.

Oligomer	RNase A in:									
	5% acetone	10% acetone	30% acetone	40% acetone	ddH_2O	Borate buffer (pH 10)	Gly-HCl buffer (pH 3)	20% ethanol	40% ethanol	40% TFE
N_D (%)	5.03	4.08	4.44	4.80	5.89	4.03	4.41	5.48	2.75	4.12
C_D (%)	7.36	7.18	6.70	7.21	7.85	6.82	5.49	8.24	8.62	11.21
T_1 (%)	2.33	1.25	1.06	2.20	1.53		1.24	2.45	0.71	2.10
T_2 (%)	0.57	0.40	0.37	0.68	0.81		0.44	0.83	0.27	0.98

70 °C. It might be useful to remember that the major trimer (T_1) could be a linear structure, formed via both N- and C-terminal end swapping, whereas the minor trimer (T_2) is a circular structure, formed only by the C-terminal end-swapping mechanism (6, 7). The two trimers were well separated and quantified, and the ratios of T_1 to T_2 were 2.65:1 in 40% ethanol and 2.20:1 in 40% TFE. These values are the closest possible to the 1.5:1 ratio (typical for the two trimers, T_1/T_2) constantly found with the lyophilization procedure (4). The data presented in Table IV are generally consistent. In conclusion, although the amount of T_1 slightly exceeds that of T_2 under severe unfolding conditions, the equilibrium between the two trimeric conformers is always definitely shifted toward the major trimer (T_1) under mild unfolding conditions: the gel-filtered trimers obtained under mild incubation conditions (23 °C, H_2O, NaCl, borate buffer at pH 10, etc.) (see Table I), analyzed by nondenaturing gel electrophoresis, showed in fact a remarkable prevalence of T_1 over T_2 (data not shown).

Why Do Different Amounts of the Two Dimers Form under Different Experimental Conditions?—The results reported indicate that mild unfolding conditions allow the production of higher amounts of the N-dimer, whereas strong unfolding conditions allow the prevailing production of the C-dimer. This means that, under mild unfolding conditions, the opening and swapping of the N-terminal peptide (residues 1–15) definitely prevails over the opening and swapping of the C-terminal peptide (residues 116–124). The results concerning the two trimeric conformers, T_1 and T_2, as previously discussed, support the view that the opening of the N terminus of RNase A is easier than the opening of its C terminus. In this context, it is worth mentioning that the dimension of the "closed interface" of the C-dimer is 1716 Å^2 and that of the N-dimer is 1592 Å^2 (6). This difference is in line with the idea that a higher energy contribution is necessary for the opening of the C-terminal strand.

Although the opening of the N or C terminus of RNase A can be considered as a first step in the oligomerization process of the protein, a second step should be responsible for the swapping and stabilization of the oligomer(s) formed. According to Chiti *et al.* (19), the regions of a protein inducing its aggregation are those relatively rich in hydrophobic amino acids and prone to form β-sheet structures, which, at the same time, however, do not participate in the formation of the folding nucleus. The N-terminal α-helix (residues 1–15) of RNase A contains a higher number of hydrophilic than hydrophobic amino acids. In contrast, the C-terminal β-strand (residues 116–124) is richer in hydrophobic amino acids. Under vigorous unfolding conditions, the hydrophobic residues present in definitely higher proportion in the C terminus could (becoming exposed in the partially denatured or unfolded state of the protein) induce the aggregation event and shift the equilibrium of the process toward the production of the C-dimer. Moreover, in the C-dimer, Asn^{113}, present in one strand of the hinge loop (residues 112–115), forms three additional hydrogen bonds with Asn^{113} in the other strand (6), further stabilizing the

structure. Under mild experimental conditions, the unfolding of the N-terminal region, richer in hydrophilic residues, should require a lower energy contribution, therefore prevailing over the unfolding of the RNase A C terminus. This would then lead to the production of a higher amount of the N-dimer compared with the C-dimer. Intermediate conditions (20% ethanol at 60 °C or 40% ethanol at 50 °C) may represent a sort of transition between the unfolding conditions favoring the formation of the C-dimer and those favoring the formation of the N-dimer. This interpretation is supported by comparison of the results originally obtained under mild experimental conditions (all media at 23 °C, NaCl, water, and borate buffer at 60 °C) (Fig. 1, *A* and *B*, *green lines*; and Table I) with those obtained under the same ionic conditions, but with an increase in the incubation temperature from 60 to 70 °C (Table IV).

It is appropriate to mention that the per residue stability of ribonuclease A has been studied by hydrogen exchange measurements in folded monomeric RNase A (20). The results obtained revealed that the C-terminal β-strand forms multiple strongly protected hydrogen bonds with the rest of the protein. In contrast, most of the hydrogen bonds formed by the N-terminal α-helix are local, *i.e.* within the helix, and weak, whereas only one or two weak to moderately strong tertiary hydrogen bonds connect the helix to the rest of the protein. Moreover, recent kinetic unfolding experiments (21) showed that the backbone NH groups in the N-terminal α-helix show significant unfolding in the burst phase and exchange locally, whereas the NH groups in the C-terminal β-strand are remarkably more resistant to local unfolding, even under vigorous unfolding conditions (5.2 M guanidine hydrochloride (pH 8) at 10 °C), and break only when the protein globally unfolds. These data are consistent with the results presented in this work, *viz.* that more vigorous unfolding conditions are required to loosen the C-terminal β-strand for swapping and oligomerization in comparison with the conditions sufficient to unfold the weakly attached N-terminal α-helix.

It is also worth pointing out that because the global stability of RNase A increases sharply from pH 2 to 5 and shows a broad maximum over pH 7–10 (22), it is surprising that the amount of the N-dimer increases at high pH values (Table I, columns 1 and 2), where the protein is more stable. A likely explanation for these results could be the strong ion pair interaction between Asp^{14} ($pK < 2$) and His^{48} ($pK = 5.9$) (23, 24), which links the N-terminal helix to the protein core and breaks at neutral and high pH values when His^{48} titrates.

In conclusion, the results of this work appear to be in line with the already advanced ideas that every protein can form aggregates, provided its concentration is high and the environmental conditions are such that they destabilize its native structure (6, 25–27). These results contribute to the understanding of the mechanism of RNase A oligomerization by 3D domain swapping (28) and of the role that 3D domain swapping has in protein aggregation.

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Thermal Aggregation of Ribonuclease A: A CONTRIBUTION TO THE UNDERSTANDING OF THE ROLE OF 3D DOMAIN SWAPPING IN PROTEIN AGGREGATION

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