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On the thermal stability of the two dimeric forms of ribonuclease A

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Abstract

The thermal stability of the two dimers of RNase A with N- or C-terminal swapped ends is investigated by means of dissociation kinetics, differential scanning calorimetry, and circular dichroism measurements. The data indicate that the dimer characterized by the swapping of the N-terminal α-helices is less prone to monomerize when compared to the dimer characterized by the swapping of the C-terminal β-strands. This finding is correlated to the structural features of the so-called open interface of the dimeric forms.

Keywords: Domain swapping; Circular dichroism; Differential scanning calorimetry; Structure–stability relationship; Protein aggregation; Open and closed interface

1. Introduction

Bovine pancreatic ribonuclease (RNase A) molecules associate into two distinct dimeric forms in various experimental conditions [1]. Initially, they were detected upon lyophilization from concentrated solutions of acetic acid [2,3]. Recently, RNase A dimers were also observed from highly concentrated protein solution under mild or strong denaturing conditions [4]. Small amounts of dimers were also found in physiological conditions [5]. As originally claimed and elegantly demonstrated by Crestfield and co-workers [6], both dimers are kept together by non-covalent interactions exploiting the 3D domain-swapping mechanism. The seminal X-ray investigations by Eisenberg and colleagues have discovered that the protomers swap different domains in the two dimers (see Fig. 1). In one dimer (AA–CS), the two monomers swap the C-terminal β-strand (residues 116–124) [7]; while in the other (AA–NS) the two monomers mutually exchange the N-terminal α-helix (residues 1-15) [8]. The structures of AA–CS and AA–NS provide the important evidence that both the N-terminal and C-terminal arms of a globular protein can be displaced from the main body and swapped between two partner monomers [7]. The occurrence of domain swapping requires a remarkable conformational change of the peptide (hinge peptide) that connects the swapped domain to the main body of the protein. The hinge peptide region of AA–NS and AA–CS corresponds to the residues 16–22 and 112–115, respectively. As a consequence of the swapping of two different fragments, AA–NS and AA–CS present completely different quaternary structures [7,8]. While AA–NS exhibits a significantly extended intersubunit interface, AA–CS shows a rather loose structure characterized by very few intermolecular contacts.

Since the catalytic residues, His12 and His119, of RNase A belong, respectively, to the N- and C-terminal regions of the protein, the occurrence of 3D domain-swapping gives rise to composite active sites in both AA–CS and AA–NS. Nevertheless, structural data [7,8] along with measurements of catalytic activity on yeast RNA and single-stranded substrates [9] suggest that the composite active sites of both AA–CS and AA–NS have a strong similarity to that of RNase A. The higher catalytic activity of AA–CS on double-

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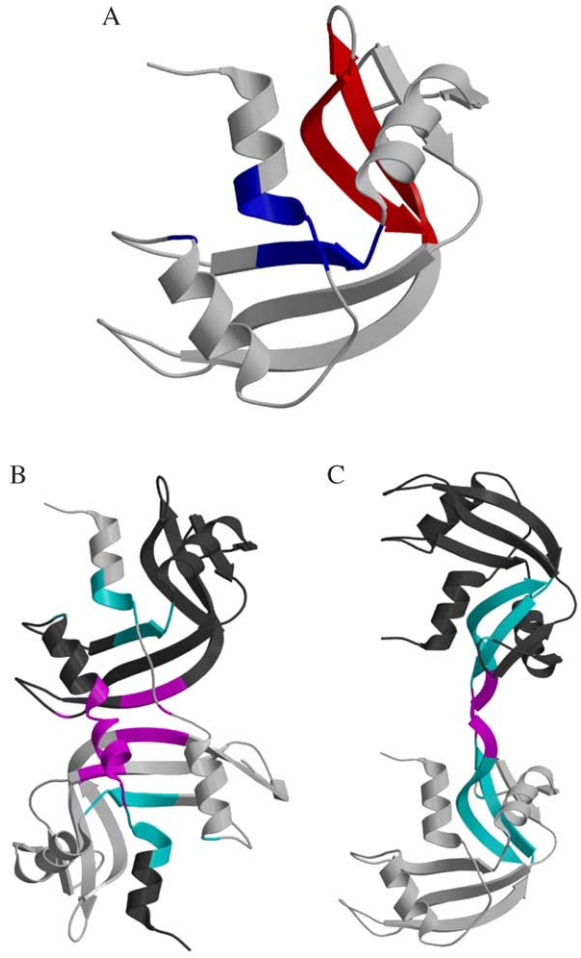

Fig. 1. 3D structures of (A) monomeric RNase A (Protein Data Bank code 7RSA) and its (B) AA–NS (PDB code 1A2W) and (C) AA–CS (PDB code 1F0V) dimeric forms. A qualitative representation of C- and O-interfaces is also given. In the monomer, the C-interfaces located at the N- and C-terminal region of the protein are coloured in blue and in red, respectively. In the dimers, the two subunits are coloured in light and dark grey whereas the C- and the O-interfaces are coloured in cyan and in magenta, respectively. The picture was generated using the programs MOLSCRIPT [26] and Raster3D [27]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

stranded substrates when compared to AA–NS should not be due to structural differences in the active sites, but it has been ascribed to the more basic nature of AA–CS [9].

Since the two dimeric forms of RNase A have gained general attention as prototypes of both 3D domain-swapped dimers [10] and in vitro protein aggregates [7,10], we performed an investigation of their thermal stability. This was also prompted by the recent finding [11] that AA–CS and AA–NS are endowed with cytotoxic and aspermatogenic effects, which have been attributed to the dimeric nature of the two proteins. Furthermore, the study would be also of interest from the methodological point of view. Since both dimers are metastable structures, their stability cannot be characterized on simple thermodynamic grounds. By combining dissociation kinetics results with differential scanning calorimetry and circular dichroims data, it emerges that AA–NS is more stable than AA–CS with respect to monomerization. Analysis of the data in conjunction with the structural information available allows a rationalization of this finding.

2. Experimental

2.1. Proteins

Bovine pancreatic RNase A (type XII-A) was purchased from Sigma. Dimers of RNase A were obtained as previously described [9]. The protein, lyophilized from 40% acetic acid [2], was dissolved in 0.2 M sodium phosphate buffer pH 6.55 and fractionated on a Sephadex G-75 column (1.5 × 105 cm) equilibrated with the same buffer. The dimeric fraction, concentrated and dialyzed against buffer A (40 mM sodium phosphate buffer, pH 6.55) with Centriprep concentrators (Amicon), was resolved into AA–CS (previously called D-I [9]) and AA–NS (previously called D-II [9]) dimers on a Pharmacia FPLC system equipped with a Source 15 S HR 10/10 column equilibrated with buffer A and run with a phosphate gradient using 200 mM sodium phosphate, pH 6.55 as buffer B. Protein concentration was determined spectrophotometrically, using ε_{278}=9800 M^{-1} cm^{-1} for monomeric and dimeric RNase A. The pH of all samples was measured before each measurement, at 25 °C, with a Radiometer pHmeter (model PHM93).

2.2. Dissociation kinetics

Samples of each RNase A dimer (0.4 mg mL^{-1}), equilibrated in 100 mM phosphate buffer, pH 6.55 by dialysis at 4 °C, were incubated at 37 °C. At appropriate time intervals, aliquots were analyzed by gel filtration with a flow-rate of 0.6 ml min^{-1} on a Pharmacia FPLC system equipped with a Superdex 75 10/30 column equilibrated in the same phosphate buffer. The amounts of monomer and dimer were estimated by measuring the areas of their absorbance profiles at 278 nm.

2.3. Scanning calorimetry

DSC measurements were performed with a Setaram instrument, interfaced with a data translation A/D board for automatic data accumulation, using a scan rate of 1.0 °C min^{-1}. All data analyses were accomplished using in-house software [12]. The raw data were converted to an apparent molar heat capacity by correcting for the instrument calibration curve and the buffer–buffer scanning curve and by dividing each data point by the scan rate and the number of moles of protein in the sample cell. The excess heat capacity function $<\Delta C_p>$ was obtained by means of baseline subtraction, assuming that the baseline is given by the linear temperature dependence of native state heat capacity [13]. The calorimetric enthalpy $\Delta_d H$ was determined by direct integration of the area under the curve, and the van't Hoff

enthalpy was calculated with the standard formula [14]:

$$\Delta_d H_{vH} = mRT_{max}^2 < \Delta C_p(T_{max}) > / \Delta_d H \quad (1)$$

where $m=4$ or 6 depending on the stoichiometry of the process, $N \Leftrightarrow U$ or $N_2 \Leftrightarrow 2U$, respectively; T_{max} is the temperature at which occurs the maximum of the DSC peak and corresponds to the denaturation temperature; $<\Delta C_p(T_{max})>$ is the value of the excess molar heat capacity function at T_{max}; R is the gas constant. The close correspondence between the calorimetric enthalpy $\Delta_d H$ and the van't Hoff enthalpy $\Delta_d H_{vH}$ is a necessary condition to state that the denaturation is a two-state transition [14,15].

2.4. Circular dichroism

CD measurements were made on a J715 Jasco spectropolarimeter equipped with a PTC-348 WI thermostat, under a constant nitrogen flow. Hellma quartz cells of 0.1 cm path length and a protein concentration of about 0.13 mg mL^{-1} were used for the far-UV region (190–240 nm). For the near-UV region (240–320 nm) the protein concentration was 1.3 mg mL^{-1} with a path length of 1 cm. All samples were dissolved in 100 mM sodium phosphate buffer, pH 6.55. In the melting experiments temperature was increased at a scan rate of 1.0 °C min^{-1}, and the CD signals at 222 or 242 nm were recorded.

3. Results

3.1. Dissociation kinetics

The dissociation kinetics of the two dimers at 37 °C and pH 6.55, 100 mM phosphate buffer, were studied by means of gel filtration. The process proved to be first-order for both dimers. However, the $t_{1/2}$ values were found to be different: 510 h for AA–CS and 640 h for AA–NS (see Fig. 2). This finding demonstrates that: (a) both dimers are metastable forms which spontaneously and irreversibly dissociate into RNase A monomers; (b) AA–CS is more prone than AA–NS to dissociate. The difference between AA–CS and AA–NS should be a consequence of the different quaternary architecture of the two dimers.

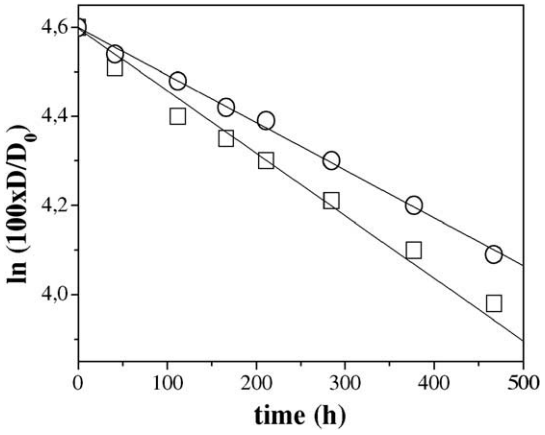

Fig. 2. Time course of dissociation of AA–NS (circles) and AA–CS (squares). At various times the samples were analyzed to detect monomer formation. D_0 and D represent dimer concentrations at time t_0 and at the indicated time, respectively.

The stability against thermal dissociation was also investigated by heating samples of AA–CS and AA–NS at about 1.0 °C min^{-1}, mimicking the temperature increase in DSC or CD heating runs, at pH 6.55, 100 mM phosphate buffer. No differences were found between AA–CS and AA–NS. When heated up to 45 °C, about 50% of either dimer was found to be dissociated; heated up to 55 °C, 80% of either dimer dissociated into monomers. These results are in agreement with those originally reported by Fruchter and Crestfield [3].

3.2. DSC data

DSC measurements on RNase A and its two dimeric forms were performed at pH 6.55, 100 mM phosphate buffer, with a scan rate of 1.0 °C min^{-1}. The DSC profiles are shown in Fig. 3, and the parameter values determined are listed in Table 1. The temperature-induced unfolding of RNase A is reversible, according to the re-heating criterion, and well represented by the two-state $N \Leftrightarrow U$ transition model, because the cooperative unit, given by the calorimetric to van't Hoff enthalpy ratio, is close to one. The thermodynamic parameters are: $T_{max}=66.5$ °C, $\Delta_d H=540$ kJ mol^{-1} and $\Delta_d C_p=5.3$ kJ K^{-1} mol^{-1}. The values of T_{max} and $\Delta_d H$ are higher than those measured for RNase A at the same pH, but in the absence of phosphate [12]. The stabilizing effect of phosphate due to its binding in the active site of RNase A is well established [16].

For the two dimeric forms it is meaningless to test the reversibility of the overall process because monomeric and unfolded RNase A chains—the only species present at high temperature—do not reconstitute the dimers on cooling the solution. Thus, in the second heating of DSC solutions, no dimeric structure was present, and we simply observed the behavior of RNase A monomers. Therefore, in order to gain insight into the thermal stability of AA–CS and AA–NS with respect to monomerization, we analyzed only the profiles recorded in the first heating of the samples containing AA–CS or AA–NS.

Let us analyze in detail such profiles, keeping in mind that the thermodynamic quantities are normalized per mole of monomer (i.e., the monomer concentration was in the range 1.2–2.5 mg mL^{-1} in DSC measurements). For AA–CS, there is a single peak characterized by $T_{max}=66.4$ °C, $\Delta_d H=515$ kJ mol^{-1} and $\Delta_d C_p=5.2$ kJ K^{-1} mol^{-1}. These values practically correspond to those determined for RNase A. In the case of AA–NS, there are two peaks: a small hump centred at 50 °C, and a large peak centred at 66.4 °C. The area of the first peak amounts to 150 kJ mol^{-1}, while that of the second one amounts to 480 kJ mol^{-1}; the overall

$\Delta_d C_p$=5.5 kJ K^{-1} mol^{-1}. All of such peaks can be represented as two-state transitions, as the cooperative unit is close to one.

The DSC heating profiles of both AA–CS and AA–NS show a large peak centred at 66.4 °C: such a peak exactly corresponds to that associated with the temperature-induced unfolding of RNase A monomers, and should represent the same phenomenon. This means that, on raising temperature, both dimers dissociate to monomers, which, in turn, unfold in an all-or-none manner, as temperature is further increased. DSC data suggest that above 55 °C there exist only monomers in the solutions containing initially the dimeric forms, in line with the results of thermal dissociation obtained by means of gel filtration.

The overall process occurring in the case of the two dimers should be described by the following two-step, dissociation/unfolding, mechanisms:

$$AA - CS \Rightarrow 2A_{CS} \Leftrightarrow 2A_U \quad (2)$$

$$AA - NS \Rightarrow 2A_{NS} \Leftrightarrow 2A_U . \quad (3)$$

On raising temperature, both dimers dissociate irreversibly to monomers, denoted as A_{CS} for AA–CS and A_{NS} for AA–NS, to underscore that they could be not completely folded and could be different between each other. Note, in fact, that in order to occur dissociation has to involve not only the disruption of the interactions at the open interface, but also the displacement of the swapped domains: the C-terminal β-strand for AA–CS, and the N-terminal α-helix for AA–NS. On further raising temperature, A_{CS} and A_{NS} unfold, in an all-or-none manner, at the same temperature of monomeric RNase A.

Even though dissociation is an irreversible step, it appears to be qualitatively different for the two dimers: (a) it is characterized by a DSC peak in the case of AA–NS, and so can be considered a cooperative phenomenon; (b) no DSC peak is present in the case of AA–CS, and so the dissociation appears as a non-cooperative phenomenon.

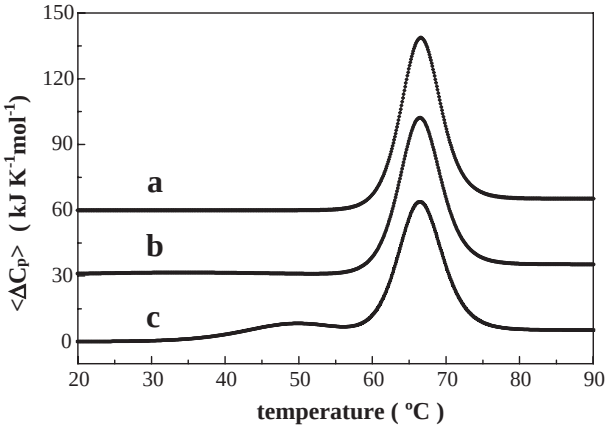

Fig. 3. DSC melting profiles at pH 6.55, 100 mM phosphate buffer, of RNase A (curve a) and RNase A dimers AA–CS (curve b), and AA–NS (curve c). The curves have been shifted along the *y* axis for ease of presentation.

Table 1
Parameters of the temperature-induced denaturation of RNase A, AA–CS and AA–NS at pH 6.55, 100 mM phosphate buffer, derived from DSC measurements

	T_{max} °C	$\Delta_d H$ kJ mol^{-1}	$\Delta_d C_p$ kJ K^{-1} mol^{-1}	CU
RNase A	66.5	540	5.3	0.96
AA–CS	66.4	515	5.2	1.04
AA–NS, 1st peak	50.0	150	–	0.92
AA–NS, 2nd peak	66.4	480	5.5	1.05

Note: CU refers to the cooperative unit, given by the calorimetric to van't Hoff enthalpy ratio. Each figure is the mean value of four measurements. The error in T_{max} does not exceed 0.2 °C, whereas those in $\Delta_d H$ and $\Delta_d C_p$ do not exceed 5% and 10%, respectively, of reported values.

This would suggest that the non-covalent interactions keeping together the two chains are stronger and more effective in AA–NS than in AA–CS.

Finally, if the hump in the DSC profile of AA–NS would represent a dissociation process, the location of its maximum should increase on raising the protein concentration. However, we could not observe such a dependence, because the peak is very flat and, in the concentration range investigated, the shift of the maximum is expected to be small. In any case, the maximum at 50 °C corresponds to about 50% dissociation, and is in line with the data obtained from the study of dissociation kinetics.

3.3. CD data

To further characterize the thermal stability of the two dimers, the CD signal at 222 nm was recorded as a function of temperature, at pH 6.55, 100 mM phosphate buffer, with a heating rate of 1.0 °C min^{-1} (i.e., the same conditions used for DSC measurements). A raise of the molar ellipticity at 222 nm indicates a loss of secondary structure. The three melting profiles are shown in Fig. 4. The profile of RNase A shows a sharp sigmoidal transition centred at 65 °C, representative of the temperature-induced unfolding of the

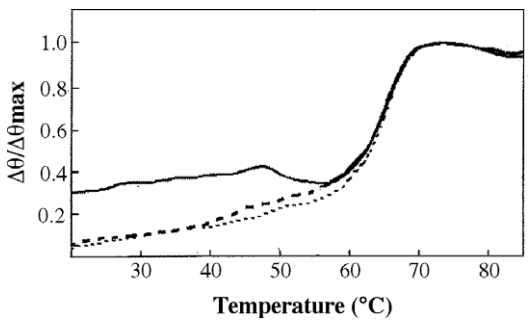

Fig. 4. Far-UV CD melting profiles recorded at 222 nm for RNase A (dotted line) and RNase A dimers AA–CS (solid line), and AA–NS (dashed line). $\Delta\theta$ represents the difference between the molar ellipticity at a given temperature and the minimum value of the molar ellipticity measured for the three proteins at 20 °C. $\Delta\theta_{max}$ is the difference between the molar ellipticity at 85 °C, which is identical for the three proteins, and the molar ellipticity at 20 °C.

native structure [12,17]. The melting profile of AA–NS is similar to that of RNase A, with a sigmoidal transition centred at the same temperature. However, a closer inspection of the curve in comparison to that of RNase A indicates that the two CD signals are different over the temperature range 40–60 °C that precedes the sigmoidal transition. The CD signal of AA–NS shows a pronounced increase, indicative of a loss of secondary structure, absent in the case of RNase A. This increase happens over the same temperature range of the small peak recorded in DSC heating profiles of AA–NS. Therefore, it should represent the same phenomenon, namely, the cooperative dissociation of AA–NS in two A_{NS} monomers.

The melting profile of AA–CS starts at a slightly different level than the others because the secondary structure content of AA–CS is different from that of the other two proteins [7,8]. On raising the temperature, the CD signal of AA–CS increases very gradually up to about 48 °C, then decreases over the range 48–56 °C, and, finally, shows a sigmoidal transition centred at 65 °C. This trend can be explained on the basis of the two-step mechanism presented above. Over the temperature range up to about 48 °C, AA–CS dissociates to A_{CS} monomers in a gradually and non-cooperative manner. Since over this temperature range the structure of RNase A is still folded, the A_{CS} monomers partially refold, causing the decrease of the CD signal over the 48–56 °C range. This partial refolding stops around 56 °C because this temperature is too high to initiate the cooperative unfolding of the RNase A structure. The latter process is manifested by the sigmoidal transition centred at 65 °C, that is common to all three protein systems investigated.

Far-UV CD data appear to confirm that the dissociation of AA–CS differs from that of AA–NS: the former is a non-cooperative phenomenon, while the latter is cooperative, suggesting, in line with DSC data, that the non-covalent interactions keeping together the two chains are less effective in AA–CS than in AA–NS.

Melting profiles for the three proteins were also determined by recording the CD signal at 242 nm, in exactly the same conditions used for DSC and far-UV CD measurements. The positive band occurring at 242 nm in the near-UV CD spectrum of RNase A and both dimers is specifically attributed to Tyr25 [18,19]. The temperature dependence of the CD signal at 242 nm should be of interest for the topological relevance of Tyr25: (a) it is close to the hinge loop of AA–NS, which includes residues 16–22 of each chain; (b) it interacts with the N-terminal region, the swapping fragment of AA–NS, of RNase A chain for the H-bond between the side-chains of Tyr25 and Asp14 [20,21]. The exposure of Tyr25 side-chain should be affected by the dissociation of AA–NS, but not by the dissociation of AA–CS. The recorded melting curves, shown in Fig. 5, emphasize that: (a) the temperature increase has identical effects on the Tyr25 exposure in both RNase A and AA–CS; (b) a significantly higher exposure of Tyr25 over the range 40–60 °C is observed in AA–NS. These results are in line with the dissociation/unfolding pathways proposed because: (a) the dissociation of AA–CS does not alter the exposure of Tyr25, structurally distant from the region where the swapping occurs; thus the melting profile recorded at 242 nm corresponds to that of RNase A; (b) the dissociation of AA–NS does alter the exposure of Tyr25 and so the melting profile recorded at 242 nm differs from those of RNase A and AA–CS.

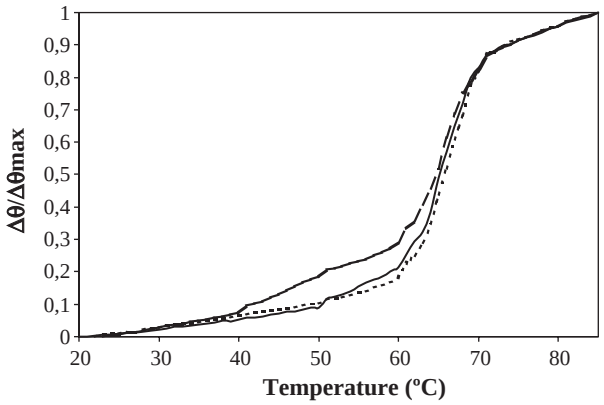

Fig. 5. Near-UV CD melting profiles recorded at 242 nm for RNase A (dotted line), and RNase A dimers AA–CS (solid line), and AA–NS (dashed line). See the legend of Fig. 3 for the definition of $\Delta\theta$ and $\Delta\theta_{max}$.

4. Discussion

According to the notation of Eisenberg and coworkers [22], the overall intermolecular interface of a domain-swapped dimer can be divided in two distinct parts. The closed interface is the intermolecular interface that pre-exists in the monomer and that is formed by the region which undergoes the swap and the rest of the protein. The open interface is the new intermolecular interface, which corresponds to a region that is open to solvent contact in each of the parent monomers. Therefore, the oligomerization through domain swapping relies on both existing and newly formed interfaces. However, since the closed interface does already exist in each monomer, the stability of the dimer against dissociation is largely dependent on the features of the open interface. A well-developed open interface, characterized by effective interactions, would lead to stable domain-swapped dimers. A stable interface is usually formed by the docking of two rather rigid secondary structure elements of the two monomers, possessing complementary interactions. The rigidity of the two faces serves to confer rigidity to the interface itself.

In this respect the structure of AA–CS [7] shows peculiar features: (a) the open interface and the closed one are spatially contiguous and cannot be topologically dissected one from the other (usually, as in the AA–NS structure, the two interfaces are spatially distant); (b) the

open interface is formed by the juxtaposition of the two hinge loops. The latter interface (residues 112–115 of each chain) forms a very short two-stranded antiparallel β-sheet, that includes Pro114, which interrupts the pattern of the β-sheet H-bonds. This short β-sheet is stabilized by the formation of three H-bonds between Asn113 of one chain with the corresponding residue of the other chain: two H-bonds between main-chain atoms and one H-bond between side-chain atoms. It is also worth noting that these hinge loops are very flexible. Indeed, residues 112–115 assume different conformations in the X-ray structure of monomeric RNase A [21], in the X-ray structure of AA–CS [7], and in the X-ray structure of the minor trimer of RNAse A [23]. Therefore, the open interface of AA–CS can be considered as a weak interface because it is not large (only 200 $Å^2$ of ASA buried) [7], and it is characterized by a limited number of H-bonds between two flexible chain segments, that surely undergo large fluctuations also at room temperature. These considerations are in line with the results of a molecular dynamics simulation study carried out on AA–CS, in which the structure of this dimer was found to undergo large fluctuations [24]. These structural features suggest that the thermal dissociation should be gradual and non-cooperative in the case of AA–CS. DSC and CD melting profiles of AA–CS, showing no peak or abrupt rise over the temperature range preceding the unfolding transition of RNase A monomers, are in line with such conclusion.

In contrast, the open interface of AA–NS is formed by the juxtaposition of the β-strands 97–103 of the two monomers (560 $Å^2$ of ASA buried), with the establishment of several H-bonds between the strands. It should be underlined that these interactions lead to the formation of a six-stranded antiparallel β-sheet extending across the open interface [8]. As shown by a molecular dynamics simulation carried out on AA–NS, the open interface of this dimer is very stable [25]. Therefore, the thermal disruption of this dimer should be a cooperative process. DSC and CD melting profiles of AA–NS, showing the hump and the rise, respectively, centred at 50 °C, confirm this expectation. These findings show that even an interface that has not been scrutinized by evolution may significantly contribute to the overall stability of the dimer. In this context, it is not surprising that new, non-native interfaces, generated through domain swapping, may play important roles in the aggregation processes responsible for neurodegenerative diseases [22].

However, since both dimers are metastable structures, their dissociation could be expected to be a kinetically-controlled process: on raising temperature monomerization occurs because the dissociation rate increases with temperature, as predicted by the Arrhenius equation with a positive activation energy. A temperature-induced kinetically-controlled process can have an almost zero enthalpy change and cannot be detected by DSC. This is not feasible for an equilibrium transition because, according to the second law of thermodynamics, any equilibrium process driven by a temperature increase must be endothermic. In this respect one has to consider that the two dimers have different structures and thus their energetic content should be different: the two open interfaces differ in both their surface area and the type and number of interactions, as pointed out above. Therefore, even though the dissociation could be a kinetically-controlled process, we propose that: (a) it should be endothermic for both dimers, because energy is required to disrupt both open interfaces; (b) it should be cooperative for AA–NS and non-cooperative for AA–CS due to the structural features of the two distinct open interfaces. It should be noted that the difference in the open interfaces manifests itself also in the largest molecular dimension of the two dimers: 85 Å for AA–CS and 77 Å for AA–NS.

A final point is related to the finding that the molar ratio between the two dimeric forms depends upon the solvent medium in which RNase A is dissolved and the selected buffer, co-solvent and temperature [3,4]. This datum can be rationalized by considering that: (a) AA–CS and AA–NS are two distinct kinetic traps in which RNase A chains fall in non-equilibrium conditions; (b) the kinetic barriers to be overcome to reach the two traps are necessarily influenced by the external conditions.

In conclusion, by combining dissociation kinetics, DSC and CD measurements of the two dimers of RNase A, AA–CS and AA–NS, a quite detailed picture has emerged. It results that: (a) the two dimers are metastable and dissociate spontaneously to monomers with different kinetics; (b) AA–NS is less prone to monomerize with respect to AA–CS; (c) thermal denaturation of both dimers can be described by a two-step dissociation/unfolding mechanism; (d) the structural determinants for the higher stability of AA–NS should reside in its open interface.

Acknowledgments

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