Thermochimica Acta, 199 (1992) 189–196
Elsevier Science Publishers B.V., Amsterdam

Conformational stability of proteins and peptide–peptide interactions in the presence of carbohydrates [1]

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(Received in final form 4 October 1991)

Abstract

The denaturation temperatures and enthalpies of bovine pancreatic RNAase A in the presence of different amounts of D-glucose or its oligomers have been determined from DSC measurements and compared with literature results for other globular proteins in the presence of oligosaccharides or polyhydroxylated compounds. Both parameters increase almost proportionally for RNAase A at increasing sugar concentration (the denaturation appearing as a reversible, one-step process) and the evaluated Gibbs energy–temperature plots show an expansion of the stability range and an increase in relative stability.

Isothermal measurements were also obtained by dilution-flow calorimetry to determine the virial coefficients of the excess enthalpies for aqueous solutions of some model peptides (*N*-acetylamides of simple amino acids) in the presence of 1 M D-glucose. These results provide an insight into the role of sugars in preventing peptide–peptide interactions.

INTRODUCTION

Concentrated aqueous solutions of mono-and oligosaccharides and other polyhydroxylated compounds are known to stabilize proteins against thermal denaturation [1–8]. Here we report a preliminary DSC study of the thermal denaturation of Ribonuclease A (RNAase A) in the presence of D-glucose, maltose and trehalose.

The mechanism through which polyhydroxylated co-solutes stabilize proteins has not yet been determined. Strengthening of hydrophobic intramolecular interactions [7], preferential solvation [4] and changes of water structure [3,9] have been put forward to explain the increase of the midpoint denaturation temperature T_d. For this reason, we have included a set of calorimetric measurements of the enthalpy of dilution of some model

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[1] Presented at the 12th National Conference on Calorimetry and Thermal Analysis, Bari, Italy, 11–13 December 1990.

peptides in the presence of 1 M D-glucose in order to obtain information on peptide–peptide interaction in this stabilizing medium.

The model peptides studied were the *N*-acetylamides of the following amino acids: glycine (NAGA), L-alanine (NAAA), L-valine (NAVA) and L-leucine (NALA); the *N'*-methylated amides of glycine (NAGAM) and L-alanine (NAAAM) were also investigated.

EXPERIMENTAL

Materials

The bovine pancreatic RNAase A used was a Sigma product (Fraction XII-A) containing less than 0.01% phosphate (in moles).

The sugars were also crystalline Sigma products of the maximum purity grade, used without purification. The *N*-acetylamides and their *N'*-methylated derivatives of amino acids were all Bachem products. Recrystallization from ethanol–ether mixtures was carried out in most cases.

RNAase A solutions were prepared in sodium acetate buffer 0.1 M, pH 5.0, using deionized, twice-distilled, degassed water. The protein concentration was determined spectrophotometrically ($\varepsilon_{278} = 9800$ l cm^{-1} mol^{-1}) [10]. Sugar concentrations were calculated by weighing the dried substances, which is adequate for the purpose of this study. The pH was finally adjusted and checked with a Radiometer instrument.

Peptide solutions were prepared by weighing water and each of the solutes or co-solutes.

Instrumentation

The microcalorimeter used was a second-generation Setaram Micro-DSC, designed for the study of dilute aqueous solutions of biological macromolecules. Its temperature program covers the range 273–373 K and the scan rate can be changed to suit widely differing conditions. After some trial experiments, a scan rate of 0.5 K min^{-1} was chosen for this study.

The enthalpies of dilution $\Delta_{dil}H$ (in J kg^{-1} of mixed solvent in the final solution) of model peptides, at constant 1 M aqueous D-glucose, were determined with an LKB 10700-1 standard flow microcalorimeter at 298.15 $\pm$ 0.02 K, as described elsewhere [11–13].

RESULTS AND DISCUSSION

Thermodynamics of RNAase A denaturation in the presence of sugars

Bovine RNAase A is a hydrolytic enzyme (124 residues, $M_r = 13\,600$) and one of the better characterized proteins. Its thermal denaturation was

first studied calorimetrically by Privalov and Khechinashvili [14] and more recently by Fujita and Noda [15].

The direct DSC signal, when normalized for the electrical calibration, gives some important parameters, such as the initial (T_i) and final (T_f) temperatures of the denaturation process, the temperature of the maximum heat absorption (T_d) (assumed to correspond to the midpoint denaturation) and the changes in the denaturation enthalpy ($\Delta_d H$) and the apparent molar heat capacity (C_p). $\Delta_d H$ is obtained directly by integrating the area under the denaturation peak

$$\Delta_d H = \int_{T_i}^{T_f} C_p \, dT - \int_{T_i}^{T_d} C_p^N \, dT - \int_{T_d}^{T_f} C_p^D \, dT \tag{1}$$

where C_p^N and C_p^D are the apparent molar heat capacities below and above the denaturation range and are extrapolated into this range up to T_d.

The preliminary results of this study on the thermal denaturation of RNAase A in water and in the presence of sugars at pH 5.0, give an average $\Delta_d H$ of 457 kJ mol^{-1}, in good agreement with results reported by Fujita and Noda [15] (453 kJ mol^{-1} at the same pH and 428 $\pm$ 17 kJ mol^{-1} at pH 5.8) and by Privalov and Khechinashvili [14] (486 kJ mol^{-1} at pH 5.5). However, freshly prepared solutions of RNAase A contain some percentages of aggregates; a thermal pretreatment for breaking them has been described [16]. After following this procedure, a value of 383 $\pm$ 24 kJ mol^{-1} was obtained from more than 10 measurements for our sample of RNAase A. A mean value of -380 kJ mol^{-1} was obtained during the cooling of some untreated solutions, and a mean value of 366 kJ mol^{-1} was obtained for other less pure RNAase A samples from Sigma, after chromatographic purification and without thermal pretreatment. Finally, we assumed that the procedure described is correct and does not introduce partial denaturation of enzyme. A slower rate of scanning (0.1 K min^{-1}) was rejected because a prolonged residence of RNAase A solutions at 340–355 K promotes irreversible transformations and inactivation.

A van't Hoff analysis of data as suggested by Privalov and Khechinashvili [14], gives

$$\Delta_d H(\text{cal})/\Delta_d H(\text{v.H.}) \approx 1$$

for all the conditions explored, confirming that the two-state model for the denaturation process is realistic and no stable intermediates are present. No deconvolution procedure is needed, therefore, for evaluating the thermodynamic parameters.

The thermal denaturation results obtained in pure water and in the presence of the same sugars are given in Table 1 and summarized in Figs. 1 and 2. Changes in the denaturation enthalpies and heat capacities obtained by the heat flow/temperature plots and calibrations, are used to deter-

TABLE 1

Thermal denaturation of RNAase A in the presence of oligosaccharides in 0.1 M sodium acetate buffer at pH 5.0

	Sugar C (mol l^{-1})	T_d [a] (K)	$\Delta_d H$ [b] (kJ mol^{-1})
Water	–	334.0	398 (9)
Glucose	1.0	338.5	437
Glucose	1.6	340.5	451
Glucose	2.7	346.0	495
Glucose	3.6	350.0	509
Maltose	0.95	340.0	431
Trehalose	0.95	340.5	409

[a] Uncertainty ±0.2 K.
[b] Uncertainty ±10 kJ mol^{-1}.

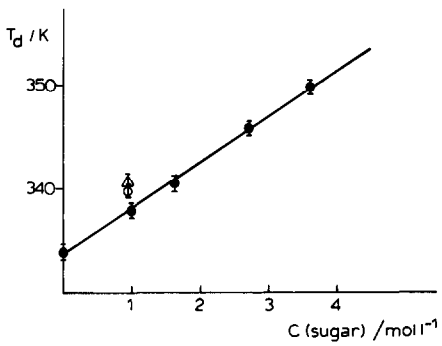

Fig. 1. Dependence of the denaturation temperature of RNAase A on the sugar concentration: ●, D-glucose; △, tetrahalose; ○, maltose.

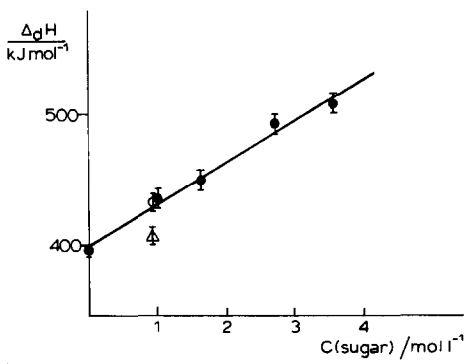

Fig. 2. Dependence of denaturation enthalpies of RNAase A on the sugar concentration (for symbols see Fig. 1).

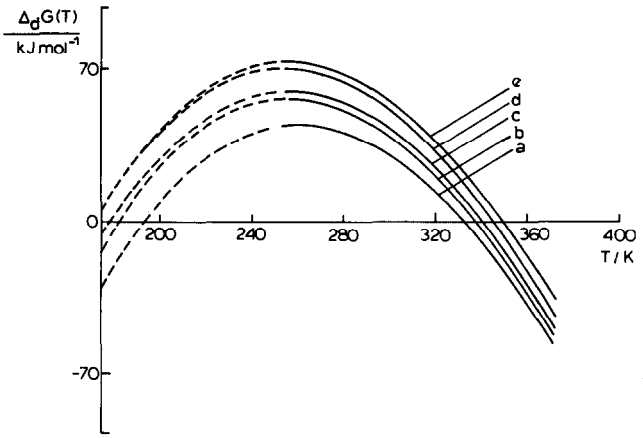

Fig. 3. Gibbs energies for the denaturation of RNAase A as a function of temperature for different concentrations of D-glucose: curve a, 0.0 M; curve b, 1.0 M; curve c, 1.6 M; curve d, 2.7 M; curve e, 3.6 M. $\Delta_d C_p = 4.6$ kJ mol^{-1} K^{-1} was assumed for all glucose concentrations.

mine, from standard thermodynamic relationships, the dependence on the temperature of enthalpy, entropy and Gibbs energy

$$\Delta_d H(T) = \Delta_d H(T_d) - \int_T^{T_d} \Delta_d C_p \, dT \tag{2}$$

$$\Delta_d S(T) = \frac{\Delta_d H(T_d)}{T} - \int_T^{T_d} \frac{\Delta_d C_p}{T} \, dT \tag{3}$$

$$\Delta_d G(T) = \Delta_d H(T) - T \, \Delta_d S(T) \tag{4}$$

It is worth noting that the thermodynamic procedure combined with the DSC measurements does not actually require any model to represent the denaturation process because eqns. (2)–(4) are solely based on the reversibility of the process, experimentally ascertained. In the case of RNAase A, moreover, the process seems to be essentially a two-state model, which facilitates application of these equations.

The trend of the Gibbs energy of denaturation with the temperature and D-glucose concentration is reported in Fig. 3, which gives an overview of the relative stability increase.

For the evaluation of $\Delta_d G(T)$, we assumed the same $\Delta_d C_p$ average value for all the sugar concentrations. The uncertainty of $\Delta_d C_p$ for each sugar concentration falls in the range of uncertainty evaluated for water (± 0.4 kJ mol^{-1} K^{-1}). The curves in Fig. 3 are reasonably affected by a systematic error, but this is confidently the same for all conditions. The independence of $\Delta_d C_p$ on sugar concentrations is a proof, in our opinion, that sugars do not interact directly with native or denatured forms of RNAase A.

Enthalpic virial coefficients of model peptides in 1 M D-glucose at 298.15 K

According to the McMillan–Mayer theory of solutions, as modified by Kauzmann, Friedman and other authors [17–21] for non-electrolyte solutions, excess enthalpies can be expressed as a power expansion series

$$H^E = h_{xx}m^2 + h_{xxx}m^3 + \ldots \quad (5)$$

where the coefficients h are the enthalpic part of the virial coefficients of the Gibbs excess energies, for example

$$g_{xx} = h_{xx} - Ts_{xx} \quad (6)$$

The virial coefficients depend on the solute–solute potential of average force W, which itself depends on the distance and all possible reciprocal orientations of two solute molecules [13,19]. However, these quantities also depend on the mean orientation of all the solvent molecules involved, because the solute–solute potential of average force is perturbed by the dynamic structure of the interposed medium. Consequently, the h values also depend on the changes in the solute–solvent and solvent–solvent interactions from extremely dilute solutions up to the actual concentrations considered. The aqueous 1 M D-glucose medium can be considered as a mixed solvent, using the same rationale.

The h_{xx} and h_{xxx} values are reported in Table 2, together with the corresponding values of h_{xx} found in water and in 7 M urea.

The most important result is that all the h_{xx} coefficients in 1 M D-glucose (both negative and positive) are smaller in absolute value than in water. The trends of the excess enthalpies also display a marked curvature, h_{xx} being smaller than h_{xxx}.

TABLE 2

Enthalpic virial coefficients for *N*-acetylamides and *N*-acetyl-*N'*-methylamides of some amino acids in 1 M D-glucose, water and 7 M urea at 298.15 K

	W (Gluc 1M)		W	W (U 7M)
	h_{xx} (J kg mol^{-2})	h_{xxx} (J kg^2 mol^{-3})	h_{xx} (J kg mol^{-2})	h_{xx} (J kg mol^{-2})
NAGA	−186 (24)	−29 (23)	−220 (9) [a,b]	290 (22) [b,c]
NAAA	30 (14)	414 (43)	273 (5) [c]	624 (10) [c]
NAVA	575 (188)	4320 (1642)	1259 (44) [a]	969 (12) [d]
NALA	1528 (52)	194 (107)	1969 (28) [c]	1430 (21) [d]
NAGAM	423 (13)	69 (31)	585 [a]	968 (8) [d]
NAAAM	388 (81)	1321 (306)	1181 [a]	1319 (14) [d]

[a] Ref. 22.

[b] h_{xxx} (W) = 48 (5) and h_{xxx} (W/U 7M) = 72 (35) J kg^2 mol^{-3} were also found for NAGA. The 95% confidence limits are given in parentheses.

[c] Ref. 23.

[d] Ref. 24.

The number of systems examined is limited, and a statistical analysis, such as that of Savage and Wood [20] cannot yet be attempted. However, these results could be rationalized by assuming that D-glucose and other saccharides compete for hydration with both the polar and the hydrophobic framework of the peptides, so that both the peptide–peptide interactions (driven mainly by the coalescence of the hydration cospheres [11,23]), and the hydrophobic interactions [25] seem to become weaker in the presence of concentrated carbohydrates, because both the peptidic and alkylic groups are less hydrated and less effective in perturbing water. Moreover, because of the different geometries, the hydration co-spheres of peptidic groups and sugars are not compatible, and a sugar–peptide interaction, direct or water-mediated, seems unlikely [26]. In conclusion, neither preferential solvation of protein by the sugars nor strengthening of the hydrophobic intramolecular interactions can be invoked to explain the increased stability of RNAase A and other proteins. A more important effect will probably be the diminished activity of water in the presence of concentrated carbohydrates. This makes water–protein interactions less effective and, hence, relatively stabilizes the protein structures. In fact, in anhydrous organic solvents, the T_d of suspended RNAase A increases considerably [27].

ACKNOWLEDGEMENTS

This work was financially supported by the Italian Consiglio Nazionale delle Ricerche (C.N.R.), Rome, and Ministero dell'Università e della Ricerca Scientifica e Tecnologica (M.U.R.S.T.).

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