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Effect of monomeric and oligomeric sugar osmolytes on ΔG_D, the Gibbs energy of stabilization of the protein at different pH values: Is the sum effect of monosaccharide individually additive in a mixture?

Nitesh Kumar Poddar [a], Z.A. Ansari [a], R.K. Brojen Singh [a], A.A. Moosavi-Movahedi [b], Faizan Ahmad [a,*]

[a] Centre for Interdisciplinary Research in Basic Sciences, Jamia Millia Islamia, New Delhi-110025, India
[b] Institute of Biochemistry and Biophysics, University of Tehran, Iran

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ABSTRACT

Thermal denaturation curves of ribonuclease-A were measured by monitoring changes in the far-UV circular dichroism (CD) spectra in the presence of different concentrations of six sugars (glucose, fructose, galactose, sucrose, raffinose and stachyose) and mixture of monosaccharide constituents of each oligosaccharide at various pH values in the range of 6.0-2.0. These measurements gave values of T_m (midpoint of denaturation), ΔH_m (enthalpy change at T_m), ΔC_p (constant-pressure heat capacity change) under a given solvent condition. Using these values of ΔH_m, T_m and ΔC_p in appropriate thermodynamic relations, thermodynamic parameters at 25 °C, namely, ΔG^o_D (Gibbs energy change), ΔH^o_D (enthalpy change), and ΔS^o_D (entropy change) were determined at a given pH and concentration of each sugar (including its mixture of monosaccharide constituents). Our main conclusions are: (i) each sugar stabilizes the protein in terms of T_m and ΔG^o_D, and this stabilization is under enthalpic control, (ii) the protein stabilization by the oligosaccharide is significantly less than that by the equimolar concentration of the constituent monosaccharides, and (iii) the stabilization by monosaccharides in a mixture is fully additive. Furthermore, measurements of the far- and near-UV CD spectra suggested that secondary and tertiary structures of protein in their native and denatured states are not perturbed on the addition of sugars.

1. Introduction

When it needs to maintain the osmotic pressure of living cells, nature has (through evolutionary selection) opted to do this by incorporating a number of compounds known as osmolytes into the cells. It is remarkable that the small numbers of these compounds span cellular organisms, plants, and animal vertebrates and invertebrates [1–3]. These compounds comprise polyols, sugars, methylamines, amino acids and their derivatives, and in some cases urea in combination with methylamines [2]. Among these chemical categories, carbohydrates are usually dominant solutes accumulated in organisms to protect the proteins in terms of loss of activity [4,5] and chemical [6,7] and thermal denaturations [8–12]. They have also been found to be effective stabilizers of proteins and biological assemblies when added at high concentrations [13–18].

There are various mechanisms that have been used to explain the observation on the effect of sugars on the protein denaturation equilibrium, native (N) state ↔ denatured (D) state [19–23]. According to one mechanism sugars stabilize N state because they are preferentially excluded from the protein surface, for the preferential exclusion increases the chemical potential of the protein proportionately to the solvent exposed surface area. Thus, by Le Chatelier's principle, sugar osmolytes favor more compact state, i.e., the N state over the structurally expanded state, i.e., D state. Hence according to this mechanism ΔG_D, the Gibbs free energy change associated with the denaturation process, N state ↔ D state, should increase in the presence of osmolytes, for $\Delta G_D = -RT\ln([D]/[N])$, where square bracket represents concentration. According to the most recent mechanism of sugar osmolytes stabilization of proteins, Bolen and colleagues [24] used apparent water-to-osmolyte solution transfer free energies for side-chain and backbone models to interpret the increase in stability. They concluded that unfavorable interactions between the fully unfolded protein backbone and the osmolyte solution drive folding. That is, the decreased exposure of the backbone on folding is the major driving force for osmolyte-induced stabilization.

A few studies have reported the effect of these osmolytes, singly and in combination on the denaturation equilibrium of proteins [25 and Refs. therein, 26]. The main conclusion of these studies is that all osmolytes act independently on the protein, i.e., none of the osmolytes alters the efficacy of the other in forcing the protein to fold or unfold. However, yet it is difficult to predict the effects of mixture of osmolytes in protein

Abbreviations: ΔG_D, Gibbs free energy change; ΔG^o_D, Gibbs free energy change at 25 °C; ΔC_p, constant-pressure heat capacity change; T_m, midpoint of thermal denaturation; ΔH_m, enthalpy change at T_m; CD, circular dichroism; $[\theta]_{222}$, mean residue ellipticity at 222 nm; RNase-A, ribonuclease-A; Glc, glucose; Fru, fructose; Gal, galactose.

* Corresponding author. Tel.: +91 11 26981733; fax: +91 11 26983409.
E-mail address: faizan_ahmad@yahoo.com (F. Ahmad).

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stabilizing. To investigate the effects of mixture of osmolytes on protein, we have carried out measurements of thermal denaturation of RNase-A in the absence and presence of six sugars (glucose, fructose, galactose, sucrose, raffinose and stachyose) and mixtures of monosaccharide constituents of each oligosaccharide at different pH values in the range 6.0–2.0. In this paper, we report values of thermodynamic parameters, T_m (midpoint of thermal denaturation), ΔH_m (enthalpy change at T_m), ΔC_p (constant-pressure heat capacity change), and ΔG_D^o (value of ΔG_D at 25 °C) obtained from these measurements. It has been observed that an equimolar mixture of monosaccharide constituents has more effect on

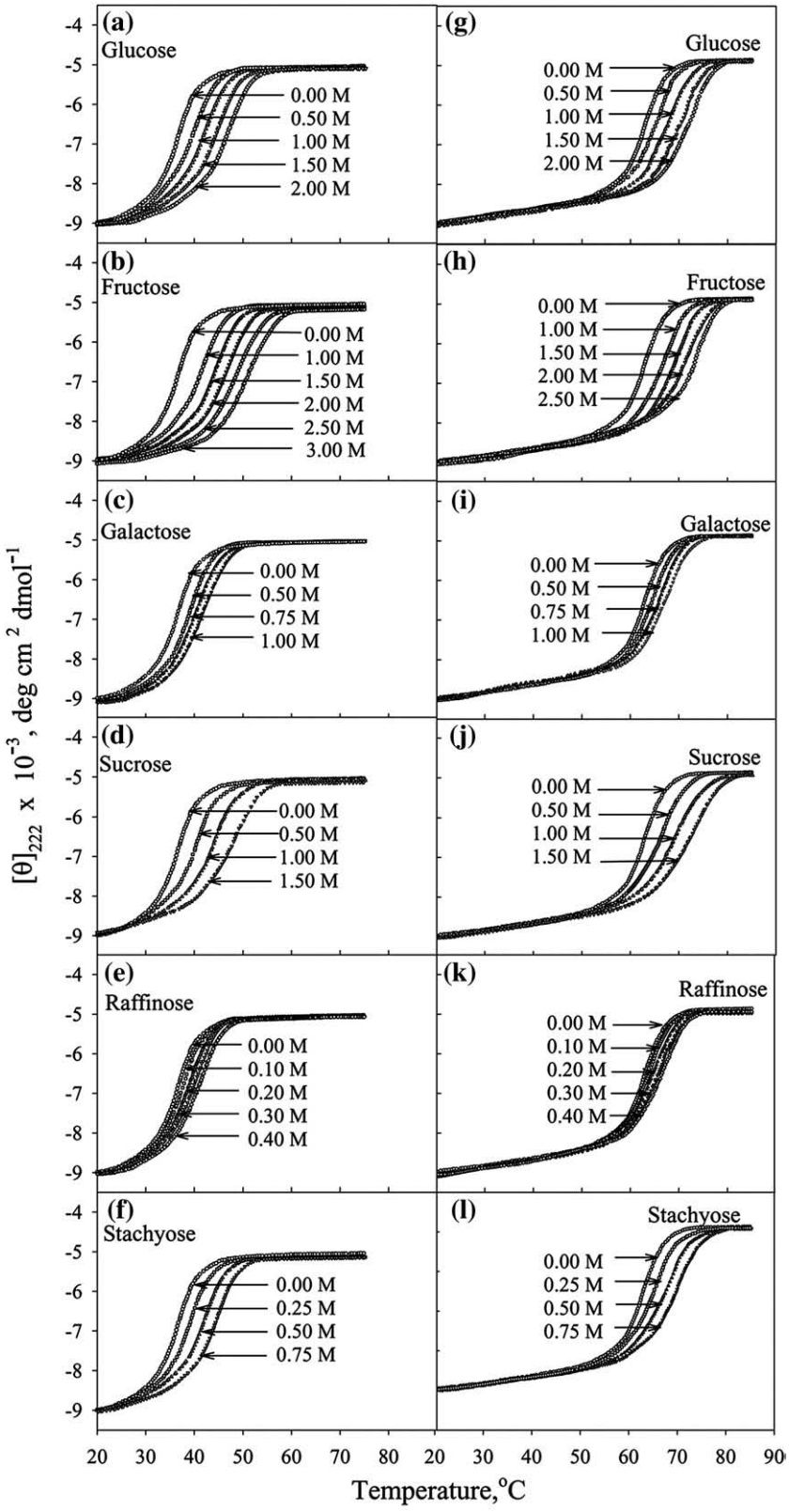

Fig. 1. Representative thermal denaturation profiles of RNase-A in the presence of different indicated concentrations of sugar(s) at pH 2.0 (a–f) and pH 6.0 (g–l). In order to maintain clarity all data points are not shown.

ΔG_D^0 of protein than the oligosaccharide. We also report the effect of oligosaccharides as well as their monosaccharide constituents alone and in combinations on the secondary and tertiary structures of the two end states of the equilibrium, N state ↔ D state.

2. Materials and methods

RNase-A (type III-A) was commercially purchased from Sigma Chemical Company (USA). D-glucose, D-fructose, D-galactose, D-sucrose, D-raffinose and D-stachyose were also from the Sigma. All the chemicals were of analytical grade and used without further purification.

RNase-A solution was dialyzed extensively against 0.1 M KCl solution (pH 7.0) at about 4 °C. Protein stock solutions were filtered using 0.45-μm millipore filter paper. The protein gave single band during the native and SDS polyacrylamide gel electrophoresis. Concentration of the protein stock solution was determined experimentally using a value of 9800 at 277.5 nm for ε, the molar absorption coefficient (M^{-1} cm^{-1}) [27]. All solutions for optical measurements were prepared in the desired degassed buffer containing 0.1 M KCl, and protein concentration was in the range 25–40 μM. Various buffers used were 0.1 M KCl–HCl buffer for pH 2.0, 0.05 M glycine–HCl for pH 3.0, 0.05 M sodium acetate buffer for pH 4.0 and 0.05 M cacodylic acid buffer for a pH range 5.0–6.0. Concentration of stock solutions of glucose, fructose and sucrose were determined by measuring the difference refractive index of each solution and respective buffer containing 0.1 M KCl [28] while that of other stock solutions of sugars for which refractive indices are not known, represent the amount of dry sample that were carefully weighed. Since pH of the protein solution may change on heating, pH of the solution was, therefore, measured after the denaturation experiment as well. It was observed that the change in pH was not significant. It should, however, be noted that no corrections were made for the possible effect of co-solutes on the pH of the protein solution.

2.1. Thermal denaturation measurements

Thermal denaturation studies were carried out in Jasco J-715 spectropolarimeter equipped with a peltier type temperature controller (PTC-348-WI) with a heating rate of 1 °C/min. This scan rate was found to provide adequate time for equilibration. Change in CD at 222 nm of the protein solution was measured in the temperature range 20–85 °C. About 650 data points of each transition curve were collected. After denaturation, the sample was immediately cooled down to measure reversibility of the reaction at different temperatures. It was observed that data from renaturation experiments fell on the denaturation curve. All solution blanks showed negligible change in CD with temperature and were, therefore, neglected during the data analysis. The raw CD data were converted into mean residue ellipticity (deg cm^2 $dmol^{-1}$) at a given wavelength λ using the relation,

$$[\theta]_\lambda = \theta_\lambda M_O / 10lc \quad (1)$$

where θ_λ is the observed ellipticity (millidegrees) at wavelength λ, M_o is the mean residue weight of the protein, c is the protein concentration (mg/cm^3), and l is the path length (cm).

Each heat-induced transition curve was analysed for T_m (midpoint of denaturation) and ΔH_m (enthalpy change at T_m) using a non-linear least-squares analysis according to the relation,

$$y(T) = \frac{y_N(T) + y_D(T)\exp[-\Delta H_m/R(1/T - 1/T_m)]}{1 + \exp[-\Delta H_m/R(1/T - 1/T_m)]} \quad (2)$$

where $y(T)$ is the optical property at temperature T (K), $y_N(T)$ and $y_D(T)$ are the optical properties of the native and denatured protein molecules at T K, respectively, and R is the gas constant. In the analysis of the transition curve, it was assumed that a parabolic function describes the dependence of the optical properties of the native and

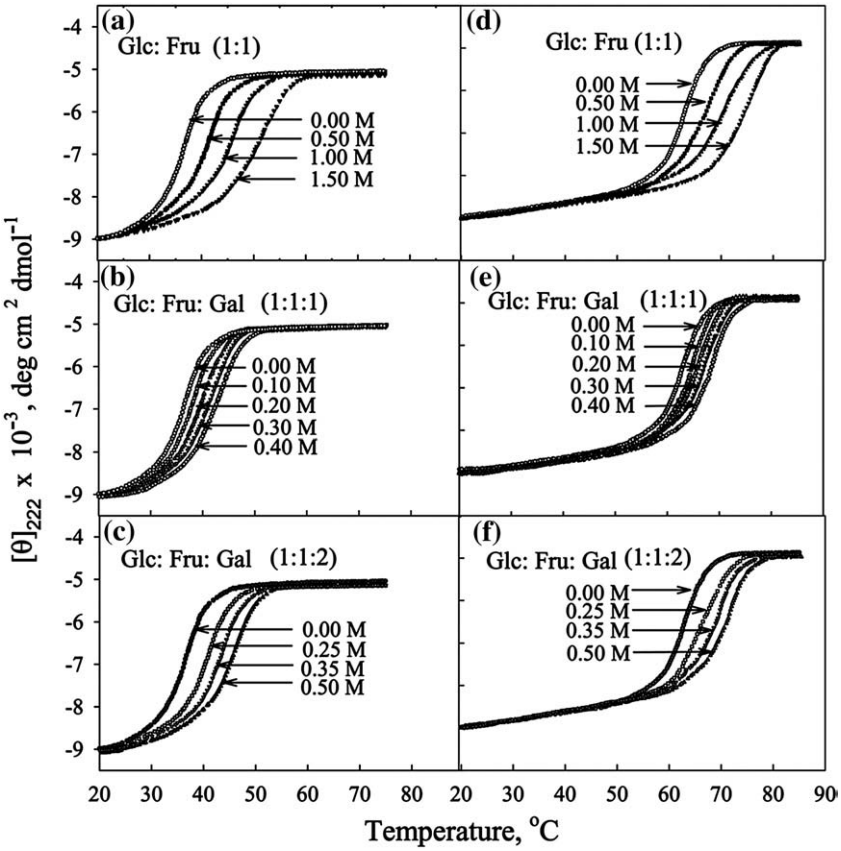

Fig. 2. Representative thermal denaturation profiles of RNase-A in the absence and presence of different indicated concentration ratios in mixtures of monosaccharide constituents of oligosaccharides at pH 2.0 (a–c) and pH 6.0 (d–f). In order to maintain clarity all data points are not shown.

denatured protein molecules (i.e., $y_N(T)=a_N+b_NT+c_NT^2$ and $y_D(T)=a_D+b_DT+c_DT^2$, where a_N, b_N, c_N, a_D, b_D, and c_D are temperature-independent coefficients) [29,30]. A plot of ΔH_m versus T_m gave the value of ΔC_p, the temperature-independent heat capacity change at constant pressure. $\Delta G_D(T)$, the value of ΔG_D at any temperature T was estimated using Gibbs–Helmholtz equation with values of T_m, ΔH_m and ΔC_p,

$$\Delta G_D(T) = \Delta H_m\left(\frac{T_m - T}{T_m}\right) - \Delta C_p\left[(T_m - T) + T\ln\left(\frac{T}{T_m}\right)\right] \quad (3)$$

3. Results

3.1. Thermal denaturation of RNase-A

Heat-induced denaturation of RNase-A in the presence of different concentrations of glucose (Glc), fructose (Fru), galactose (Gal), sucrose, raffinose and staychose individually and mixtures of monosaccharide constituents of oligosaccharides were measured at five pH values (2.0, 3.0, 4.0, 5.0 and 6.0). The heat denaturation was monitored by measuring $[\theta]_{222}$ (a probe to measure change in peptide backbone conformation [31]). It has been observed that the thermal denaturation of RNase-A in the presence and absence of all sugars is reversible.

Figs. 1 and 2 show the representative denaturation curves of RNase-A in the presence and absence of glucose, fructose, galactose, sucrose, raffinose and stachyose, and equimolar mixture of glucose and fructose (constituents of sucrose), equimolar mixture of glucose, fructose and galactose (constituents of raffinose), 1:1:2 molar ratio of glucose: fructose: galactose (constituents of stachyose), respectively. Each denaturation curve of a protein at a given pH and [sugar (s)], the molar concentration of sugar(s), was analyzed for ΔH_m and T_m using a non-linear least-squares method that involves fitting the entire ($[\theta]_{222}$, T) data of the transition curve to Eq. (2) with all eight free parameters (a_N, b_N, c_N, a_D, b_D, c_D, ΔH_m, and T_m). Table 1 shows values of ΔH_m and T_m at each pH and sugar(s) concentration. It should be noted that each thermodynamic parameter represents the mean of the triplicate measurements. At a given [sugar(s)], values of ΔH_m versus T_m obtained at different pH values were used to construct a plot of ΔH_m versus T_m. The linear least-squares analysis of such a plot gave ΔC_p ($=\delta\Delta H_m/\delta T_m)_p$) at this given [sugar(s)]. Values of ΔC_p with their standard deviations at each [sugar(s)] are also given in Table 1.

Fig. 3 shows plots of ΔT_m versus [sugar] at different pH values, where ΔT_m is the difference between T_m (in the presence of the sugar) and T_m (in the absence of sugar).

The value of ΔG_D^o (ΔG_D value at 25 °C) of RNase-A in a given solvent condition was determined using Eq. (3) with values of T_m, ΔH_m and ΔC_p given in Table 1. Values of ΔG_D^o obtained in different solvent conditions are also shown in Table 1. This table also shows $\%\Delta\Delta G_D^o$, the percent change in ΔG_D^o of the protein due the presence of sugars(s); $\%\Delta\Delta G_D^o = 100\,[\Delta G_D^o$ (in the presence of sugar(s)) $- \Delta G_D^o$ (in the absence of sugar)$]/\Delta G_D^o$ (in the absence of sugar). Fig. 4 (a–f) shows the representative plots of $\Delta\Delta G_D^o$ of RNase-A in the presence of various molar concentrations of di-, tri-, and tetrasaccharides with their free monosaccharides constituents individually and in mixtures at different pH values.

3.2. Effect of sugars on N and D states of RNase-A

Fig. 5 a and b show respectively the far- and near-UV CD spectra of the native RNase-A in the absence and presence of the highest concentration of each sugar individually and in the presence of oligosaccharide constituents mixtures at pH 6.0 and 25 °C (curves grouped as 1). It is seen in these figures that there is no significant change in the secondary and tertiary structures of the native protein on the addition of sugar osmolytes. These figures also shows the far- and near-UV CD spectra of the heat-denatured RNase-A in the absence and presence of the highest concentration of all sugars studied at pH 2.0 and 85 °C (curves grouped as 2). These measurements suggest that there is no significant difference in the characteristic CD spectra of the heat-denatured protein in the absence and presence of all sugars studied. It should be noted that due to a very strong absorbance of the free fructose in the near-UV range [32], measurements of the protein solution were not possible. Hence, no near-UV CD spectra are shown here.

4. Discussion

All thermodynamic quantities, given in Table 1, were obtained from the analysis of heat denaturation curves of RNase-A in the presence and absence of different sugars at different pH values (e.g., see Figs. 1 and 2). This analysis according to Eq. (2) assumes that the transition between the native and denatured states is a two-state process. This is indeed true for RNase-A in the absence of sugars [33]. Furthermore, a comparison of T_m and ΔH_m of RNase-A in the absence of osmolytes given in Table 1 with those obtained from differential scanning calorimetry (DSC) measurement [33] gave excellent agreement. This agreement leads us to believe that our measurements of transition curves and their analysis for thermodynamic parameters are authentic and accurate. However, DSC data of RNase-A in the presence of sugars studied here are not reported. In order to check whether the two-state assumption is also valid in the presence of sugars, thermal denaturation curves were also monitored by $\Delta\varepsilon_{287}$, the difference molar absorption coefficient at 287 nm, which is a probe to measure change in the environment of tyrosine residues in RNase-A (denaturation curves not shown). It should be noted that such measurements were not possible in the presence of fructose individually or in a mixture containing other monosaccharides. This is due to the fact that fructose has a very strong absorption in the near-UV region [32]. The denaturation curves monitored by $\Delta\varepsilon_{287}$ were analysed for T_m and ΔH_m using the same procedure used for the analysis of transition curves monitored by $[\theta]_{222}$. Values of T_m and ΔH_m are given in braces in Table 1. It has been observed that both measurements in a given solvent condition gave, within experimental errors, identical values of T_m and ΔH_m. This comparison suggests that heat-induced denaturation of RNase-A in the presence of sugars is a two-state process.

To compare a thermodynamic quantity of a protein in the presence of sugar with that in its absence, it is necessary to show that the structural characteristics of the two end states, i.e., N and D states of the heat denaturation equilibrium of RNase-A are not affected by the addition of sugars as well as mixtures of monosaccharide constituents of oligosaccharide. It is seen in Fig. 5 that the far- and near-UV CD spectra of both the native and denatured states in the absence and presence of sugars and the respective monosaccharide mixtures of oligosaccharide are, within experimental errors, identical. This observation for the native protein is consistent with X-ray diffraction [34] and CD [35] and dimension [24,36,37] measurements showing that the native structure of a protein is not perturbed in the presence of osmolytes. The near-UV CD spectrum of the heat/acid (pH 2.0 and 85 °C) denatured state is not perturbed in the presence of sugar(s). This observation is also in agreement with the earlier CD data on chymotrypsinogen [35]. Hence, a comparison of a thermodynamic property of denaturation in the presence and absence of sugar(s) is valid.

It is seen in Fig. 3 (also see Table 1) that T_m of RNase-A at each pH increases linearly with an increase in the concentration of individual sugar. It has been observed that an oligosaccharide has more stabilizing effect than the individual monosaccharide constituent, and that, on the molar scale, the order of stabilization is: stachyose>raffinose>sucrose>glucose, fructose, galactose (see Fig. 3). It is noteworthy that cytochrome-c was also shown to follow the same ranking, i. e., the order of stabilization was tetra->tri->di->mono-saccharide [19]. We have also observed that the extent of stabilization of RNase-A is pH dependent, that is, it is more at lower pH than that at higher pH. It has been argued that the effect of osmolytes is predominantly on the

Table 1
Stability parameters of RNase-A in the presence of different concentrations of sugars at various pH values[a,b,c]

[Sugar], M	pH 6.0				pH 5.0				pH 4.0				pH 3.0				pH 2.0				ΔC_p
	T_m	ΔH_m	ΔG^o_D	$\%\Delta\Delta G^o_D$	T_m	ΔH_m	ΔG^o_D	$\%\Delta\Delta G^o_D$	T_m	ΔH_m	ΔG^o_D	$\%\Delta\Delta G^o_D$	T_m	ΔH_m	ΔG^o_D	$\%\Delta\Delta G^o_D$	T_m	ΔH_m	ΔG^o_D	$\%\Delta\Delta G^o_D$	
0.00	62.4 {62.5}	468 {468}	41.1	0	60.7 {60.6}	460 {460}	39.0	0	55.5 {55.7}	435 {431}	32.8	0	45.4 {45.6}	385 {376}	21.2	0	35.8 {35.9}	330 {334}	10.6	0	5.14±0.22
Glucose																					
0.50	64.6 {64.5}	472 {477}	43.1	5	62.8 {62.9}	464 {464}	40.9	5	57.5 {57.7}	447 {443}	35.5	8	48.0 {48.2}	397 {393}	24.1	14	38.5 {38.4}	339 {343}	13.2	25	5.10±0.15
1.00	66.3 {66.1}	477 {481}	45.1	10	64.9 {64.7}	468 {472}	43.3	11	59.8 {59.6}	447 {447}	37.5	14	50.2 {50.3}	401 {405}	26.3	24	40.9 {41.0}	351 {355}	15.8	49	4.89±0.25
1.50	68.4 {68.5}	477 {472}	47.3	15	67.1 {67.0}	468 {464}	45.4	17	62.2 {62.2}	447 {443}	39.8	21	52.6 {52.7}	405 {397}	28.8	36	43.5 {43.3}	359 {359}	18.5	75	4.60±0.20
2.00	71.0 {70.8}	468 {472}	49.2	20	69.2 {69.0}	460 {464}	47.0	21	64.4 {64.2}	443 {443}	41.8	27	55.0 {55.1}	405 {405}	31.2	47	46.0 {45.8}	364 {368}	21.0	99	4.14±0.22
Galactose																					
0.50	64.3 {64.4}	468 {464}	43.1	5	62.4 {62.7}	460 {456}	40.8	5	57.5 {57.4}	439 {439}	35.2	7	47.7 {47.6}	389 {389}	23.5	11	38.1 {38.0}	343 {339}	13.1	24	4.81±0.26
0.75	65.3 {65.0}	468 {468}	44.1	8	63.4 {63.3}	460 {460}	41.9	7	58.4 {58.3}	439 {439}	36.1	10	48.8 {48.9}	393 {393}	24.9	17	39.2 {39.3}	347 {351}	14.3	35	4.64±0.23
1.00	66.2 {66.3}	468 {464}	44.9	9	64.6 {64.6}	460 {456}	42.8	10	59.4 {59.5}	439 {435}	36.9	12	49.9 {50.2}	397 {389}	26.1	23	40.3 {40.1}	347 {347}	15.2	43	4.60±0.25
Fructose[d]																					
1.00	66.4	472	45.3	10	64.8	464	43.2	11	59.7	443	37.4	14	50.0	397	26.1	23	40.6	351	15.6	48	4.68±0.26
1.50	68.5	472	47.6	16	66.8	460	44.9	15	61.8	443	39.5	21	52.4	401	28.6	35	43.0	359	18.2	72	4.35±0.20
2.00	70.4	472	49.3	20	68.8	464	47.2	21	63.8	447	41.8	27	54.6	405	30.9	46	45.5	368	20.9	97	4.18±0.17
2.50	72.3	477	50.9	24	70.9	468	48.9	25	65.9	451	43.6	33	57.0	414	33.4	57	47.8	372	22.9	117	4.22±0.19
3.00	–	–	–	–	72.8	464	50.5	30	67.9	451	45.7	39	59.3	418	35.9	69	50.3	376	25.4	140	3.93±0.20
Sucrose																					
0.50	65.7 {65.5}	468 {472}	44.1	7	63.8 {63.6}	460 {464}	41.9	7	58.7 {58.5}	435 {439}	35.7	9	49.1 {49.0}	389 {393}	24.7	16	39.4 {39.3}	343 {347}	14.2	34	4.77±0.19
1.00	68.8 {68.6}	464 {468}	46.7	14	67.1 {66.9}	456 {460}	44.6	14	62.0 {61.8}	435 {439}	38.8	18	52.6 {52.4}	393 {393}	28.1	32	42.8 {42.7}	351 {355}	17.6	66	4.35±0.23
1.50	71.8 {71.6}	468 {468}	49.9	21	70.2 {70.0}	460 {460}	47.8	23	65.2 {65.3}	439 {439}	42.0	28	56.2 {56.0}	401 {405}	31.8	50	46.3 {46.2}	364 {364}	21.3	101	4.10±0.21
[Glc]: [Fru] (1:1)[d]																					
0.50	66.3	472	45.5	11	64.7	464	43.5	11	59.6	439	37.2	13	50.0	393	25.9	22	40.5	355	15.8	49	4.56±0.23
1.00	70.3	468	48.8	19	68.6	460	46.7	20	63.7	443	41.3	26	54.3	401	30.3	43	45.0	364	20.2	91	4.14±0.17
1.50	74.3	472	53.5	30	72.6	464	51.3	31	67.9	447	45.9	40	58.9	414	35.7	68	49.6	380	25.5	141	3.68±0.15
Raffinose																					
0.10	63.1 {62.9}	468 {468}	41.8	2	61.4 {61.2}	460 {460}	39.7	2	56.3 {56.1}	435 {439}	33.6	2	46.2 {46.4}	389 {385}	22.2	5	36.7 {36.5}	334 {339}	11.5	9	5.02±0.10
0.20	63.9 {64.1}	468 {468}	42.6	4	62.1 {62.2}	460 {460}	40.5	4	57.0 {57.1}	435 {435}	34.3	4	47.1 {47.3}	389 {385}	23.0	8	37.6 {37.7}	339 {339}	12.5	18	4.89±0.13
0.30	64.7 {64.7}	468 {468}	43.4	6	62.9 {62.9}	460 {460}	41.3	6	57.8 {58.0}	435 {431}	35.1	7	47.9 {48.1}	389 {385}	23.7	12	38.4 {38.4}	343 {343}	13.3	26	4.77±0.20
0.40	65.3 {65.5}	468 {464}	43.9	7	63.6 {63.8}	460 {456}	41.7	7	58.6 {58.6}	439 {439}	36.1	10	48.7 {48.9}	393 {389}	24.7	16	39.3 {39.4}	343 {343}	14.1	33	4.77±0.10
[Glc]:[Fru]:[Gal] (1:1:1)[d]																					
0.10	63.6	464	42.2	3	61.9	456	40.1	3	56.8	435	34.4	5	46.8	385	22.6	7	37.2	339	12.2	15	4.77±0.24
0.20	64.9	468	43.6	6	63.1	460	41.4	6	58.1	439	35.7	9	48.2	393	24.3	15	38.7	343	13.6	28	4.77±0.19
0.30	66.1	468	44.8	9	64.3	460	42.6	9	59.4	439	36.9	12	49.6	397	25.8	22	40.2	347	15.1	43	4.60±0.14
0.40	67.2	472	45.8	12	65.5	464	43.7	12	60.6	443	38.0	16	50.9	401	27.1	28	41.6	351	16.4	55	4.68±0.20
Stachyose																					
0.25	64.7 {64.5}	464 {464}	43.4	6	63.1 {63.0}	456 {456}	41.3	6	57.9 {57.7}	431 {435}	35.0	7	48.1 {47.9}	385 {389}	23.7	12	38.2 {38.2}	343 {343}	13.2	25	4.60±0.13
0.50	66.9 {67.1}	464 {460}	45.4	10	65.4 {65.6}	456 {451}	43.4	11	60.2 {60.5}	435 {431}	37.5	14	50.8 {50.8}	389 {385}	26.3	24	41.0 {41.1}	351 {347}	16.1	52	4.39±0.16
0.75	69.2 {69.1}	464 {468}	47.7	16	67.8 {67.6}	456 {460}	45.7	17	62.6 {62.8}	435 {435}	39.7	21	53.5 {53.3}	393 {397}	29.1	37	43.6 {43.4}	359 {359}	18.8	78	4.10±0.18
[Glc]:[Fru]:[Gal] (1:1:2)[d]																					
0.25	66.3	464	44.7	9	64.6	456	42.6	9	59.5	435	36.8	12	49.8	393	25.8	22	40.5	347	15.4	46	4.47±0.23
0.35	67.8	468	46.6	13	66.1	460	44.4	14	61.0	439	38.5	17	51.6	401	28.0	32	42.3	355	17.4	64	4.35±0.14
0.50	70.1	468	49.0	19	68.4	460	46.7	20	63.4	443	41.3	26	54.2	401	30.4	43	45.1	368	20.6	95	4.06±0.10

[a] T_m is in °C, ΔH_m and ΔG^o_D are in kJ mol^{-1}, and ΔC_p is in kJ mol^{-1} K^{-1}.
[b] From triplicate measurements values of maximum errors from the mean are 0.1–0.6% and 2–5% in T_m and ΔH_m, respectively. The error in ΔG^o_D is in the range of 4–7%.
[c] A value in the parenthesis is obtained from the transition curve monitored by $\Delta\varepsilon_{287}$.
[d] $\Delta\varepsilon_{287}$ measurements were not possible (see the text).

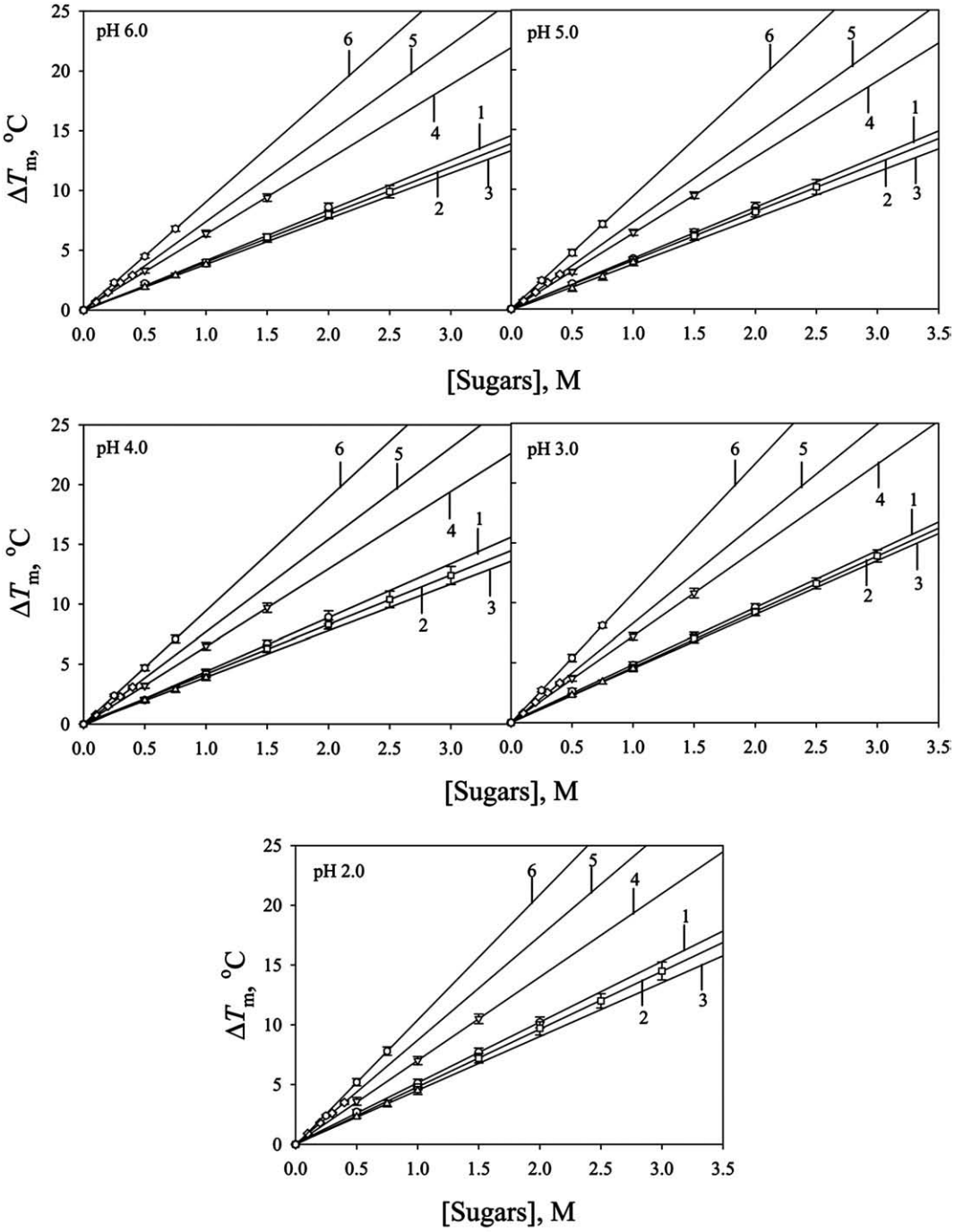

Fig. 3. Values of ΔT_m of RNase-A as a function of molar concentration of sugars at various pH values. Curve numbers 1, 2, 3, 4, 5 and 6 in all the panels represent Glc, Fru, Gal, sucrose, raffinose and stachyose, respectively. The solid lines represent the best fit of (ΔT_m, [sugar]) data according to the linear least-squares method.

denatured molecules, for it is very little on the native molecule [22,36]. If this is the case, the stabilization of the protein by a sugar will be more at lower pH, for lowering the pH will increase the number of denatured molecules. It is noteworthy that the earlier studies have also reported that osmolytes have more stabilizing effect on proteins with the decrease in pH [14–16,38–40] with a notable exception in the case of trimethylamine N-oxide (TMAO) [41].

The ΔH_m values of many proteins remains unchanged in the presence of various osmolytes [12,14–16,35,39,40,42]. We have also observed that the ΔH_m of RNase-A in the presence of different sugars shows insignificant dependence on type and concentration of the sugar. This and earlier observations suggest that sugar osmolytes have no significant affinity on the protein.

We have determined ΔC_p ($=(\delta\Delta H_m/T_m)_p$) from the linear plot of ΔH_m and T_m values by varying pH, for ΔC_p is independent of pH [43]. Values of ΔC_p in the presence of different concentrations of sugars are presented in Table 1. The ΔC_p value of 5.14 kJ mol^{-1} K^{-1} for RNase-A in the absence of sugars (see Table 1) is found to be in good agreement with the calorimetric value of ΔC_p for RNase-A [33]. The molecular interpretation of the origin of ΔC_p of proteins is that it mainly reflects the interactions with solvent water of the polar and apolar groups, which are exposed upon protein denaturation [44]. It is seen in Table 1 that in general there is a very small decrease in ΔC_p of RNase-A in various sugars as well as the mixtures of the monosaccharide constituents of the oligosaccharide with an increase in the concentration of the respective sugar and mixture. This observation is in good agreement with those reported earlier [9,16,39].

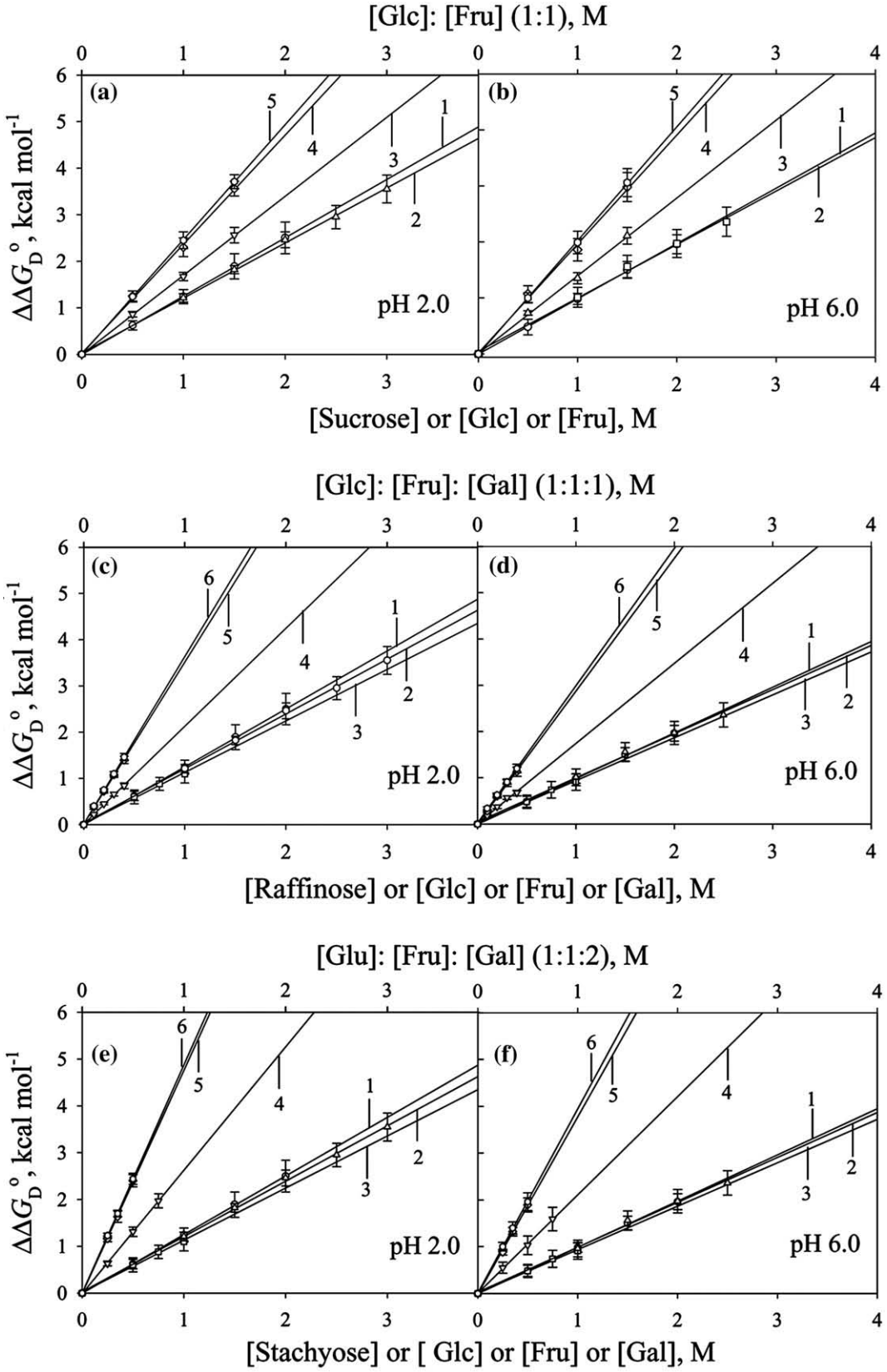

Fig. 4. Representative plots of $\Delta\Delta G^{o}_{D}$ of RNase-A versus [sugars (s)] at two extreme pH values. (a) and (b): Glc (1); Fru (2); sucrose (3); and 1:1 mixture of Glc and Fru, observed (4) and predicted (5). (c) and (d): Glc (1); Fru (2); Gal (3); raffinose (4); and 1:1:1 mixture of Glc, Fru and Gal, observed (5) and predicted (6). (e) and (f): Glc (1); Fru (2); Gal (3); stachyose (4); and 1:1:2 mixture of Glc, Fru and Gal, observed (5) and predicted (6). The solid lines represent the best fit of ($\Delta\Delta G^{o}_{D}$, [sugar]) data according to the linear least-squares method.

The effect of sugars on protein stability have been explained in terms of preferential binding and preferential exclusion of these co-solutes [18,22,23], which is supported by recent observations on the transfer-free energy of protein groups from the solvent water to the co-solvent aqueous solutions [24]. Both Timasheff's and Bolen's group have argued that the source of stabilization of protein by sugars is the

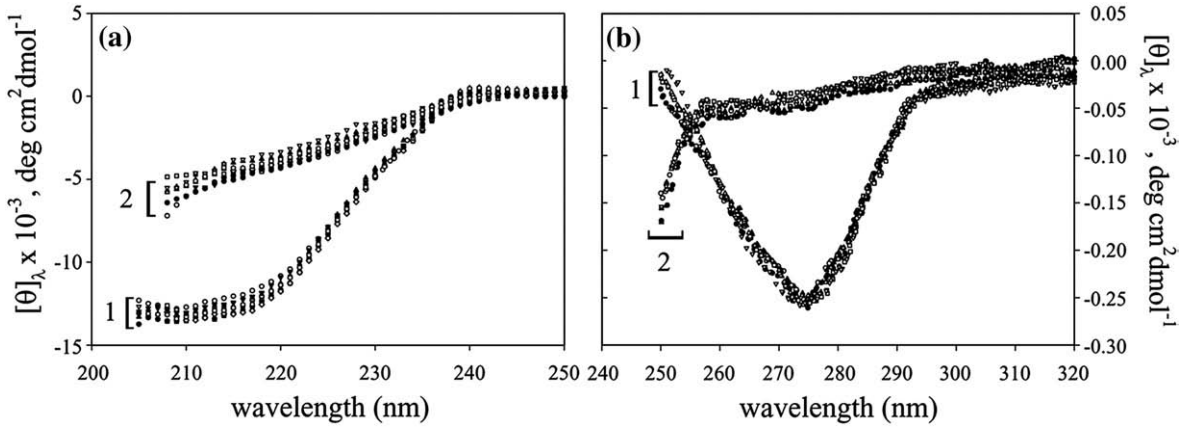

Fig. 5. Effect of sugars on the secondary and tertiary structures of the native and heat-denatured states of RNase-A. (a): The far-UV CD spectra of the protein in the absence (○) and presence of 2.0 M Glc (●), 3.0 M Fru (■), 1.0 M Gal (▲), 1.5 M sucrose (□), 0.4 M raffinose (△), 0.75 M stachyose (▽), 1.5 M Glc+1.5 M Fru (◊), 0.4 M Glc+0.4 M Fru+0.4 M Gal (⬠) and 0.50 M Glc+0.50 M Fru+1.0 M Gal (▼). (b): The near-UV CD spectra of the protein in the absence (○) and presence of 2.0 M Glc (●), 1.0 M Gal (▲), 1.5 M sucrose (□), 0.4 M raffinose (△), 0.75 M stachyose (▽). Spectra grouped as 1 and 2 were measured at pH 6.0 and 25 °C and at pH 2.0 and 85 °C, respectively.

shifting of denaturation equilibrium towards the N state [9,24]. Thus, what effects co-solvents will have on the denaturation equilibrium, N state ↔ D state under the native condition will be known only by measuring ΔG^o_D. Since ΔH_m, T_m and ΔC_p are known in a particular solvent condition (Table 1), we determined ΔG^o_D using Eq. (3).

It is seen from Table 1 that the effect of sugars and the mixtures of monosaccharide constituents of oligosaccharide on ΔG^o_D of protein increases with increasing sugar concentrations at all pH values. However, more effect is observed at lower pH than at higher pH values. This pH dependence of ΔG^o_D of the protein can be explained in the light of the report that different charge states of a protein affect its hydrophobicity [45]. Kunh et al. [45] have argued that the protein hydrophobicity increases with a decrease in pH due to the protonation of COO^- groups, and hence the increased exclusion (repulsion) of sugars from the vicinity of the denatured protein as compared with the native protein, results in larger stabilization effect at low pH. Interestingly, it has been reported that the RNase-A is stabilized by trehalose and the stabilization increases with decreasing pH [39].

Fig. 4 shows the plots of $\Delta\Delta G^o_D$ (=ΔG^o_D in the presence of the sugar (s)−ΔG^o_D in the absence of the sugar) versus [sugar(s)]. It is seen in Fig. 4 a and b that the $\Delta\Delta G^o_D$ increases linearly with the molar concentration of the additive. Curves 1–3 of this figure show the concentration-dependence of $\Delta\Delta G^o_D$ on glucose, fructose and sucrose, respectively. Curve 4 shows the dependence of $\Delta\Delta G^o_D$ on 1:1 molar mixture of glucose: fructose as constituents of sucrose. It should be noted that all data points shown in this figure are observed points, and each straight line represents the least-squares best fit of the observed data points ($\Delta\Delta G^o_D$, [sugar]). Value of $\Delta\Delta G^o_D$ of RNase-A in the presence of sucrose is higher than that obtained in the presence of the individual monomer (glucose or fructose). This means that, on the molar scale, sucrose is better stabilizer than its individual constituents. However, $\Delta\Delta G^o_D$ of the protein in a given concentration of sucrose is less than $\Delta\Delta G^o_D$ of the protein obtained in the presence of 1:1 mixture of glucose and fructose (see Fig. 4a and b, and also see Table 1). Thus, mixture of the monomers provides better stability to the protein in comparison to disaccharide osmolyte. This suggests that mixture behaves differently than two monomers individually as an osmolyte. It is interesting to note that the observed $\Delta\Delta G^o_D$ in the presence of 1:1 molar mixture is, within experimental errors, equal to the algebraic sum of $\Delta\Delta G^o_D$ of the protein obtained in the presence of individual monomer osmolyte (curve 5, Fig. 4a and b).

It is seen in Fig. 4 (c–f) (also see Table 1) that (i) trisaccharide (or tetrasaccharide) has more stabilizing effect than each individual constituent, (ii) the sum of $\Delta\Delta G^o_D$ values in the presence of a mixture of equimolar constituents is larger than that in the presence of the trisaccharide (or tetrasaccharide), and (iii) in a mixture, the stabilizing effect of one monomer on the stability is independent of the effect of the other monomer(s). This observation is found to be similar with the earlier report that two osmolytes have independent effect on the protein stability [25,26]. A plot of $\Delta\Delta G^o_D$ versus [oligosaccharide] shows that, on the molar scale, $\Delta\Delta G^o_D$ increases with the increase in the oligosaccharide size. Although this plot is not shown here, this observation can be seen in Table 1.

Although there is no universal molecular theory that can explain the mechanism by which these stabilizing osmolytes interact with proteins to affect their stability. However, a new mechanism based on the observation of transfer-free energy of the protein backbone from water to aqueous osmolyte solution predicts that the interaction between the protein backbone and osmolytes polar group is more favourable than the corresponding interaction with non-polar groups [46]. Thus stabilizing/destabilizing osmolytes will be preferentially excluded/accumulated around protein backbone. This prediction is consistent with the thermodynamics of preferential interaction of stabilizing and destabilizing osmolytes [9,47,48]. This new molecular mechanism for osmolyte-induced protein stability also predicts that osmolytes having the same fraction of the polar contact surface area will have the same effect on the protein denaturation equilibrium [46]. Since glucose, fructose and galactose have the same fraction of polar contact area, the observed effect of these sugars at a given molar concentration is almost identical. This new molecular mechanism also predicts that an oligosaccharide will be more stabilizing than individual monomers [46]. Results shown in Table 1 are consistent with this prediction.

The earliest thermodynamic mechanism of stabilization [18,22,23] suggests that the degree of exclusion of sugars depends on the nature of molecules that maximally hydrate the protein domain. The preferential hydrations of RNase-A in sucrose and glucose are 350 and 130 [9,10], respectively. This suggests that sucrose is more excluded from the protein domain, leading more stabilization of the protein. Although no preferential hydrations of RNase-A in the presence of raffinose and stachyose are reported, our results suggest that the degree of hydration will increase with an increase in the number of monomer in the oligosaccharide, for ΔG^o_D increases with an increase in the length of the oligosaccharide (see Table 1).

The stabilization of the protein in terms of ΔG^o_D by sugars under physiological conditions, depends on two factors namely, ΔH^o_D (enthalpy change at 25 °C) and ΔS^o_D (the denaturational entropy change at 25 °C). We estimated enthalpy and entropy contributions to ΔG^o_D in a given solvent condition using the values of ΔT_m, ΔH_m and ΔC_p (given in Table 1) in equations, $\Delta H^o_D = \Delta H_m - \Delta C_p\ (T_m - 298.15)$ and $\Delta S^o_D = (\Delta H_m/T_m) + \Delta C_p \ln (298.15/T_m)$. For each sugar, plots of ΔH^o_D

Table 2
Stability parameters of RNase-A in the presence of 1M sugar at various pH values and 25 C[a]

[Sugar], 1M	pH 6.0			pH 5.0			pH 4.0			pH 3.0			pH 2.0		
	ΔH_D^o	$T\Delta S_D^o$	ΔG_D^o	ΔH_D^o	$T\Delta S_D^o$	ΔG_D^o	ΔH_D^o	$T\Delta S_D^o$	ΔG_D^o	ΔH_D^o	$T\Delta S_D^o$	ΔG_D^o	ΔH_D^o	$T\Delta S_D^o$	ΔG_D^o
Control	276	235	41.0	276	237	39.0	278	245	32.8	280	258	21.2	275	264	10.6
Glc	275	229	45.2	273	230	43.2	277	240	37.6	278	252	26.3	273	258	15.8
Gal	279	234	44.8	278	235	42.8	281	244	37.0	283	257	26.0	277	261	15.2
Fru	279	233	45.3	278	235	43.1	281	243	37.4	280	254	26.1	278	262	15.6
Sucrose	274	227	46.7	273	228	44.5	274	235	38.8	273	245	28.1	274	256	17.6
Raffinose[b]	279	231	48.3	278	232	46.1	280	239	40.8	279	249	29.5	279	259	19.5
Stachyose[b]	285	235	49.8	281	233	47.9	282	240	42.0	274	243	31.5	286	265	21.6

[a] ΔH_D^o, $T\Delta S_D^o$, ΔG_D^o are in kJ mol^{-1}.
[b] Represents the extrapolated values.

versus [sugar] and ΔS_D^o versus [sugar] are linear (plots not shown). Such plots are used to estimate ΔH_D^o and ΔS_D^o in the presence of 1 M sugar. These values are given in Table 2. It has been observed that at each pH both ΔH_D^o and $\Delta S_D{}^o$ are positive and $\Delta H_D{}^o > T\Delta S_D{}^o$ in the presence of all sugars (see Table 2). Thus the stabilization of RNase-A by sugars is under enthalpic control. It is noteworthy that Lee and Timasheff [9] have reported that the stabilization of α-chymotrypsin and chymotrypsinogen by sucrose is under enthalpic control.

The mixture of different kinds of osmolytes may be helpful in different types of applications in biological systems. An equimolar mixture of arginine and glutamic acid have been found to have better solubilization than the sum effect of each component individually [49]. Furthermore, mixtures of TMAO and trehalose can be more efficient in refolding proteins than expected from simple additive effect [50]. However, mixtures containing arginine or TMAO have limited uses, for it has shown that arginine and TMAO behave as denaturants at high concentrations [51] and low pH [41], respectively. On the other hand, this study shows that an equimolar mixture of monosaccharide constituents is not only a better protein stabilizer (folder) than the oligosaccharide but their stabilizing effect is predictable.

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