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DOMAINS IN BOVINE SEMINAL RIBONUCLEASE

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Bovine seminal ribonuclease is the only pancreatic-type ribonuclease to possess a dimeric structure: the two identical subunits are covalently linked by two disulfide bridges. Actually, the protein exists in two different dimeric structures owing to the possibility of swapping the N-terminal α-helical segments: the swapped MxM dimer, and the non-swapped M=M dimer. The thermal denaturation of the two separated forms is investigated by differential scanning calorimetry. The process is reversible and can be represented by two sequential two-state transitions, indicating the presence of two domains in BS-RNase, regardless of the swapping phenomenon. Inspection of the structural models leads to the tentative identification of an external domain and a core domain, the latter more stable.

Keywords: *differential scanning calorimetry, domain swapping, structural domain, thermodynamic domain*

Introduction

Bovine seminal ribonuclease, BS-RNase, is the only dimeric ribonuclease of the pancreatic-type superfamily discovered so far [1, 2]. Its two identical subunits are linked by two disulfide bridges pairing Cys31 and Cys32 of one chain with Cys32 and Cys31, respectively, of the other chain. Each subunit possesses more than 80% of sequence homology with pancreatic ribonuclease, RNase A: 101 out of 124 residues are identical and the four intrachain disulfide bridges occupy the same sequence positions. More important, BS-RNase does exist in two dimeric structures [3]: the MxM form in which there is the swapping of the N-terminal α-helical segment between the two subunits, and the M=M form in which the swapping does not occur and each N-terminal segment packs against the remaining part of its own subunit (Fig. 1). The biochemical evidences have definitely been supported by the X-ray structures of the two dimeric forms [4, 5]. The swapping ability is related to the conformational properties of a small peptide, the segment 16–22, called hinge peptide, that connects the N-terminal α-helix to the second α-helix in each subunit [4–6]. The two dimeric structures prove to be in equilibrium between each other in the natural protein isolated from bull seminal plasma: the molar ratio [M=M]/[MxM]=1/2 at 37°C [3].

To the extraordinary property of populating two dimeric structures, it is necessary to add that BS-RNase displays special biological actions, and the two dimeric forms are endowed with distinct biological functions [1, 2]. While M=M possesses only the normal catalytic reactivity toward RNA, MxM is allosterically regulated in the second, rate-determining step of the catalytic reaction, and more important, it is a cytotoxic agent selective for tumoral cells [7–9].

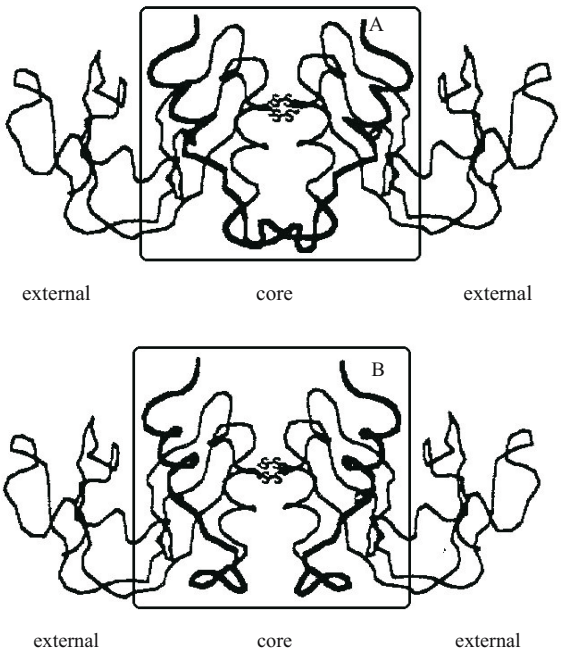

Fig. 1 Structural models of the two dimeric forms of BS-RNase; panel A, MxM (PDB entry code, 1BSR); panel B, M=M (PDB entry code, 1R3M). The two thermodynamic domains suggested by the analysis of DSC curves are indicated

In the last years we have investigated the thermal denaturation of several ribonucleases [10–16], also BS-RNase [17]. In particular, Barone and colleagues

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studied the thermodynamic stability of the two dimeric forms of BS-RNase, performing differential scanning calorimetry, DSC, and circular dichroism measurements [18]. Thermal denaturation proved to be reversible for both MxM and M=M, but not well represented by a simple two-state transition model. Deconvolution of DSC curves indicated the presence of two domains in the BS-RNase structure, regardless of the swapping phenomenon [18]. In the present work we analyze a different set of DSC measurements at pH 5.0, recorded some years ago, by means of a different deconvolution procedure, with the aim to provide an alternative identification of thermodynamic domains in BS-RNase. Data obtained by deconvolution allow also the calculation of the Gibbs energy difference between M=M and MxM: the swapped dimer proves to be more stable by 1.5 kJ mol^{-1} at 37°C, in line with the estimate coming from the equilibrium constant determined by means of gel filtration experiments [3].

Materials and methods

Protein preparation

The two dimeric forms of BS-RNase can be separated according to the following procedure. Selective cleavage of the two intersubunit disulfide bridges leads to monomers in the case of M=M, and to non-covalent dimers in the case of MxM, owing to the noncovalent interactions existing between the swapped N-terminal α-helical segment and the main body of the other subunit [3]. The X-ray structures of both monomer and non-covalent dimer have been solved by Mazzarella and co-workers [19, 20]. Pure M=M and MxM forms are obtained by air reoxidation of monomers and non-covalent dimers, respectively. Doubly deionized water is used throughout. Before DSC measurements, sample solutions are dialyzed against the buffer solution at 4°C for 24 h, using dialysis tubes with a cutoff limit of 15000 Da. The same value of extinction coefficient, ε_{278}= 12740 M^{-1} cm^{-1}, is used for both MxM and M=M [13]. All the solutions contain 100 mM sodium acetate buffer at pH 5.0. The pH is measured before each DSC scan, at 25°C, with a Radiometer pHmeter (model PHM93).

Scanning calorimetry

Calorimetric measurements are carried out on a Setaram Micro-DSC instrument, interfaced with a data translation A/D board for automatic data acquisition. Data analysis is accomplished with in-house programs [10, 11], and the excess heat capacity function, ΔC_p, is obtained by subtraction of the baseline, given by linear extrapolation of the native state heat capacity [21]. The van't Hoff enthalpy is calculated using the formula [22]:

$$\Delta_d H_{vH}=4RT_{max}^2\,\Delta C_p(T_{max})/\Delta_d H \qquad (1)$$

where T_{max} is the temperature corresponding to the maximum of DSC peak, $\Delta C_p(T_{max})$ is the height of the excess heat capacity at T_{max}, $\Delta_d H$ is the total denaturation enthalpy change calculated by direct integration of the DSC peak and R is the gas constant. The close correspondence between $\Delta_d H$ and $\Delta_d H_{vH}$ is a necessary condition to state that the denaturation is a two-state transition [22, 23].

Thermodynamic model

Complex DSC profiles have to be deconvoluted according to definite statistical thermodynamic approaches [24–28]. Two sequential two-state transitions give rise to three states: the native state N, an intermediate, indicated by I, in which part of the molecule is unfolded, and the denatured state D. K_1 is the equilibrium constant of the first conformational transition N⇔I, and is temperature dependent according to:

$$K_1=\exp-\{[\Delta_d H_1(T_{d,1})/R][(1/T)-(1/T_{d,1})]+(\Delta_d C_{p,1}/R)[1-(T_{d,1}/T)-\ln(T/T_{d,1})]\} \qquad (2)$$

where $T_{d,1}$ is the denaturation temperature at which K_1=1, $\Delta_d H_1(T_{d,1})$ is the enthalpy change associated with the first transition and $\Delta_d C_{p,1}$ is the corresponding heat capacity change. K_2 is the equilibrium constant of the second conformational transition I⇔D, and is temperature dependent according to:

$$K_2=\exp-\{[\Delta_d H_2(T_{d,2})/R][(1/T)-(1/T_{d,2})]+(\Delta_d C_{p,2}/R)[1-(T_{d,2}/T)-\ln(T/T_{d,2})]\} \qquad (3)$$

where $T_{d,2}$ is the denaturation temperature at which K_2=1, $\Delta_d H_2(T_{d,2})$ is the enthalpy change associated with the second transition and $\Delta_d C_{p,2}$ is the corresponding heat capacity change. Equations (2) and (3) are exact in the assumption that the heat capacity changes are temperature independent. By assuming the native state as reference, the macroscopic canonical partition function is [21, 27]:

$$Q_N(T)=1+K_1+K_1K_2 \qquad (4)$$

The population fractions of the three macroscopic states are $f_N=1/Q_N$, $f_I=K_1/Q_N$, and $f_D=K_1K_2/Q_N$. The excess enthalpy function with respect to the native state is:

$$\Delta H(T)=[\Delta_d H_1(T_{d,1})+\Delta_d C_{p,1}(T-T_{d,1})]f_I+[\Delta_d H_1(T_{d,1})+\Delta_d C_{p,1}(T-T_{d,1})+\Delta_d H_2(T_{d,2})+\Delta_d C_{p,2}(T-T_{d,2})]f_D \qquad (5)$$

The excess heat capacity function, ΔC_p, which is the physical observable of DSC measurements, is given by the temperature derivative of $\Delta H(T)$. According to Privalov *et al.* [28], the contributions of the first and second transitions to ΔC_p are given by:

$$\Delta C_{p,1}=[\Delta_d H_1(T_{d,1})/RT^2]\Delta H f_N \quad (6)$$

$$\Delta C_{p,2}=[\Delta_d H_2(T_{d,2})/RT^2] [\Delta_d H_1(T_{d,1})+\Delta_d H_2(T_{d,2})-\Delta H]f_D \quad (7)$$

and $\Delta C_p=\Delta C_{p,1}+\Delta C_{p,2}$. DSC curves can be directly simulated by using as input parameters the values of $T_{d,1}$, $\Delta_d H_1(T_{d,1})$, $\Delta_d C_{p,1}$, $T_{d,2}$, $\Delta_d H_2(T_{d,2})$ and $\Delta_d C_{p,2}$: these are the parameters to be determined by the deconvolution analysis of experimental DSC curves. The total heat capacity change associated with denaturation, $\Delta_d C_p=\Delta_d C_{p,1}+\Delta_d C_{p,2}$, can be eliminated by means of an iterative procedure [29]. In this manner, the number of parameters to be determined by deconvolution reduces to four: $T_{d,1}$, $\Delta_d H_1(T_{d,1})$, $T_{d,2}$ and $\Delta_d H_2(T_{d,2})$. Deconvolution of experimental peaks is performed by means of a nonlinear regression, using the Levenberg-Marquardt algorithm, as implemented in the Optimization Toolbox of MATLAB [27].

Since $\Delta_d C_p$ is eliminated from the excess heat capacity profiles, the values of $\Delta_d C_{p,1}$ and $\Delta_d C_{p,2}$ can be calculated by assuming that the heat capacity changes associated with the two transitions are proportional to the corresponding enthalpy changes [28]:

$$\Delta_d C_{p,i}=[\Delta_d H_i(T_{d,i})/\Delta_d H]\Delta_d C_p \quad (8)$$

When thermal denaturation is described by two sequential two-state transitions, the total denaturation Gibbs energy change is the sum of the individual contributions associated with the first and second conformational transitions [28]:

$$\Delta_d G=\Delta_d G_1+\Delta_d G_2 \quad (9)$$

where each contribution can be calculated by means of the well-known relationship [22]:

$$\Delta_d G_i=\Delta_d H_i(T_{d,i})[1-(T/T_{d,i})]+ \Delta_d C_{p,i}[T-T_{d,i}-T\ln(T/T_{d,i})] \quad (10)$$

which is exact in the assumption that the heat capacity change is temperature independent.

Results and discussion

DSC measurements on the two dimeric forms of BS-RNase at pH 5.0, 100 mM acetate buffer, using a scan rate of 0.5°C min^{-1}, indicate that the denaturation process is reversible, according to the reheating criterion. A second heating of a sample previously heated up to 85°C and then cooled to 20°C, gives a DSC peak superimposable on that obtained in the first scan. The process is not affected by protein concentration in the range 1.0–3.0 mg mL^{-1}. Calorimetric profiles of MxM and M=M are shown in Fig. 2: they are different, because the DSC peak of MxM has, in the low-temperature side, a longer tail than that of M=M. The reliability of such curves may be questioned on the basis of the well-established fact that each of the two dimers, spontaneously, interconverts into the other in order to reconstitute the natural equilibrium mixture [3]. However, by heating protein solutions up to 75°C using different scanning rates, over the range 0.3–1.2°C min^{-1}, superimposable DSC profiles are obtained for MxM and M=M samples, respectively. Kinetic data showed that the two forms are stable at 37°C for about 10 h [3]. Therefore, it is possible to assume that the interconversion process is so slow that, during DSC measurements, each dimer does not transform into the other in appreciable quantity, and denaturation is a reversible process [18].

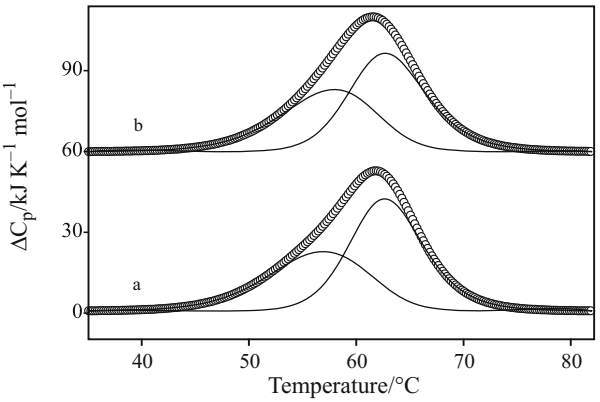

Fig. 2 Experimental DSC profiles, at pH 5.0, 100 mM acetate buffer, of MxM (c=1.50 mg mL^{-1}, curve a), and M=M (c=1.45 mg mL^{-1}, curve b). Excess heat capacity values are shifted along the y-axis for ease of presentation. The full lines represent the two sequential two-state transition curves calculated by deconvolution. See text for further details

Thermodynamic parameters obtained directly from the analysis of DSC curves are collected in Table 1. The temperature corresponding to the maximum of DSC peaks is practically identical for the two forms: T_{max}=61.4°C for MxM and 61.3°C for M=M. On the contrary, the total calorimetric enthalpy change is different: $\Delta_d H$=650 kJ mol^{-1} for MxM, and 620 kJ mol^{-1} for M=M [17,18]. This finding emphasizes that the two dimeric forms should have a different energetic content. Even though caution is necessary because the difference is of the order of the experimental uncertainty (i.e., approximately 5% of reported values), nevertheless it is statistically significant, in view of the large number of DSC measurements performed. On the other hand, as suggested by the asymmetric shape of DSC profiles shown in Fig. 2, the thermal denaturation does not correspond

Table 1 Thermodynamic parameters of the thermal denaturation of the two dimeric forms of BS-RNase at pH 5.0, 100 mM acetate buffer. The values of $\Delta_d H_{vH}$ are calculated according to Eq. (1)

	T_{max}/ °C	$\Delta_d H$/ kJ mol^{-1}	$\Delta_d H_{vH}$/ kJ mol^{-1}	$\Delta_d C_p$/ kJ K^{-1} mol^{-1}
MxM	61.4	650	330	8.0
M=M	61.3	620	310	8.0

Each figure represents the value averaged over ten measurements at different scanning rates, in the range 0.3–1.2°C min^{-1}. The error in T_{max} does not exceed 0.2°C. The estimated relative uncertainties in $\Delta_d H$ and $\Delta_d C_p$ amount approximately to 5 and 10%, respectively, of reported values

Table 2 Thermodynamic parameters calculated by deconvolution of the experimental DSC curves of MxM and M=M forms of BS-RNase at pH 5.0 according to the sequential model. The values of the heat capacity changes are calculated by means of Eq. (8)

	MxM	M=M
$T_{d,1}$/°C	56.3±0.3	57.4±0.3
$\Delta_d H_1(T_{d,1})$/kJ mol^{-1}	270±8	270±9
$\Delta_d C_{p,1}$/kJ K^{-1} mol^{-1}	3.3	3.5
$T_{d,2}$/°C	62.4±0.3	62.4±0.3
$\Delta_d H_2(T_{d,2})$/kJ mol^{-1}	380±12	350±14
$\Delta_d C_{p,2}$/kJ K^{-1} mol^{-1}	4.7	4.5

to a two-state N⇔D transition for both MxM and M=M forms. The calorimetric to van't Hoff enthalpy ratio is close to two for both the dimeric forms of BS-RNase, indicating the presence of two thermodynamic domains in the BS-RNase structure, regardless of the N-terminal α-helical segment swapping. Finally, $\Delta_d C_p$ amounts to 8.0±1.0 kJ K^{-1} mol^{-1} for both proteins.

A comparison of these values with those of RNase A in the same experimental conditions indicates that: T_{max} corresponds exactly to the denaturation temperature of RNase A, T_d=61.3°C; the values of both $\Delta_d H$ and $\Delta_d C_p$ of MxM and M=M are less than twice those of RNase A, 465 kJ mol^{-1} and 5.5 kJ K^{-1} mol^{-1}, respectively [12, 13]. A further comparison can be performed with the thermal denaturation of the monomeric and carboxyamidomethylated form of bovine seminal ribonuclease, MCAM-BS-RNase, in the same experimental conditions. The process was a reversible two-state transition characterized by T_d=55.2°C, $\Delta_d H$=380 kJ mol^{-1} and $\Delta_d C_p$= 4.7 kJ K^{-1} mol^{-1} [13]. Therefore, on forming a dimeric structure, either swapped or non-swapped, the denaturation temperature increases.

To shed further light on the thermodynamic stability of the two forms of BS-RNase, a deconvolution analysis of the DSC curves for both MxM and M=M forms is performed according to the sequential model described in the Materials and Methods section. The results are collected in Table 2, and the two sequential two-state transition curves are shown in Fig. 2. The value of $T_{d,1}$=56.3±0.3°C for MxM, and 57.4±0.3°C for M=M, while $T_{d,2}$=62.4±0.3°C for both the forms. Therefore, the mid-point denaturation temperatures, characterizing the two sequential transitions, are practically equal for the two dimeric forms; the small difference in $T_{d,1}$ values will be shortly discussed in the following. The value of $\Delta_d H_1(T_{d,1})$=270 kJ mol^{-1} for both forms: it is smaller than half of the total area of DSC peak. This, on the basis of Eq. (8), reflects in $\Delta_d C_{p,1}$=3.3 kJ K^{-1} mol^{-1} for MxM, and 3.5 kJ K^{-1} mol^{-1} for M=M. The value of $\Delta_d H_2(T_{d,2})$= 380 kJ mol^{-1} for MxM, and 350 kJ mol^{-1} for M=M; it is larger than half of the total area of DSC peak. On the basis of Eq. (8), $\Delta_d C_{p,2}$=4.7 kJ K^{-1} mol^{-1} for MxM, and 4.5 kJ K^{-1} mol^{-1} for M=M. A thermodynamic model with two independent two-state transitions also fits the experimental DSC profiles, but not as well as the proposed sequential model.

Having determined these parameters, it is possible to calculate the denaturation Gibbs energy change at any temperature, according to Eqs (9) and (10), for both MxM and M=M. Performing the calculation at 37°C, it results: (a) for MxM, $\Delta_d G_1$=14.0 kJ mol^{-1} and $\Delta_d G_2$=24.2 kJ mol^{-1}, so that $\Delta_d G$=38.2 kJ mol^{-1}; (b) for M=M, $\Delta_d G_1$=14.5 kJ mol^{-1} and $\Delta_d G_2$= 22.2 kJ mol^{-1}, so that $\Delta_d G$=36.7 kJ mol^{-1}. Therefore, at 37°C, the difference in $\Delta_d G$ between MxM and M=M amounts to 1.5 kJ mol^{-1}. This value has to be considered an estimate of the standard Gibbs energy change associated with the interconversion MxM⇔M=M. This assumption is supported by the fact that the denatured states of both MxM and M=M should be identical and the differences in thermodynamic parameters should be due to the structural features of the folded forms. The interconversion process, at 37°C, has an equilibrium constant K= [M=M]/[MxM]=1/2 [3], which corresponds to a standard Gibbs energy change of 1.8 kJ mol^{-1}. The close agreement between the latter value and that estimated from the analysis of DSC data seems to indicate the reliability of our results. The MxM form is more stable, in thermodynamic terms, than the M=M form; however, since the Gibbs energy difference is very small, both dimeric forms are significantly populated in equilibrium conditions at 37°C.

Deconvolution analysis of DSC profiles at pH 5.0 of the two dimeric forms of BS-RNase indicates that the thermal denaturation can be described by two sequential two-state transitions which should be ascribed to the sequential unfolding of two distinct

thermodynamic domains. In general, a thermodynamic domain is that part of a globular protein that unfolds cooperatively, i.e., according to the two-state transition model. Usually, a thermodynamic domain should correspond to a structural domain of the protein. Inspection of the X-ray structure of MxM and M=M, shown in Fig. 1, suggests the presence of two structural domains in these molecules: (a) a 'core domain' comprising the central region of the dimer and containing six disulfide bridges, i.e., the two disulfide bridges connecting the two subunits and the two bridges Cys26–Cys84 and Cys40–Cys95 in each subunit; (b) an 'external domain' comprising the peripheral regions of the dimer and containing four disulfide bridges, i.e., the two bridges Cys58–Cys110 and Cys65–Cys72 in each subunit. A peculiar feature of these two domains, in both MxM and M=M, is the fact that each one is constituted by parts belonging to both subunits.

Each subunit of the dimers possesses three α-helical segments, spanning residues 3–13, 24–34 and 50–60, respectively; and two antiparallel β-sheets, that give rise to the V-shaped motif characteristic of the pancreatic ribonuclease folding pattern [4, 5]. The first β-sheet has three strands comprising residues 42–48, 79–87 and 97–104, respectively; the second β-sheet is less regular and is made up of two long strands comprising residues 105–113 and 114–124, and two short strands comprising residues 61–64 and 71–75. We propose that the binding clefts, constituting the active sites of BS-RNase and originating from the packing of the two N-terminal α-helical segments against the V-shaped motif made up of the two β-sheets in each subunit, dissect the dimer in two domains, regardless of the swapping phenomenon. Such domains are schematically indicated in Fig. 1 for both MxM and M=M.

The core domain, in each subunit, is formed by the polypeptide segments containing residues 1–49 and 79–104; these two regions are covalently linked by means of the disulfide bridges Cys26–Cys84 and Cys40–Cys95. Therefore, the core domain is made up of the first and second α-helices and the first β-sheet. Furthermore, Mazzarella and co-workers [4] pointed out that the two N-terminal segments in the structure of MxM interact via H-bonds only with residues belonging to the proposed core domain, namely with residues 25, 33, 44, 45, 47, 48 and 49 of the primary structure; actually, there are also two H-bonds, mediated by water molecules, between Ala4 and Val118, and Ala5 and Pro117, respectively. The external domain, in each subunit, is formed by the polypeptide segments containing residues 50–78 and 105–124; these two regions are covalently linked by means of the disulfide bridge Cys58–Cys110, whereas the bridge Cys65–Cys72 serves to confer rigidity and stability to a loop exposed to the solvent. Following the same assignment of secondary structure elements [4], the external domain is made up of the third α-helix and the second β-sheet. Clearly, due to the connectivity of the polypeptide chain and the specific features of BS-RNase, it is not correct to consider the two domains as real independent units.

Therefore, the core domain would comprise about 150 residues, whereas the external domain would comprise about 100 residues. Since there is an approximate direct proportionality between the denaturation enthalpy change and the molecular mass of the polypeptide chain forming a thermodynamic domain [30], the two transition enthalpies should be in a ratio roughly equal to 2/3=0.67, in both the dimeric forms of BS-RNase. In fact, we have found: $\Delta_d H_1(T_{d,1})/\Delta_d H_2(T_{d,2})$=0.71 for MxM and 0.77 for M=M. The above discussion points out that the core domain should be more stable, in terms of transition temperature, than the external domain in both MxM and M=M. The first transition should correspond to the unfolding of the external domain with $T_{d,1}$=56.3°C in MxM and 57.4°C in M=M; while the second transition should correspond to the unfolding of the core domain with $T_{d,2}$=62.4°C for both MxM and M=M.

The finding that the transition temperature of the core domain is the same in both MxM and M=M seems physically reliable, because the interface region between the two subunits is practically identical in the two dimeric forms [5]. Structural differences between the core domain in MxM and M=M should originate in the distinct packing of the N-terminal segments and hinge peptides against the main body of the subunits [4, 5]. This should explain the difference of about 30 kJ mol^{-1} found between the values of $\Delta_d H_2(T_{d,2})$ for MxM and M=M, respectively. But, such a difference in denaturation enthalpy is largely compensated for by a corresponding difference in denaturation entropy, and the $T_{d,2}$ value proves to be the same for both MxM and M=M. According to the $T_{d,i}$ values obtained by deconvolution (i.e., 56.3 and 62.4°C for MxM *vs.* 57.4 and 62.4°C for M=M), the two domains appear to be slightly more coupled in M=M than in MxM. In fact, the X-ray structure of M=M shows a closure of the binding cleft with respect to the X-ray structure of MxM [5].

Conclusions

In conclusion, DSC measurements and their analysis point out that the two dimeric forms of BS-RNase have a similar thermodynamic stability. In terms of Gibbs energy, the swapped MxM form is more stable than the non-swapped M=M form, but the difference is so small that the two structures are both significantly populated in the bull seminal plasma.

References

1 G. D'Alessio, A. Di Donato, A. Parente and R. Piccoli, Trends Biochem. Sci., 16 (1991) 104.
2 G. D'Alessio, A. Di Donato, L. Mazzarella and R. Piccoli, in Ribonucleases: Structures and Functions (G. D'Alessio and J. F. Riordan, Eds), Academic Press, New York 1997, p. 383.
3 R. Piccoli, M. Tamburrini, G. Piccialli, A. Di Donato, A. Parente and G. D'Alessio, Proc. Natl. Acad. Sci. USA, 89 (1992) 1870.
4 L. Mazzarella, S. Capasso, D. Demasi, G. Di Lorenzo, C. A. Mattia and A. Zagari, Acta Cryst., D49 (1993) 389.
5 R. Berisio, F. Sica, C. De Lorenzo, A. Di Fiore, R. Piccoli, A. Zagari and L. Mazzarella, FEBS Lett., 554 (2003) 105.
6 L. Mazzarella, L. Vitagliano and A. Zagari, Proc. Natl. Acad. Sci. USA, 92 (1995) 3799.
7 V. Cafaro, C. De Lorenzo, R. Piccoli, A. Bracale, R. Mastronicola, A. Di Donato and G. D'Alessio, FEBS Lett., 359 (1995) 31.
8 B. S. Murthy, C. De Lorenzo, R. Piccoli, G. D'Alessio and R. Sirdeshmukh, Biochemistry, 35 (1996) 3880.
9 R. J. Youle and G. D'Alessio, in Ribonucleases: Structures and Functions (G. D'Alessio and J. F. Riordan, Eds), Academic Press, New York, 1997, p. 491.
10 F. Catanzano, C. Giancola, G. Graziano and G. Barone, Biochemistry, 35 (1996) 13378.
11 G. Graziano, F. Catanzano, C. Giancola and G. Barone, Biochemistry, 35 (1996) 13386.
12 F. Catanzano, G. Graziano, S. Capasso and G. Barone, Protein Sci., 6 (1997) 1682.
13 F. Catanzano, G. Graziano, V. Cafaro, G. D'Alessio, A. Di Donato and G. Barone, Biochemistry, 36 (1997) 14403.
14 F. Catanzano, G. Graziano, V. Cafaro, G. D'Alessio, A. Di Donato and G. Barone, Int. J. Biol. Macromol., 23 (1998) 277.
15 E. Notomista, F. Catanzano, G. Graziano, F. Dal Piaz, G. Barone, G. D'Alessio and A. Di Donato, Biochemistry, 39 (2000) 8711.
16 E. Notomista, F. Catanzano, G. Graziano, S. Di Gaetano, G. Barone and A. Di Donato, Biochemistry, 40 (2001) 9097.
17 G. Barone, F. Catanzano, P. Del Vecchio, C. Giancola and G. Graziano, Pure Appl. Chem., 69 (1997) 2307.
18 C. Giancola, P. Del Vecchio, C. De Lorenzo, R. Barone, R. Piccoli, G. D'Alessio and G. Barone, Biochemistry, 39 (2000) 7964.
19 F. Sica, A. Di Fiore, A. Zagari and L. Mazzarella, Proteins, 52 (2003) 263.
20 F. Sica, A. Di Fiore, A. Merlino and L. Mazzarella, J. Biol. Chem., 279 (2004) 36753.
21 E. Freire and R. L. Biltononen, Biopolymers, 17 (1978) 463.
22 P. L. Privalov, Adv. Protein Chem., 33 (1979) 167.
23 Y. Zhou, C. K. Hall and M. Karplus, Protein Sci., 8 (1999) 1064.
24 A. Michnik, K. Michalik and Z. Drzazga, J. Therm. Anal. Cal., 80 (2005) 399.
25 A. Michnik, K. Michalik, A. Kluczewska and Z. Drzazga, J. Therm. Anal. Cal., 84 (2006) 113.
26 A. Michnik and Z. Drzazga, J. Therm. Anal. Cal., 88 (2007) 449.
27 F. Catanzano, A. Gambuti, G. Graziano and G. Barone, J. Biochem., 121 (1997) 568.
28 J. H. Carra, E. C. Murphy and P. L. Privalov, Biophys. J., 71 (1996) 1994.
29 P. L. Privalov and S. A. Potekhin, Methods Enzymol., 131 (1986) 4.
30 A. D. Robertson and K. P. Murphy, Chem. Rev., 97 (1997) 1251.

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