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The influence of glycosylation on the thermal stability of ribonuclease

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Abstract

Using DSC, the thermal unfolding of RNase A, RNase B, and two partly deglycosylated RNase B forms was studied. The oligosaccharide side chain leads to slight protein stabilization. The conformational stability at pH 4.0 amounts to $\Delta G^{25\,^{\circ}C} = 34.5$, 34.6, 33.7, and 32.8 kJ mol^{-1} for RNase B, Man_1-RNase, $GlnNAc_1$-RNase, and RNase A, respectively. The heat capacity remains the same for glycosylated and deglycosylated protein. These results are consistent with a proposed hydrogen bond of Lys37 with GlnNAc-1.

Keywords: Ribonuclease; Glycosylation; Differential scanning microcalorimetry

1. Introduction

Glycosylation is the most frequently occurring natural modification of proteins. The carbohydrate moiety of a glycoprotein may enable specific recognition capabilities of the protein and alter other biochemical properties of the protein [1,2]. However, side effects of glycosylation, in particular the influence of the carbohydrate moiety on the conformational stability of the protein, are still poorly understood.

The enzyme ribonuclease (RNase) is a protein which occurs in vivo in both glycosylated (RNase B) and non-glycosylated (RNase A) forms. RNase B exhibits a single glycosylation site at Asn34 which is connected with a mannose oligosaccharide $Man_{5-9}GlcNAc_2$. RNase A and B show comparable enzymatic activity [3]. A comparison of the X-ray structures of RNase A and B shows no significant differences in the peptide [4]. Vice versa, the oligosaccharide exhibits in native and denatured RNase B, the same conformational properties as the free oligosaccharide [5,6].

In optically monitored thermal denaturation studies of RNase B, a moderate increase in thermostability of $\Delta T = 1.5^{\circ}C$ compared with RNase A was shown [7]. However, an unusual heat capacity change was reported in that study. In the present paper, direct measurements by highly sensitive differential scanning microcalorimetry will be applied to RNase A and B as well as to partly deglycosylated forms of RNase B to determine a complete set of thermodynamic quantities related to protein unfolding.

2. Experimental

RNase A (Type II-A) and RNase B (purified by affinity chromatography) from bovine pancreas, and α-mannosidase from jack beans were purchased from

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Sigma (Deisenhofen, Germany). Endoglycosidase H was purchased from Boehringer (Mannheim, Germany). All other reagents were from Sigma (Sigma ultra quality).

Partly deglycosylated RNase B was prepared according to Arnold et al. [8] with minor modifications in the procedure [9]. All proteins were checked by mass spectrometry (MALDI-TOF MS). By α-mannosidase treatment Man_1-RNase was produced (observed molar mass 14.249 kDa) containing traces of Man_2-RNase. By endoglycosidase H treatment, a homogeneous preparation of GlcNAc-RNase (observed molar mass 13.885 kDa) was obtained.

Prior to the calorimetric measurements, all proteins were applied to rechromatography in 20 mM MOPS with 0.1 M NaCl, pH 7.0, on a HiLoad 16/60 Sephacryl S 100 column (Pharmacia, Sweden). Then the samples were extensively dialyzed against the measuring buffer (40 mM glycine/HCl for pH 2.5–3.6, and 40 mM acetate for pH 4.0–4.5) and carefully degassed.

Calorimetric measurements were performed at the VP-DSC instrument (MicroCal, Northampton, USA), and on the DASM-4 instrument (Biopribor, Pushchino, Russia) equipped with a DASM-1 gold cell. Temperature calibration of both instruments was performed using the MicroCal standards, and heat calibration was done using the in-built calibration heaters. Protein concentration was 0.5–2.5 mg ml^{-1}. All scans were recorded under the same conditions from 5 to 90 °C at a heating rate of 1 K min^{-1}. Data evaluation was performed with the ORIGIN software package (MicroCal, Northampton, USA), and with a non-linear regression procedure for two-state transitions that approximates pre- and postdenaturational slopes by polynomial expressions [10]. Refolding was assessed by reheating of the sample and comparison of the areas under the peak at first and second heating.

Protein concentration was determined spectrophotometrically using a molar extinction coefficient of 10,020 M^{-1} cm^{-1} at 276 nm according to Pace et al. [11].

3. Results

A representative scanning calorimetric recording of RNase B at pH 4.5 along with the scan obtained at reheating are shown in Fig. 1. The residuals of the raw data relative to a two-state transition are small at first heating of the sample but increase significantly when reheating the sample. Similarly, as originally stated by Privalov for RNase A, RNase B thermal unfolding can be regarded as a reversible two-state process up to the first approximation [12].

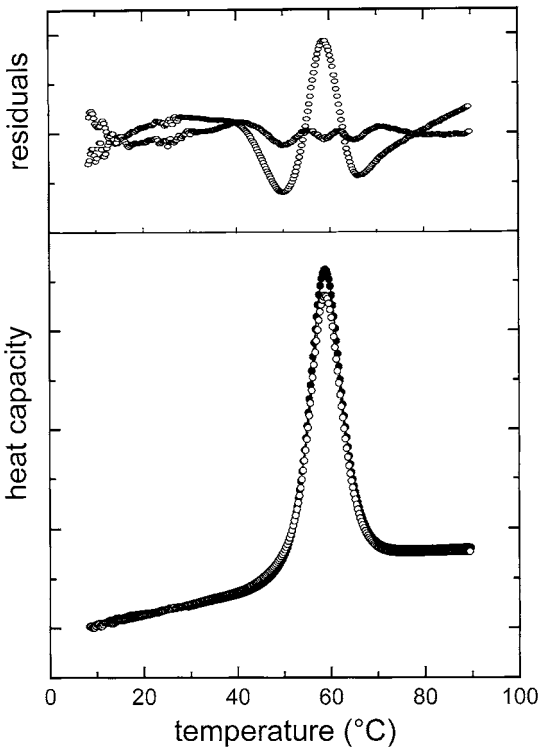

Fig. 1. Scanning calorimetric recordings of RNase B. First scan (filled circles), second scan (open circles). The residuals in the upper part show the deviation of the raw data compared with a two-state fit. Experimental conditions: 1.264 mg ml^{-1} RNase B in 40 mM acetate buffer. Instrument: VP-DSC.

The oligosaccharide of RNase B (Fig. 2) is composed of two *N*-acetylglucosamine units and a branched high-mannose *N*-glycan Man_{5-9} with varying mannose content (about 60% Man_5 and 30% Man_6 [6]). From RNase B, two partly deglycosylated proteins were obtained enzymatically: Man_1-$GlcNAc_2$-RNase (called here Man_1-RNase) and $GlcNAc_1$-RNase.

Thermodynamic data from DSC measurements of RNase B, Man_1-RNase, $GlcNAc_1$-RNase, and RNase A are listed in Table 1 along with values for the conformational stability of the proteins ($\Delta G^{25\,°C}$)

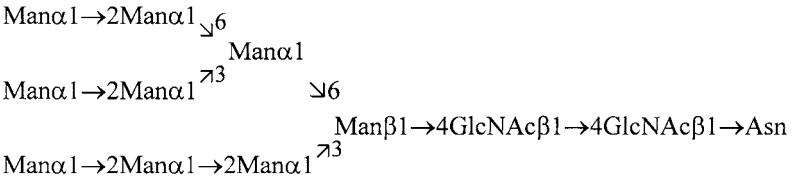

Fig. 2. Structure of the Man_9-$GlcNAc_2$ oligosaccharide isomer in RNase B.

calculated by using Eq. (1) [12].

$$\Delta G^{25\,^{\circ}C} = \Delta H^{cal}\frac{(T_{trs}-T)}{T_{trs}} - \Delta C_p(T_{trs}-T) + T\Delta C_p \ln\left(\frac{T_{trs}}{T}\right) \quad (1)$$

In Eq. (1), the determination of $\Delta G^{25\,^{\circ}C}$ is based on directly measured calorimetric data: T_{trs}, the transition temperature, ΔH^{cal}, the transition enthalpy (calorimetric value) at T_{trs}, and ΔC_p, the heat capacity change (for more details on the determination of ΔC_p see Table 2).

The DSC data show, for RNase B compared with RNase A, a slightly increased thermal transition temperature and enthalpy change, thus confirming the previous spectroscopic data [7]. Accordingly, Eq. (1) yields a moderate increase in the conformational stability of RNase B, Man_1-RNase, and $GlnNAc_1$-RNase compared with RNase A.

Recently, the validity of heat capacity data for RNase A has been questioned [11,13]. In fact, the data reported so far for ΔC_p range from about 2.8 to 8.8 kJ mol^{-1} K^{-1} [14]. Therefore, a careful reinvestigation using various approaches was done by Pace et al. [11] which led to $\Delta C_p = 4.81 \pm 0.33$ kJ mol^{-1} K^{-1}. The present data listed in Table 2 confirms that value. Glycosylation of RNase does not affect the heat capacity change. ΔC_p has, within experimental error, the same value for RNase B and the partly deglycosylated proteins.

It has been proposed that the efficiency of protein refolding might be increased by glycosylation [15,19]. Thermally denatured RNase is able to refold with high yield (Fig. 1). Recovery of $95.3 \pm 2.0\%$ ($n = 8$) was found for RNase A, and $92.3 \pm 2.6\%$ ($n = 12$) for RNase B. These data are specific for the VP-DSC instrument. The lower recovery of $89.0 \pm 1.9\%$ ($n = 4$) found for RNase A in the DASM-4 instrument is probably due to the lower cooling rate between the scans.

Table 1
Thermodynamic quantities obtained by DSC at two pH values for RNase B, Man_1-RNase, $GlnNAc_1$-RNase, and RNase A, respectively

Protein	pH	n[a]	T_{trs}[b] (°C)	ΔH^{cal}[c] (kJ mol^{-1})	ΔC_p[d] (kJ mol^{-1} K^{-1})	$\Delta G^{25\,^{\circ}C}$[e] (kJ mol^{-1})
RNase B	4.0	16	57.5	433.6	4.92	34.5
	2.5	12	41.7	357.7		16.7
Man_1-RNase	4.0	2	56.9	434.5	4.61	34.6
	2.5	2	40.2	359.0		15.7
$GlnNAc_1$-Rnase	4.0	2	57.0	430.2	4.80	33.7
	2.5	2	40.7	343.4		15.2
RNase A	4.0	10	56.5	426.9	5.16	32.8
	2.5	10	40.1	341.6		14.6

[a] Number of measurements.
[b] T_{trs}, transition temperature, uncertainty ±0.3 °C.
[c] ΔH^{cal}, transition enthalpy, calorimetric value at T_{trs}, uncertainty ±7.9 kJ mol^{-1}.
[d] ΔC_p, heat capacity change derived from ΔH versus T_{trs}, see Table 2.
[e] $\Delta G^{25\,^{\circ}C}$, conformational protein stability at 25 °C from Eq. (1).

Table 2
The heat capacity change of RNase B, Man_1-RNase, $GlnNAc_1$-RNase, and RNase A, respectively, obtained by DSC using different procedures for the data treatment

Protein/method	pH	ΔC_p (kJ mol^{-1} K^{-1})	n
RNase B			
From ΔH versus T_{trs}	2.5–4.5	4.92 ± 0.25	50
From individual scans[a]	2.5–4.5	4.51 ± 0.63	46
From individual scans[a]	4.0	4.45 ± 0.56	16
From manual curve analysis	4.0	4.72 ± 0.86	16
From individual scans[a,b]	2.5	4.63 ± 0.77	12
Man_1-Rnase			
From ΔH versus T_{trs}	2.5–4.5	4.61 ± 0.11	6
From individual scans[a]	2.5–4.5	4.48 ± 0.14	6
$GlcNAc_1$-Rnase			
From ΔH versus T_{trs}	2.5–4.5	4.80 ± 0.34	5
From individual scans[a]	2.5–4.5	4.75 ± 0.24	5
Rnase A			
From ΔH versus T_{trs}	2.5–4.5	5.16 ± 0.21	27
From individual scans[a]	2.5–4.5	4.59 ± 0.62	27
From individual scans[a]	4.0	4.58 ± 0.67	10
From manual curve analysis	4.0	4.26 ± 0.61	10
From individual scans[a,b]	2.5	4.88 ± 0.49	10

[a] Data obtained by curve fit based on two-state unfolding (see Section 2).
[b] At pH 2.5 the determination of ΔC_p by manual curve analysis is insecure due to the predenaturational slope of the scans

4. Discussion

The importance of glycosylation for the conformational stability of proteins is still unclear. Comparative studies of glycosylated and non-glycosylated proteins reported so far do not allow to draw general conclusions. Glycosylation may lead to a minor increase in conformational stability by about $\Delta G = 0-5$ kJ mol^{-1} and an increase in thermostability by about 0–2 °C [7,16–20]. A more significant effect of glycosylation was found for immunoglobulin IgG2b C_H2 domain [21].

RNase (Fig. 3) offers the possibility to determine complete thermodynamic data sets for equilibrium unfolding on a glycosylated protein that shows structural integrity of the oligosaccharide at partial or complete deglycosylation [4–6]. The present scanning calorimetric data obtained on two different instruments show minor but significant differences in thermal transition temperature and enthalpy change for RNase B, Man_1-RNase, $GlnNAc_1$-RNase, and RNase A. At the same time, the heat capacity change remains unaltered and confirms data recently reported by Pace et al. [11].

Comparing the conformational stability it follows at pH 4.0 $\Delta G^{25\,°C} = 34.5$, 34.6, 33.7, and 32.8 kJ mol^{-1} for RNase B, Man_1-RNase, $GlnNAc_1$-RNase, and RNase A, respectively. There may be two different explanations for these results: (i) volume effects of the attached oligosaccharide which reduce the conformational entropy of the unfolded protein, and (ii) specific interactions of the oligosaccharide with the protein. Volume effects cannot be excluded completely but seem to be of minor importance. The reduction of the oligosaccharide in size at partial deglycosylation does not parallel the decrease of protein conformational stability. This might be due to the presence of intact disulfide bridges in the thermally denatured RNase which considerably restrict the expected increase in Stokes radius compared with random coil [22,23]. On the other hand, $GlnNAc_1$-RNase with only one *N*-acetylglucosamine attached to Asn34 shows still enhanced conformational stability compared with RNase A. This result

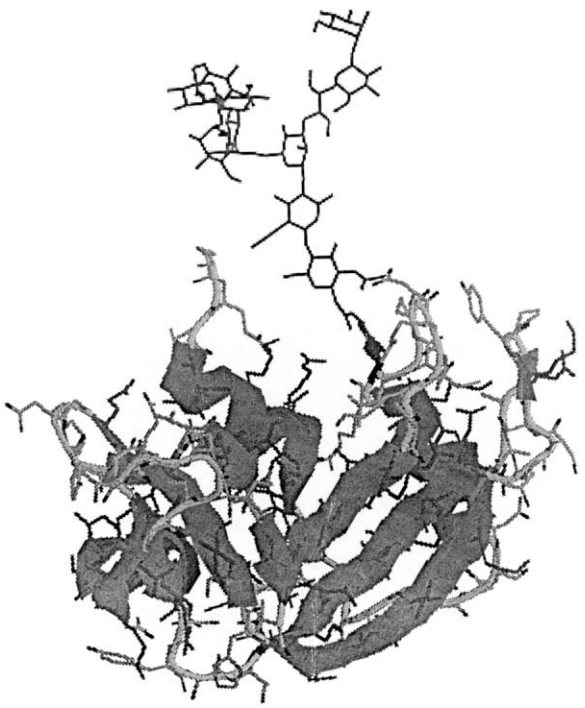

Fig. 3. Structure of RNase B. The folded protein is shown in the lower part, the oligosaccharide attached to Asn34 (thick line) rises up. The protein structure was drawn using the PDB file 1RBB and RASMOL.

is consistent with a hydrogen bond of Lys37 with GlnNAc-1 proposed by molecular dynamics simulations [24].

To sum up, the present data support the current view on the stabilizing effect of *N*-glycosylation of a folded protein [4,25].

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References

[1] T.W. Rademacher, R.B. Parekh, R.A. Dwek, Annu. Rev. Biochem. 57 (1988) 785.
[2] H. Lis, N. Sharon, Eur. J. Biochem. 218 (1993) 1.
[3] P.M. Rudd, H.C. Joao, E. Coghill, P. Fiten, M.R. Saunders, G. Opdenakker, R.A. Dwek, Biochemistry 33 (1994) 17.
[4] R.L. Williams, S.M. Greene, A. McPherson, J. Biol. Chem. 262 (1987) 16020.
[5] M.R. Wormald, R.A. Dwek, Structure 7 (1999) R155.
[6] L. Gonzalez, M. Bruix, T. Diaz-Maurino, T. Feizi, M. Rico, D. Solis, J. Jimenez-Barbero, Arch. Biochem. Biophys. 383 (2000) 17.
[7] U. Arnold, R. Ulbrich-Hofmann, Biochemistry 36 (1997) 2166.
[8] U. Arnold, A. Schierhorn, R. Ulbrich-Hofmann, Eur. J. Biochem. 259 (1999) 470.
[9] M. Fukuda, A. Kobata (Eds.), Glycobiology—a Practical Approach, IRL Press, Oxford University Press, Oxford, 1993.
[10] T.V. Burova, V. Beckert, H. Uhlmann, O. Ristau, R. Bernhardt, W. Pfeil, Protein Sci. 5 (1996) 1890.
[11] C.N. Pace, G.D. Grimsley, S.T. Thomas, G.I. Makhatadze, Protein Sci. 8 (1999) 1500.
[12] P.L. Privalov, N.N. Khechinashvili, J. Mol. Biol. 86 (1974) 665.
[13] C.N. Pace, E.J. Hebert, K.L. Shaw, D. Schell, V. Both, D. Krajcikova, J. Sevcik, K.S. Wilson, Z. Dauter, R.W. Hartley, G.R. Grimsley, J. Mol. Biol. 279 (1998) 271.
[14] W. Pfeil, Protein Stability and Folding—a Collection of Thermodynamic Data, Springer, Berlin, 1998, and Supplement 1, Springer, Berlin, 2001.
[15] G. Kern, N. Schülke, F.X. Schmid, R. Jaenicke, Protein Sci. 1 (1992) 120.

[16] G. Williamson, N.J. Belshaw, T.R. Noel, S.G. Ring, M.P. Williamson, Eur. J. Biochem. 207 (1992) 661.
[17] P.M.M. Weers, C.M. Kay, K. Oikawa, M. Wientzek, D.J. van der Horst, R.O. Ryan, Biochemistry 33 (1994) 3617.
[18] T. Ueda, H. Iwashita, Y. Hashimoto, T. Imoto, J. Biochem. 119 (1996) 157.
[19] C. Wang, M. Eufemi, C. Turano, A. Giartosio, Biochemistry 35 (1996) 7299.
[20] G.T. DeKoster, A.D. Robertson, Biochemistry 36 (1997) 2323.
[21] V.M. Tishchenko, J. Lund, M. Goodall, R. Jefferis, in: J.E. Ladbury, B.Z. Chowdry (Eds.), Biochemistry: Applications of Calorimetry in the Biological Sciences, Wiley, New York, 1998, p. 267.
[22] A. Nöppert, K. Gast, M. Müller-Frohne, D. Zirwer, G. Damaschun, FEBS Lett. 380 (1996) 179.
[23] K. Gast, D. Zirwer, H. Damaschun, U. Hahn, M. Müller-Frohne, M. Wirt, G. Damaschun, FEBS Lett. 403 (1997) 245.
[24] R.J. Woods, C.J. Edge, R.A. Dwek, Struct. Biol. 1 (1994) 499.
[25] B. Imperiali, S.E. O'Connor, Curr. Opinion Chem. Biol. 3 (1999) 643.