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Thermodynamics of protein stability: A family of ribonucleases

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Abstract. Ribonucleases constitute a ubiquitous superfamily of enzymes. Besides the well known hydrolytic activity against the RNAs, recently some of the proteins of this group, the so called RISBASE, have shown to possess a multiplicity of biological functions. In this article a short review is reported concerning systematic thermodynamic studies carried out at our laboratory mainly by means of differential scanning calorimetry on the well known ribonuclease A, RNase A, and its congeners. Among them, dimeric natural ribonuclease, BS-RNase, extracted from bull seminal plasma or vesicles, has shown very interesting features.

INTRODUCTION

Ribonucleases are a well known superfamily of ubiquitous proteins. Their main function is to re-utilize the nucleotides, through the hydrolysis of the RNAs, during each passage of the protein synthesis on ribosomes. In this manner an enormous sparing of materials is accomplished, the protein synthesis resulting highly efficient. The most studied and known, among these enzymes, is the group of ribonuclease A, RNase A, usually obtained from bovine pancreas, and its related homologues. The primary sequence of RNase A is exactly known from 1960 (1). The three-dimensional structure has been determined by XRD on the crystals (2,3) and substantially confirmed in solution by NMR (4). The RNase A conformation is characterized by three segments of α-helices and by several chain frameworks in β-sheets that interconnect all the protein globule. Four disulfide bridges cooperate in maintaining compact the overall molecule. The main body of the molecule presents a cleft into which is juxtaposed the N-terminal segment (i.e., 12 residues in α-helix conformation plus other 8 residues, constituting the so-called S-peptide, which is the product of mild digestion of RNase A by subtilisin). The uniqueness of RNase A tertiary structure is such that the stoichiometric recombination of S-peptide with the remaining part of the molecule, called S-protein, gives a product, ribonuclease S, which possesses, practically, the same structure and enzymatic activity of RNase A (5,6). Ribonuclease S proves only less thermally stable than RNase A (7-9). In addition, Anfinsen (10) demonstrated the reversible reconstitution of RNase A tertiary structure, after denaturation and reduction of disulfide bridges, without any assistance of enzymes or chaperonines. This is a direct proof that the physiological conformation of RNase A corresponds to a minimum of the Gibbs energy. Really, *in vivo* the reconstitution of disulfide bridges is catalyzed by the protein disulfide isomerase, PDI, that accelerates many times the process (11,12). It is possible that *in vitro* the reparation of some errors can require much time, decreasing the reaction rate. Particular experimental conditions are necessary, *in vitro,* to obtain some non-specific and inactive structures with scrambled disulfide bridges. The scrambled RNases, however, seem do not possess a unique tertiary structure.

From some years, in Naples, an interesting dimeric ribonuclease, BS-RNase, has been obtained from bull seminal vesicles or seminal plasma (13,14). This protein is formed by two identical subunits, each showing 80% of sequence homology with RNase A. The two subunits are covalently bonded by means of two disulfide bridges, at position 31 and 32, as proved by X-ray crystallography (15,16). In the extracellular fluids BS-RNase seems to display a multiplicity of biological functions: antispermatogenic, immunodepressive, and antitumoral action (17-19). It has been proposed from D'Alessio and co-workers (20) to call RISBASE the group of the superfamily of ribonucleases endowed with multiple biological capabilities (Ribonucleases with Intrinsic Special Biological Actions). The dimeric structure seems essential for the expression of immunodepressive and antitumoral activity and for transferring the molecule out of cell membrane (21). Monomers obtained by selective reduction of the two intersubunit disulfide

bridges are inactive (except for the usual biocatalytic capability). *Viceversa* artificial dimers of RNase A seem to gain immunodepressive and antitumoral activity (22).

In this paper we report the results of our investigations by means of differential scanning calorimetry on thermal denaturation of RNase A and some point mutants, RNase B, a glycosilated form of RNase A, and the dimeric BS-RNase. In particular, the very complex behaviour of BS-RNase is carefully discussed on the basis of our calorimetric data and the structural and biochemical information now available.

CALORIMETRY

Differential scanning calorimetry, DSC, gives a direct measure of the enthalpy change associated with the temperature-induced denaturation of globular proteins and any other conformational transitions of biological macromolecules. There is no need to introduce any model or use temperature derivatives (van't Hoff isocores). It is possible to determine the temperature of the maximum thermal effect, T_d, the heat capacity difference between the denatured and native states $\Delta_d C_p$, the analytical degree of progress of the transformation at increasing the temperature, $\Theta(T)$, and ascertain the process reversibility by means of the reheating criterion. From the determination of $\Theta(T)$, it is possible to obtain in the case of a two-state N $\Leftrightarrow$ D transition the apparent equilibrium constant as a function of temperature:

$$K(T) = \Theta(T)/[1 - \Theta(T)] \quad (1)$$

It is easy to verify the assumption that the process is a two-state transition,by calculating the molar denaturation enthalpy from the van't Hoff equation:

$$d\ln K/dT = \Delta_d H_{vH}/RT^2 \quad (2)$$

Usually the following expression, calculated for $\Theta = 0.5$ at $T = T_d$ is used (23):

$$\Delta_d H_{vH}(T_d) = 4RT_d^2\ [<\Delta C_p(T_d)>/\Delta_d H(T_d)] \quad (3)$$

If $\Delta_d H_{vH}(T_d)$ coincides (within ± 10%) with $\Delta_d H(T_d)$ directly measured by integration of calorimetric peak area, it is possible to assume that the unfolding is a two-state process. Really, this is only a necessary condition. To verify that the denaturation process is well represented by the two-state transition model, it is necessary to show that in the whole investigated temperature range only two macroscopic states are significantly populated. The analysis consists in determining, independently, the population fractions of native and denatured states, f_N and f_D respectively, and checking if their sum is equal to one for each temperature (i.e., no intermediates are present). When the ratio $R_{vH} \equiv \Delta_d H(T_d)/\Delta_d H_{vH}(T_d) > 1.0 \pm 0.1$ and the sum of f_N and f_D is less than one, a more complex process occurs. Other tests have also been proposed (24).

The simplest approach for describing a complex denaturation process is to consider it as a sum of a certain number of independent two-state transitions. It is possible to give an analytical expression for the excess heat capacity function referred to the native state $<\Delta C_p(T)>$ for a two state system:

$$<\Delta C_p(T)> = [(\Delta_d H^2/RT^2] * \{K(T)/[Q(T)]^2\} + \Delta_d C_p[K(T)/Q(T)] \quad (4)$$

where $Q(T) = 1+K(T)$ is the canonical partition function for a two-state system, assuming the native state as reference. This is the starting point of the deconvolution procedure. In fact, if the denaturation process can be considered as the sum of n independent two-state transitions, the overall excess heat capacity function is given by:

$$<\Delta C_p(T)> = \sum_{i=1}^{n} <\Delta C_p(T)>_i \quad (5)$$

where $<\Delta C_p(T)>_i$ is expressed as in Eq.4. The program uses as inputs the experimental $<\Delta C_p(T)>$ file, the supposed number of two-state transitions n, and the values of $T_{d,i}$, $\Delta_d H_i$ and $\Delta_d C_{p,i}$ associated with each transition. Then it performs a non-linear regression using the Levenberg-Marquardt algorithm (24), to adjust them until a best fit between the function $<\Delta C_p(T)>$ calculated by Eq.5 and the experimental one is reached.

RESULTS AND DISCUSSION

RNase A and RNase B

The thermal behaviour of bovine pancreatic RNase A, the highest purity commercial product of SIGMA, was studied in a wide range of pH values (25). The buffer solutions, at a concentration of 0.1 M, used in the increasing pH range were: glycine-HCl, sodium acetate; 2-[morpholino]ethansulphonate (MES); 3-N-[morpholino]-propanesulphonate (MOPS) and N-[hydroxyethyl]piperazine-N'-[2-hydroxypropanesulphonate] (HEPPSO). The results are reported in Table 1. In the last column is reported the van't Hoff ratio $R_{vH} \equiv \Delta_d H(T_d) / \Delta_d H_{vH}(T_d)$.

Table 1. Thermodynamic parameters of the temperature-induced denaturation of RNase A and RNase B.

	pH	T_d °C	$\Delta_d H(T_d)$ kJ mol^{-1}	$\Delta_d C_p$ kJ K^{-1}mol^{-1}	R_{vH}	
RNase A	2.0	35.2	274	4.8	0.85	
	3.0	53.9	372	6.3	0.90	
	3.7	56.8	435	6.4	1.03	
	5.0	61.3	465	5.5	1.01	
	5.5	61.8	480	6.2	1.00	
	6.0	62.2	490	6.5	0.99	
	7.0	62.8	500	5.7	1.01	
	8.0	63.0	505	5.5	1.00	irrev.
	8.4	64.1	523	6.8	1.00	irrev.
RNase B	5.0	61.5	451	6.5	0.95	
	8.0	64.5	545	5.0	1.06	irrev.

For RNase A $R_{vH} \cong 1.0$ except at pH 2.0, where $R_{vH} = 0.85$, and at pH 8.0 and 8.4, where $R_{vH} = 1.0$, but the unfolding proves irreversible. Other tests have also been accomplished to demonstrate, without doubts, that in the range of pH 3.5÷7.5 the denaturation is a two-state reversible transition. At pH 2.0 the compact physiological structure probably undergoes a partial swelling, while at pH 8.0 the process is irreversible due to side-reactions occurring at basic conditions and high temperature (26).

The analyses of DSC data concerning the thermal denaturation of RNase A in the presence of specific ligands (2' and 3' CMP) or denaturing agents (guanidinium salts and urea) were reported and discussed in previous works (7,27).

RNase B is identical in primary structure to RNase A and contains a single polysaccharide moiety attached through N-acetylglucosamine to asparagine 34 (28,29). The carbohydrate moiety consists of two N- acetylglucosamine residues and five to nine mannose groups. The mean molecular weight of RNase B, obtained from FAB mass spectrometry, is 14994 dalton. RNase B was obtained from the commercial Sigma product after purification by means of affinity chromatography on ConA-Sepharose in order to eliminate the abundant fraction of RNase A. Preliminary results concerning RNase B at two pH values are reported In Table 1. The data show that the T_d and $\Delta_d H(T_d)$ values are about the same of RNase A. At pH 8.0 RNase B thermal denaturation is also irreversible.

Deamidated isoforms of RNase A

Deamidation of asparagine side-chains is a post-translational non-enzymatic reaction that occurs frequently *in vivo* and/or *in vitro* for several proteins (30). It is suspected to play a physiological role in controlling the *in vivo* half-life of proteins (30), and to be one of the causes determining the irreversibility of thermal denaturation in basic conditions (26). In the case of RNase A, deamidation of Asn67 can be induced *in vitro* at pH 8.0, favoured by the neighbour Gly68 (31). The reaction gives rise to two products: (N67D)RNase A containing an α-aspartate at position 67, and (N67isoD)RNase A containing a β-aspartate at position 67. The most abundant form is N67isoD, the molar ratio being 3 : 2 (31).

DSC and CD experiments have been performed at pH 5.0 on the accurately separated and purified products of RNase A deamidation (32). From the values reported in Table 2 it is clear that the substitution N67D does not produce an appreciable change in the thermodynamic parameters and stability of RNase A. On the contrary, the substitution N67isoD with the introduction of a CH_2 group in the backbone, for the presence of an iso-peptide bond, causes a large decrease in the T_d and $\Delta_dH(T_d)$ values, while the van't Hoff ratio R_{vH} is always close to one.

Table 2. Thermodynamic parameters of the denaturation process of the two monodeamidated forms of ribonuclease A, at pH 5.0, 0.1 M sodium acetate buffer.

		T_d °C	$\Delta_dH(T_d)$ kJ mol^{-1}	Δ_dC_p kJ K^{-1}mol^{-1}	R_{vH}
(N67D)RNase A	DSC	61.5	460	5.5	1.03
	CD	61.6	455	--	-.-
(N67isoD)RNase A	DSC	55.0	400	5.4	0.98
	CD	55.3	390	--	--

To perform a thermodynamically correct comparison between the stability of RNase A and (N67isoD) form, we compared at the T_d value of the latter protein, the denaturation enthalpy and entropy changes of both enzymes. The analysis shows that (N67isoD)RNase A is less stable than the parent enzyme for enthalpic factors, amounting to 30 kJ mol^{-1} at 55.0°C, which are partially offset by entropic factors. The X-ray structure of (N67isoD)RNase A, recently solved at 1.9 Å (33), points out that the introduction of a CH_2 group in the external loop closed by the disulfide bridge between Cys65 and Cys72 causes the loss of two important intra-loop hydrogen bonds, and an increase in the chain flexibility. Therefore, the loss of two-hydrogen bonds well correlates to the decrease in denaturation enthalpy and the increase in denaturation entropy pointed out by DSC and CD measurements.

Bovine seminal ribonuclease

In previous works we perfomed preliminary DSC investigations on BS-RNase (25,34). The main features of DSC profiles and the values of Δ_dH and R_{vH} pointed out that the temperature-induced denaturation was not a simple two-state transition. The analysis suggested that the overall process could be treated as the sum of two independent two-state transitions. Deconvolution of DSC curves, according to Eq.5, gave enthalpy values suggesting that the cooperative melting domains are not the two subunits, but, probably, the peripheral framework and the core of the dimer stabilized by the two intersubunit disulfide bridges. These findings were in agreement with the structural information available. X-ray analysis of BS-RNase performed by Mazzarella and co-workers (15,16,35), showed that the two N-terminal segments are swapped between the two subunits, giving rise to composite active sites. Indeed the residues involved in the active site belong to different chains (Lys7, Glu11 and His12 from one chain, and Lys41, Thr45 and His119 from the other). The molecule possesses a pseudo-symmetry binary axis, as one subunit is translated of few angstroms after the 180° rotation. It is worth noting that the swapping of the N-terminal segments is the mechanism proposed to stabilize the non-covalent artificial dimer of RNase A (36,37).

Actually, D'Alessio and co-workers (38) unequivocally demonstrated that native BS-RNase in solution exists as a 2:1 mixture of two isomers: the form in which there is the swapping of the N-terminal segment, called MxM; and the form in which the swapping does not occur and the N-terminal segment is tilted on its own subunit, called M=M. The most abundant conformational isomer MxM, is the only isomer selected in the crystals. The two isomers can be separated at room temperature by suitable gel filtration procedures (38), even though they continue to interconvert between each other with a rate depending on temperature. The existence of M=M isomer was strongly supported by the fact that the monomeric derivative of BS-RNase is fully active (39): this requires that the N-terminal segment is tilted on its own subunit in order to reconstitute the active site.

Our DSC results on these isomeric forms at pH 5.0 and 8.4 are collected in Table 3, where BS-RNase stands for the natural mixture of MxM and M=M forms in a 2:1 molar ratio; and MCM-BS-RNase is the monomeric derivative of seminal ribonuclease obtained by selective reduction with dthiothreitol of the two intersubunit disulfide bridges and carboxymethylation of the free SH-groups with iodoacetic acid (39).

Temperature-induced denaturation at pH 5.0 is a reversible process for all dimeric proteins and the monomeric derivative MCM. At pH 8.4 only the monomer MCM shows a reversible denaturation, while the dimeric forms probably undergo side-reactions favoured by temperature and alkalinity, which prevent the refolding of the polypeptide chain on cooling.

Table 3. Thermodynamic parameters of the temperature-induced denaturation of BS-RNase.

	pH	T_d °C	$\Delta_d H(T_d)$ kJ mol^{-1}	$\Delta_d C_p$ kJ K^{-1}mol^{-1}	R_{vH}	
BS-RNase	5.0	61.6	633	9.3	1.7	
MxM	5.0	61.4	643	8.0	2.1	
M=M	5.0	61.3	612	6.2	2.0	
MCM-BS-RNase	5.0	55.8	363	5.2	0.95	
BS-RNase	8.4	65.7	722	9.0	1.5	irrev.
MxM	8.4	64.9	790	7.7	2.3	irrev.
MCM-BS-RNase	8.4	57.8	370	6.5	1.0	

The two forms MxM and M=M do not show remarkable differences between each other and with respect to BS-RNase. The temperatures of the maximum heat effect are practically identical to the T_d values of RNase A at the same pHs. On the contrary, the values of the denaturation enthalpy are lower than twice the $\Delta_d H$ values of RNase A, as it would be expected. This finding may be due to the fact that the presence of two intersubunit disulfide bridges makes the denatured state very different from a random coil (i.e., the dimeric forms of BS-RNase possess 10 disulfide bridges), and prevents the loss of a higher fraction of intramolecular contacts with respect to RNase A. Furthermore, the values of the van't Hoff ratio are greater than one, emphasizing that the process is not well represented by the two-state transition model. It could be accomplished a deconvolution of DSC curves according to Eq.5. However, there is strong evidence that the increase of temperature at a scan rate of 0.5 °C min^{-1} in a DSC experiment greatly enhances the rate of the interconversion MxM $\Leftrightarrow$ M=M. Therefore, all DSC measurements on dimeric forms refer, in reality, to mixtures and this makes uncertain the interpretation of the deconvolution results.

On the other hand, the monomeric derivative, whose denaturation is a reversible two-state transition both at pH 5.0 and 8.4, proves markedly less stable than the strictly homologous RNase A. For instance, at pH 5.0, the values of T_d and $\Delta_d H(T_d)$ are 5.5 °C and 100 kJ mol^{-1}, respectively, lower than the corresponding ones of RNase A. These results are in line with the structural information available on MCM derivative. NMR measurements performed by Temussi and co-workers (40) showed that in MCM the juxtaposition of the N-terminal segment into the cleft of the main body of the molecule is not so effective as in RNase A. These data seem to suggest that, in all probability, there are little but significant structural differences between the MxM and M=M isomers. This is not surprising because from a biochemical and biological point of view there are dramatic differences: only the MxM form shows allosteric regulation of the rate-determining-step of biocatalytic activity (38) and possesses antitumor capability (41).

RNase A mutants

A cDNA coding for RNase A was mutated to insert in the mutant protein chain a Pro residue at position 19, a Leu residue at position 28, and Cys residues at position 31 and 32 (42,43). These residues substitute Ala, Gln, Lys, and Ser, respectively, of wild-type RNase A. The mutant cDNA was expressed in *E.coli.* The mutant proteins, sequestered into inclusion bodies, were unfolded, purified and refolded into catalitically active proteins in the presence of a glutathione redox buffer. These four residues have a key-role in determining the dimeric structure and the N-terminal segment swapping of BS-RNase, as suggested by Mazzarella, D'Alessio and co-workers (35,42,43). Some of these mutants of RNase A can be made dimeric, with a dual quaternary structure and also active as an antitumor agent (43).

DSC measurements were performed in 0.1 M sodium acetate buffer at pH 5.0 for comparing the thermal stability of RNase A with that of P-RNase A (A19P), PL-RNase A (A19P/Q28L), MCAM-PLCC-RNase A (A19P/Q28L/K31C/S32C, monomeric and carboxyamidomethylated form) and the

carboxyamidomethylated monomer of recombinant BS RNase, MCAM-rBS-RNase. The results are collected in Table 4. In the selected experimental conditions the thermal denaturation of all these proteins is a two-state process, as confirmed by the closeness to one of the van't Hoff ratio. In addition, the transitions prove reversible according to the reheating criterion. The single substitution Ala19Pro causes a decrease of denaturation temperature of about 2 °C, T_d passes from 61.3 °C for RNase A to 59.6 °C for P-RNase A and $\Delta_d H(T_d)$ decreases from 465 to 415 kJ mol^{-1}. These findings are in contrast with the expectation that the introduction of a proline residue will cause an entropic stabilization of the native structure as a consequence of the lower conformational freedom in the denatured state. However, they are in agreement with the theoretical calculations performed by Mazzarella's group (44) using the ECEPP force field. Indeed the minimization procedure showed that proline 19 in P-RNase A assumes a trans conformation determining a decrease of the non-covalent interactions between the N-terminal segment and the rest of the protein.

Table 4. Thermodynamic parameters of the denaturation process of ribonuclease A and some mutants, at pH 5.0, 0.1 M sodium acetate buffer.

	T_d °C	$\Delta_d H(T_d)$ kJ mol^{-1}	$\Delta_d C_p$ kJ $K^{-1} mol^{-1}$	R_{vH}
RNase A	61.3	465	5.5	1.01
P-RNase A	59.6	415	5.2	1.03
PL-RNase A	59.0	410	5.0	0.99
MCAM-PLCC-RNase A	56.4	390	4.8	0.99
MCAM-rBS-RNase	55.2	380	4.7	0.97

For PL-RNase the denaturation temperature and enthalpy change slightly decrease, being T_d = 59.0 °C and $\Delta_d H(T_d)$ = 410 kJ mol^{-1}. Instead MCAM-PLCC-RNase A shows a strong decrease of thermal stability with respect to the parent enzyme. The denaturation temperature and enthalpy change result 56.4 °C and 390 kJ mol^{-1}, respectively. These values must be compared to those determined for MCAM-rBS-RNase, a monomeric derivative of dimeric BS-RNase obtained after selective cleavage of the two intersubunit disulfide bridges and carboxyamidomethylation of the free SH groups. This monomeric form has a denaturation temperature of 55.2 °C and the denaturation enthalpy change amounts to 380 kJ mol^{-1}. Therefore, MCAM-rBS-RNase, although it does possess 80 % of sequence homology with RNase A, is markedly less stable, as already found for the monomeric derivative MCM-BS-RNase. In addition, it is evident that the introduction into RNase A of the four residues, playing the key-role in protein dimerization and N-terminal segment swapping, shifts the thermal stability toward that of the monomeric form of BS-RNase. Work is in progress in our laboratory to further characterize these mutants of RNase A.

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