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THERMODYNAMIC STABILITY OF RIBONUCLEASE B

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Abstract

The thermodynamic stability of pancreatic ribonuclease B (RNase B), which possesses identical protein structure of pancreatic ribonuclease A (RNase A), but differs by the presence of a carbohydrate chain attached to Asn 34, was studied by means of differential scanning calorimetry (DSC) at different pH conditions. The comparison between the two proteins has shown a little but significant stabilization of RNase B with respect to the unglycosylated one at pH values higher than 7.0. The thermodynamic analysis reveals the carbohydrate moiety to have a small stabilization effect of 3 kJ mol^{-1} at pH 8.0 and 63°C on the protein.

Keywords: DSC, glycosylated ribonuclease, thermodynamic stability

Introduction

The role of carbohydrate moiety of glycoproteins has been related to effects on protein solubility [1], protection against the proteolitic degradation [2, 3] and thermal stability of the protein [4]. Furthermore, the N-linked and O-linked oligosaccharides have been also shown to facilitate the protein folding [5]. Glycoproteins consist of several glycosylated variants (glycoforms) which may differ from each other with respect to the number and position of the attached oligosaccharides as well as the structure and sequence of these oligosaccharides.

RNase B consists of the five glycoforms Man5-9 $GlcNAc_2$ and contains a single glycosylation site. The enzyme has a molecular mass of approximately 15.5 kDa and 124 amino acid residues and differs from RNase A (molecular mass 13.68 kDa) for the oligosaccharide moiety N-linked to Asn34 [6–8]. Both enzymes catalyze the hydrolysis of 3’-5’-phosphodiester linkages of ribonucleic acids in a two-step reaction, for both single and double-stranded RNA, or nucleotide esters at the 5’-ester bond. In the RNase B the oligosaccharide extends into the solvent and is for the most part disordered, with the only contact between the oligosaccharide and the protein being at the point of attachment [8]. A comparison of the crystal structure of RNase A and RNase B reveals no significant differences, suggesting that oligosaccharide has no influence on the conformation of protein [9].

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In the aim to elucidate the role of carbohydrate moiety on protein stability we studied by means of DSC the thermal denaturation of ribonuclease B (RNase B) at different pH conditions. All the results have been compared with those of the unglycosylated form ribonuclease A (RNase A) [10].

Materials and methods

Sample preparation and protein purification

Ribonuclease B (Type XII-B), a Sigma product containing almost 40% of RNase A, was purified by affinity chromatography on a Con-A Sepharose (Pharmacia) column. The unglycosylated protein passed through the column unretarded while the RNase B was bound selectively and eluted with 10% α-methyl-D-glucopyranoside. The column capacity was approximately 1.5 mg of enzyme/mL of gel volume of Con-A-Sepharose [11]. The obtained fractions containing RNase B were collected, exhaustively dialyzed against double distilled water at 4°C and finally lyophilized. The extent of glycosylation of our sample was determined by mass spectrometry analysis (CEINGE/SESMA-laboratories-Naples) and a molecular mass of 15210 Da was obtained. Ribonuclease A (Type XII-A) was purchased from Sigma and used without further purification.

Protein solutions for calorimetric measurements were exhaustively dialyzed against the appropriate buffer solution at 4°C by using Spectra Por MW 6000-8000 Da dialysis tubes. The protein concentrations varied from 1–2 mg mL^{-1} and were determined spectrophotometrically using an extinction coefficient of 9800 M^{-1} cm^{-1} at 278 nm for RNase A [12] and 11994 M^{-1} cm^{-1} at 280 nm for RNase B [13]. The pH of each protein solution was determined with a Radiometer pHmeter, model PHM 93, at 25°C.

Buffer solutions, purchased from Sigma, prepared at 50 mM concentration, were: glycine-HCl for pH 3.0, sodium acetate for pH 4.0 and 5.0, 2-[morpholino]-ethansulfonate (MES) for pH 6.0, 3-[N-morpholino] propanesulfonate (MOPS) for pH 7.0 and N-[hydroxyethyl] piperazine-N'-[2-hydroxypropanesulfonate] (HEPPSO) for pH 8.0. All the buffers employed had low protonation enthalpies [14] in order to reduce the pH dependence on temperature. The water used for buffer and sample solutions was double distilled.

Calorimetry

Calorimetric measurements were performed on a second generation Setaram Micro-DSC. The calorimetric unit was interfaced to an IBM PC computer for automatic data collection and analysed using the software previously described [15]. A scanning rate of 0.5°C min^{-1} was chosen for the experiments. The apparent molar heat capacity values were obtained by correcting each calorimetric curve for the instrument calibration curve and buffer-buffer scanning curve and dividing each data point by the scan rate and the number of protein moles in the sample cell. The performance of the in-

strument was calibrated periodically with an electrical pulse. The excess heat capacity curve $<\Delta C_p(T)>$ was obtained according to the procedure of Freire and Biltonen [16], assuming that the baseline is given by the linear temperature dependence of the native state heat capacity. The van't Hoff enthalpy was calculated by the calorimetric data assuming a two-state N↔D transition using the standard formula [17]:

$$\Delta_d H_{v.H.} = 4RT_d^2[<\Delta_d C_p(T_d)>/\Delta_d H(T_d)] \quad (1)$$

where T_d is the denaturation temperature and corresponds to the maximum of the DSC peak, $<\Delta_d C_p(T_d)>$ is the value of the excess heat capacity function at T_d, $\Delta_d H(T_d)$ is the calorimetric enthalpy determined by integration of the area under the curve and R is the gas constant. The number of cooperative units was calculated as the calorimetric to van't Hoff enthalpy ratio, $CU = \Delta_d H(T_d)/\Delta_d H_{v.H}$. The closeness to unity of CU is a necessary condition to state that the denaturation process is a two-state transition.

Results and discussion

In Table 1 are collected the thermodynamic parameters of thermal denaturation of RNase B in the pH range 3.0–8.0. The corresponding values for RNase A are reported in Table 2. The *CU* value was found close to unity for all the pH conditions. In the pH range 3.0–7.0 the denaturation process is completely reversible according to the reheating criterion. For both ribonucleases the denaturation temperature and enthalpy increase at increasing pH. According to the electrostatic principles, the maximum stability of globular proteins should occur at their isoelectric point [18]. The isoelectric point of RNase A is about 9.3 [19] and the same is expected for RNase B, so the maximum of stability should occur at high pH values. Above pH 7.0 the thermal denaturation of both RNase A and RNase B becomes irreversible. This was attributed to deamidation of asparagine and glutamine side chains and other side reactions occurring at high temperature and alkaline pHs [20]. However, the extent of irreversibility was found to be dependent on the time spent by the protein at high temperature and on the maximum temperature reached. This indicated that the irreversibility of temperature-induced denaturation is due to side reactions occurring after completion of the conformational transition [21]. In fact, for RNase B at pH 8.0 in our experimental conditions, we found that a partial reversibility was recovered when a scan rate of $1°C\ min^{-1}$ was used and the temperature of 75°C was not exceeded (data not shown).

In order to confirm that the temperature-induced denaturation of RNase B is a two-state N↔D transition, we perform a non-linear regression of the experimental curve with respect to the excess molar heat capacity function $<\Delta_d C_p>$ [22] using the Levenberg–Marquardt algorithm [23]. In Fig. 1 the curve calculated was superimposed on the experimental one at pH 8.0 and the agreement between both proved satisfactory. The thermodynamic parameters calculated were the following: T_d=65°C, $\Delta_d H(T_d)$=539 kJ mol^{-1}, $\Delta_d C_p$=5.8 kJ mol^{-1} K^{-1}, where the standard deviation of the fit was σ=0.96 kJ mol^{-1} K^{-1}. This procedure was repeated for all the experiments at each pH.

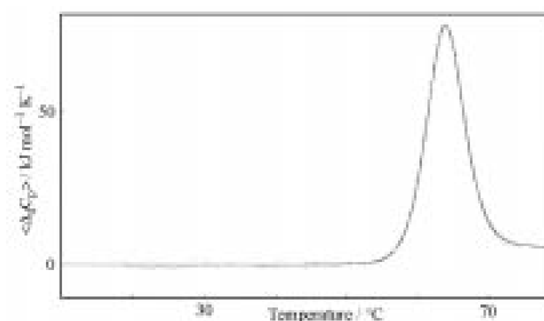

Fig. 1 Experimental DSC curve for RNase B in 50 mM HEPPSO buffer solution pH 8.0. The solid line represents the best fit according to the two-state N↔D model transition

Table 1 Thermodynamic parameters of thermal denaturation of RNase B at different pH values[a]

pH	T_d/°C	$\Delta_d H(T_d)$/kJ mol^{-1}	$\Delta_d C_p$/kJ mol^{-1} K^{-1}	CU[b]
3.0	52.6	425±12	5.1	0.98
4.0	57.2	445±13	5.3	1.00
4.3	58.3	455±13	5.2	1.00
5.0	61.9	475±14	5.6	0.97
6.0	63.0	480±14	5.4	0.97
7.0	64.2	520±15	5.6	1.07
8.0	64.9	538±15	5.9	1.06

[a]Each figure is the mean value of at least four measurements. The error in T_d does not exceed 0.2 degree. Reported errors for $\Delta_d H(T_d)$ and $\Delta_d C_p$ are the standard deviations of the mean from the multiple determinations

[b]*CU* refers to cooperative unit and is the ratio of the calorimetric to van't Hoff enthalpy

Table 2 Thermodynamic parameters of thermal denaturation of RNase A at different pH values[a]

pH	T_d/°C	$\Delta_d H$/kJ mol^{-1}	$\Delta_d C_p$/kJ mol^{-1} K^{-1}	CU
3.0	52.4	415±13	5.1	0.99
3.5	54.7	430±12	5.9	1.00
4.0	57.0	440±12	5.4	0.99
5.0	61.3	465±14	5.5	1.01
6.0	62.4	470±13	5.8	1.02
7.0	62.8	500±14	5.5	1.01
8.0	63.0	505±14	5.5	1.00

[a]Symbols and uncertainties as in Table 1

As can be seen from the comparison of the thermal denaturation parameters reported in Tables 1 and 2, the T_d and $\Delta_d H$ values for RNase B are always higher than RNase A, even if at the limits of experimental uncertainties. Above pH 6.0 these dif-

ferences are fully significant. For instance at pH 8.0 the denaturation temperature and enthalpy of RNase B are 1.9°C and 33 kJ mol^{-1} higher than the corresponding values of RNase A. In Tables 1 and 2 are also reported the experimental values of $\Delta_d C_p$ at each pH. If the $\Delta_d H$ values are plotted as a function of T_d, the slope provides a more accurate estimate of the mean value of $\Delta_d C_p$. Such plot is linear and a value of 5.5 ±0.2 kJ mol^{-1} K^{-1} for both ribonucleases is obtained. The positive value of $\Delta_d C_p$ in the protein thermal denaturation is due to the hydration of non-polar moieties before buried in the native state. As the two proteins only differ for the saccharidic part, the same value of $\Delta_d C_p$ is expected. In fact, the oligosaccharide chain, as evidentiated by the crystal structure studies [8], protrudes out of the surface of the polypeptide domain. Hence it can be assumed that it is entirely hydrated in the native form.

To correctly compare the denaturation enthalpy and entropy of the two ribonucleases, it is necessary to calculate these functions at the same temperature [24]. As reference temperature we selected the T_d value of RNase A at pH 8.0 and 63°C at which the higher difference between the two enzymes occurs. For a two-state transition, assuming that $\Delta_d C_p$ is temperature independent, the denaturation enthalpy and entropy can be calculated according to the classical relations:

$$\Delta_d H(T)=\Delta_d H(T_d)+\Delta_d C_p(T-T_d) \quad (2)$$

$$\Delta_d S(T)=[\Delta_d H(T_d)/T_d]+\Delta_d C_p \ln(T/T_d) \quad (3)$$

For $\Delta_d C_p$=5.5±0.2 kJ mol^{-1}, it results $\Delta_d H$ (63°C)=527±15 kJ mol^{-1}, for RNase B and this value is 22 kJ mol^{-1} higher than that found for RNase A at the same temperature. The values of $\Delta_d S$ (63°C) are 1.6 and 1.5 kJ mol^{-1} K^{-1} for RNase B and A respectively. In other words at the denaturation temperature of RNase A, the enthalpic contribution stabilizes the native form of RNase B, overwhelming the conformational destroying entropic factor, whereas for RNase A there is obviously a complete compensation.

The values of T_d, $\Delta_d H(T_d)$, and $\Delta_d C_p$ obtained from DSC measurements allow the estimation of Gibbs energy of unfolding of protein at a given pH as a function of temperature using the equation:

$$\Delta_d G(T)=\Delta_d H(T_d)(1-T/T_d)+\Delta_d C_p\,[T-T_d-T\ln(T/T_d)] \quad (4)$$

Application of Eqs (3) and (4) is possible only in the case of a reversible process. Since the irreversibility observed at pH 8.0 can be considered due to side reactions and not a feature of the conformational transition, we consider correct the use of the above equations. It is well known that the enthalpic factor stabilizes the native conformation for all proteins up to the denaturation temperature. The Gibbs energy difference between the two proteins amounted to 3 kJ mol^{-1} at 63°C and pH 8.0, and to 5 kJ mol^{-1} at 25°C. In fact, the values obtained at 25°C are: $\Delta_d G$=50±2 and 45±3 kJ mol^{-1} for RNase B and RNase A, respectively. Therefore, even if at the limit of the experimental uncertainty, the trend of thermodynamic properties as function of pH confirm a greater stability of RNase B with respect to that of RNase A.

This analysis led to the conclusion that the stabilization of RNase B with respect to the RNase A is due to enthalpic factor, only partially compensated by destroying entropic

factors. The slightly higher stability of RNase B is not surprising because only one site of glycosylation occurs for this protein. The presence of carbohydrates in solution or of saccharidic chains attached to the polypeptide backbone reduces the denaturing tendency promoted by the preferential hydration of the groups buried in the core of the protein [25, 26]. A recent study, moreover, has shown that the protein stabilization induced by saccharidic moieties increases at increasing the glycosylation sites [4].

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