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Thermodynamic analysis of protein unfolding in aqueous solutions as a multisite reaction of protein with water and solute molecules

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ABSTRACT

Thermal unfolding of ribonuclease A, lysozyme, and chymotrypsinogen A was analyzed as a multisite reaction of a protein molecule with water and solute molecules. The protein unfolding process in various solutions of sugars and denaturants was described well by the van't Hoff equation. The reciprocal form of the Wyman-Tanford equation, which describes the unfolded-to-folded protein ratio as a function of water activity, was successfully applied to obtain a good linear relationship. From this analysis, the role of water activity on protein stability was clearly explained and the contributions of hydration and solute binding to protein molecule were separately discussed in protein unfolding. General solution for the free energy of protein stability was obtained as a simple function of solute concentration.

1. Introduction

Protein thermal stability is ultimately important in its practical applications, especially in its use as a biocatalyst [1,2]. The thermal unfolding process of protein in an aqueous solution can be described as a multisite reaction of a protein molecule with water and solute molecules as shown in Fig. 1. Some sugars are reported to be preferentially excluded from the protein surface [3] while protein denaturants as urea and guanidine HCl are known to bind protein preferentially. Various methods have been applied to analyze the interactions of protein with solute molecules. Partial specific volume analysis was applied to evaluate the preferential binding of urea [4] and guanidine hydrochloride [5] on various proteins, and alkylureas on α-chymotrypsinogen A [6]. Vapor pressure osmometry was used to analyze the interactions of urea and guanidine HCl with bovine serum albumin [7]. Makhatadze and Privalov [8] applied calorimetric method to analyze the binding of urea and guanidine HCl to ribonuclease A (RNase). Poklar et al. [9] combined calorimetric and spectroscopic studies to analyze the binding of alkylurea to RNase.

For the theoretical analysis of solute–protein interaction, molecular dynamics (MD) have been applied. By using this method, Kuharski and Rossky [10,11] analyzed the experimentally known improved solvation of apolar molecules in an aqueous urea solution and suggested that urea displaces water molecules from the apolar solvation shell of the hydrophobic species. Carfish and Karplus [12] analyzed urea binding to RNase and found much higher density of urea molecules than the bulk when denaturation of the protein occurred. Bennion and Daggett [13] investigated the molecular basis for the chemical denaturation of proteins by urea and suggested that urea denatures proteins by direct binding and by indirect effect through water structure weakening. Mason et al. [14] analyzed interaction of guanidinium ions with a model peptide and found out that guanidinium ions bind to Arg, Trp, and Glu side-chains.

Tanford [15] extended the concept of the linked functions proposed by Wyman [16] for the analysis of protein denaturation in aqueous solutions. In this method, protein denaturation process was described as a process of hydration and solute-binding of the protein molecule. Tanford-Wyman approach has been frequently applied in the literature to analyze the effect of sugars [3,17–19] and denaturants [20] on the thermal stability of proteins. In these studies, however, the denaturation process was related and analyzed based on solute activity.

On the contrary, Jenkins [21] applied a reciprocal Tanford-Wyman equation to analyze protein solubility in various solutions. In this case, protein solubility was related to water activity as an alternative and better method to evaluate the hydration and solute-binding to protein. In the previous paper [22], this approach was applied to obtain the hydration number change upon protein unfolding.

In this paper, the reciprocal form of the Wyman-Tanford equation was also applied to analyze the effects of solutes on the unfolding of

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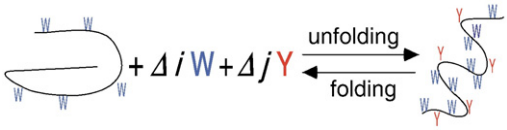

Fig. 1. Scheme of protein unfolding in aqueous solution.

ribonuclease A, lysozyme, and α-chymotripsinogen A. Thermal unfolding behaviors of these proteins were analyzed in various solutions at various concentrations.

2. Theoretical

Protein unfolding in an aqueous solution is accompanied with changes in the hydration state and solute-binding of protein as shown in Fig. 1. Tanford [15] theoretically analyzed the effect of hydration and the binding of solute on protein unfolding in a solution as a multisite reaction.

$$N + iW + jY \rightarrow NW_iY_j \tag{1}$$

$$D + i'W + j'Y \rightarrow DW_{i'}Y_{j'} \tag{2}$$

where N and D represent native and unfolded protein, X and Y represent water and solute as a ligand, i and i' are the numbers of water molecules, and j and j' are the solute molecules involved, respectively, in the process. The equilibrium constants for Eqs. (1) and (2) are:

$$K_{ij,N} = \left[NW_iY_j\right] / \left\{[N]a_W^i\, a_Y^j\right\} \tag{3}$$

$$K_{i'j',D} = \left[DW_{i'}Y_{j'}\right] / \left\{[D]a_W^{i'}a_Y^{j'}\right\} \tag{4}$$

where a_W and a_Y are activities of water and solute, respectively. Then all the forms of native (N_T) and unfolded protein (D_T) are:

$$N_T = N_f \sum\sum K_{ij,N} a_W^i\, a_Y^j \tag{5}$$

$$D_T = D_f \sum\sum K_{i'j',D} a_W^{i'}\, a_Y^{j'} \tag{6}$$

where N_f and D_f, respectively, are native and unfolded protein molecules without any bound water or solute. By applying the Wyman's linkage analysis [13], the following equation for K_D ($=D_T/N_T$) is obtained [15].

$$dlnK_D / dlna_Y = \Delta j - (X_Y / X_W)\Delta i \tag{7}$$

where X_W and X_Y are molar fractions of water and solute, respectively, Δi ($=i'-i$) is the change in hydration number per protein molecule, Δj ($=j'-j$) is the change in bound-solute molecules per protein molecule upon unfolding. The reciprocal form of the above Wyman-Tanford equation is expressed as follows [21].

$$dlnK_D / dlna_W = \Delta i - (X_w / X_Y)\Delta j \tag{8}$$

The whole unfolding process of the protein is described by the following equation.

$$N + \Delta iW + \Delta jY \rightarrow D \tag{9}$$

The equilibrium constant, K^*, of this unfolding process is described by

$$K^* = [D] / ([N]\, a_W^{\Delta i} a_Y^{\Delta j}) \tag{10}$$

$K_{D,0}$, K^* at $a_W = 1$, is equal to the ratio of the unfolded-to-folded protein molecules and is related to the unfolding ratio in the total protein molecules, x_{unf}, as follows:

$$K_{D,0} = [D] / [N] = K_D = x_{unf} / (1 - x_{unf}) \tag{11}$$

$$x_{unf} = ([D]) / ([D] + [N]) \tag{12}$$

The effect of temperature T on $K_{D,0}$ is described by the van't Hoff equation [23].

$$dlnK_{D,0} / dT = \Delta H_0 / RT^2 \tag{13}$$

where ΔH_0 is the enthalpy of protein unfolding at $a_W = 1$ and R is gas constant. This equation is based on the two-state equilibrium model, which has been proved to be applicable to the thermal unfolding of RNase and lysozyme [24].

By integration of Eq. (13), the following equation is obtained.

$$lnK_{D,0} = -(\Delta H_0 / R)(1/T - 1/T_m) \tag{14}$$

where, T_m is the melting temperature.

When $a_W \neq 1$, the integration of van't Hoff equation will be as follows:

$$lnK^* = ln([D] / [N]) - \Delta ilna_W - \Delta jlna_Y = lnK_{D,S} - \Delta ilna_W - \Delta jlna_Y \tag{15}$$

$$lnK_{D,S} = lnK^* + \Delta ilna_W + \Delta jlna_Y = -(\Delta H^* / R)(1/T - 1/T_m^*) \tag{16}$$

where, T_m^* is the melting temperature and ΔH^* is the enthalpy of protein unfolding when $a_W \neq 1$.

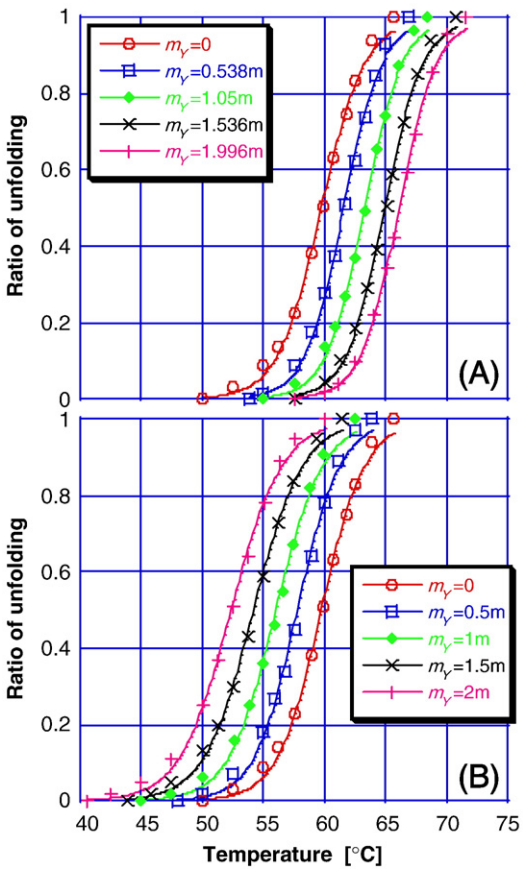

Fig. 2. Thermal unfolding of RNase in solutions of glucose (A) and urea (B) with various concentration (m_Y; mol/kg-solvent) at pH = 5.5.

Table 1
Parameters in van't Hoff equation (Eq. (19)) for the unfolding of proteins in solutions.

Protein	pH	Solute	m_Y (mol/kg-solvent)	ΔH^* (kJ/mol)	T_m^* (K)	r^*
RNase	5.5	Trehalose	0	498.0	332.8	0.9983
			0.5426	504.9	335.4	0.9987
			1.0256	509.3	337.5	0.9986
			1.4583	499.6	339.6	0.9975
			1.8481	557.8	341.1	0.9991
		Sucrose	0	516.0	332.9	0.9985
			0.5632	562.1	335.3	0.9991
			1.0801	574.0	337.2	0.9990
			1.5563	610.9	338.7	0.9993
			1.9963	693.2	340.9	0.9993
		Glucose	0	516.0	332.9	0.9985
			0.5389	591.7	334.7	0.9983
			1.0500	601.5	336.4	0.9981
			1.5356	607.3	338.1	0.9992
			1.9956	616.4	339.3	0.9988
		Ribose	0	516.0	332.9	0.9984
			0.5205	716.5	333.3	0.9992
			1.0176	810.8	333.5	0.9983
			1.4927	918.6	333.9	0.9980
			1.9473	1036.1	334.3	0.9990
		Urea	0	620.6	332.9	0.9985
			0.5	626.6	330.9	0.9986
			1.0	565.4	329.1	0.9985
			1.5	531.6	327.3	0.9991
			2.0	508.8	325.5	0.9989
		Guanidine HCl	0	620.6	332.9	0.9985
			0.5	569.7	328.6	0.9992
			1.0	522.1	324.2	0.9990
			1.5	473.7	319.9	0.9992
			2.0	456.2	315.7	0.9983
RNAse	4.2	Trehalose	0	450.7	330.6	0.9989
			0.5426	460.8	333.4	0.9987
			1.0526	464.8	335.9	0.9992
			1.4583	510.5	338.2	0.9998
			1.8481	497.3	341.1	0.9998
		Sucrose	0	460.3	331.1	0.9997
			0.5632	490.7	333.5	0.9988
			1.0801	511.7	335.8	0.9993
			1.5563	521.1	337.7	0.9994
			1.9963	547.7	339.6	0.9993
		Glucose	0	460.3	331.1	0.9997
			0.5389	518.4	332.9	0.9995
			1.0500	506.6	334.6	0.9993
			1.5356	540.9	336.1	0.9993
			1.9956	525.0	337.3	0.9991
		Ribose	0	460.3	331.1	0.9997
			0.5205	502.1	331.9	0.9994
			1.0176	553.5	332.1	0.9992
			1.4927	604.6	332.7	0.9994
			1.9473	598.1	`333.2	0.9990
		Urea	0	553.6	331.1	0.9997
			0.5	554.7	328.0	0.9984
			1.0	553.6	326.4	0.9987
			1.5	461.4	324.1	0.9987
			2.0	454.3	322.1	0.9992
		Guanidine HCl	0	553.6	331.1	0.9997
			0.5	531.2	325.2	0.9996
			1.0	516.2	321.3	0.9995
			1.5	447.5	317.2	0.9992
			2.0	414.5	313.3	0.9991
RNAse	2.8	Trehalose	0	415.3	322.2	0.9989
			0.5426	427.0	325.5	0.9996
			1.0256	448.7	329.3	0.9993
			1.4583	453.0	331.9	0.9991
			1.8481	465.0	334.0	0.9988
		Sucrose	0	403.6	322.1	0.9994
			0.5632	472.1	325.2	0.9993
			1.0801	484.6	329.1	0.9991
			1.5563	535.3	331.0	0.9988
			1.9963	541.3	333.2	0.9992
		Glucose	0	403.6	322.1	0.9994
			0.5389	431.1	324.5	0.9996
			1.0500	453.1	326.5	0.9996
			1.5356	447.7	328.5	0.9993
			1.9956	470.3	330.3	0.9997
		Ribose	0	403.6	322.1	0.9994
			0.5205	438.0	322.4	0.9992
			1.0176	465.9	323.3	0.9993
			1.4927	489.4	323.6	0.9992
			1.9473	533.7	324.2	0.9992
		Urea	0	485.5	322.1	0.9994
			0.5	474.4	316.9	0.9996
			1.0	460.8	316.3	0.9986
			1.5	438.4	309.1	0.9997
			2.0	471.1	305.5	0.9997
		Guanidine HCl	0	485.5	322.1	0.9994
			0.5	452.6	314.6	0.9994
			1.0	407.0	310.3	0.9987
			1.5	435.5	306.3	0.9996
			2.0	492.8	302.6	0.9994
Lyz	5.5	Trehalose	0	766.8	347.4	0.9990
			0.5426	752.5	350.8	0.9980
			1.0256	834.1	353.5	0.9986
			1.4583	937.9	355.6	0.9959
		Sucrose	0	712.8	347.4	0.9978
			0.5632	822.7	349.8	0.9987
			1.0801	902.7	352.1	0.9984
			1.5563	832.3	354.5	0.9967
			1.9963	958.4	357.2	0.9865
		Glucose	0	712.8	347.4	0.9978
			0.5389	933.4	348.8	0.9931
			1.0500	828.2	350.9	0.9971
			1.5356	798.4	353.4	0.9964
			1.9956	942.1	353.4	0.9972
		Urea	0	857.4	347.4	0.9978
			0.5	792.3	346.1	0.9979
			1.0	604.2	345.3	0.9979
			1.5	672.3	343.7	0.9986
			2.0	601.0	342.6	0.9994
		Guanidine HCl	0	857.4	347.4	0.9978
			0.5	845.4	341.2	0.9976
			1.0	778.7	338.0	0.9972
			1.5	558.3	336.1	0.9966
			2.0	381.6	334.6	0.9976
CTA	2.0	Trehalose	0	380.4	315.1	0.9982
			0.5426	406.6	318.2	0.9990
			1.0256	418.8	320.6	0.9986
			1.4583	431.5	320.4	0.9983
			1.8481	439.5	323.9	0.9987
		Sucrose	0	411.7	315.3	0.9984
			0.5632	431.4	317.2	0.9985
			1.0801	452.0	319.2	0.9973
			1.5563	508.5	321.4	0.9974
			1.9963	533.8	323.0	0.9971
		Glucose	0	411.7	315.3	0.9984
			0.5389	396.9	316.8	0.9989
			1.0500	417.3	318.4	0.9979
			1.5356	434.5	320.5	0.9985
			1.9956	448.4	322.4	0.9988
		Ribose	0	411.7	315.3	0.9984
			0.5205	422.7	315.1	0.9988
			1.0176	426.0	315.4	0.9987
			1.4927	436.4	316.0	0.9987
			1.9473	436.1	316.5	0.9987
		Urea	0	495.2	315.3	0.9984
			0.5	460.4	312.7	0.9972
			1.0	444.5	311.8	0.9967
			1.5	446.3	310.4	0.9967
			2.0	439.4	308.8	0.9960

* Correlation coefficient.

From this equation, x_{unf}, which corresponds to the thermal denaturation curve, is obtained as follows:

$$K_{D,S} = [D]/[N] = exp[-(\Delta H^*/R)(1/T - 1/T_m^*)] \quad (17)$$

$$x_{unf} = 1/[1 + exp\{-(\Delta H^*/R)(1/T - 1/T_m^*)\}] \quad (18)$$

3. Materials and method

3.1. Materials

Ribonuclease A (RNase, bovine pancrease, chromatographically purified), lysozyme (Lyz, chicken egg white, 3×crystallized), α-chymotripsinogen A (CTA, bovine pancrease, 6×crystallized), and ribose were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Trehalose was obtained from Acros Organics (Geel, Belgium). Sucrose, urea, and guanidine HCl were from Nacalai Tesque (Tokyo, Japan). Glucose was from Kanto Kagaku (Tokyo, Japan). These solutes were added to adjust the water activity in protein solutions, pH of which were adjusted at 5.5 (0.04 M acetate buffer), 4.2 (0.04 M acetate buffer), or 2.8 (0.04 M glycine–HCl buffer) for RNase, at 5.5 (0.04 M acetate buffer) for Lyz, and at 2.0 (0.01 M HCl) for CTA.

3.2. Measurement of protein unfolding

To measure the thermal unfolding process, the temperature of the enzyme solution was raised at 1 °C/min (ETC-505S, Jasco, Tokyo, Japan) and the optical absorbance was monitored at λ = 287 nm for RNase, at λ = 292 nm for Lyz, and at λ = 293 nm for CTA by a spectrophotometer (V-560, Jasco, Tokyo, Japan). Concentration of protein was fixed at 1 mg/ml.

3.3. Measurement of water activity

Water activity of a solution, a_W, with a solute Y is described as a function of the molar fraction of Y, X_Y, by the following equation [25,26].

$$a_W = (1 - X_Y)exp(\alpha X_Y + \beta X_Y^2 + \gamma X_Y^3) \quad (19)$$

The experimental parameters, α, β and γ, have been determined from the freezing point depression and were reported in the literature [26] for sucrose, ribose, and urea. No data were available for trehalose and guanidine HCl solutions. In this case, water activity was also measured by the freezing point depression [26].

4. Results

Fig. 2 shows the typical thermal unfolding curve of RNase at pH = 5.5 in glucose and urea solutions with concentrations varied. It is clearly seen that RNase is stabilized by glucose and destabilized by urea and the extent of stabilization or destabilization increased with an increase in solute concentration. Solid lines in Fig. 2 are the best-fitted curve of unfolding described by Eq. (18), which agreed well with the experimental results. The best-fit parameters in Eq. (18) were determined for RNase at pH 5.5, 4.2, and 2.8, Lyz at pH 5.5, and α-chmotripsinogen A (CTA) at pH 2.0, in aqueous solutions of trehalose, sucrose, glucose, ribose, urea, and guanidine HCl at various concentrations.

Table 2
Parameters in Eq. (19) to describe water activity of solutions.

Solute	α	β	γ	r^*
Trehalose	–	−6.646	164.72	0.9981
Sucrose	–	−7.405	–	0.9999
Glucose	–	−2.734	–	0.9999
Ribose	–	−1.699	–	0.9993
Urea	–	0.8309	–	0.9999
Guanidine HCl	−0.6462	6.560	−20.97	0.9997

*Correlation coefficient.

Table 1 summarizes the best-fit parameters in Eq. (18) for all the proteins tested in various solutions with concentrations varied. In general, ΔH^* and T_m^* increased with an increase in sugar concentration and decreased with an increase in denaturant concentration although the extent of increase or decrease was different among solutes that coexisted. As for the effect of pH, both of melting temperature, T_m^*, and enthalpy for unfolding, ΔH^* reduced with the reduction of pH (Table 1) showing that the acidic pH facilitates protein unfolding.

Fig. 3 shows the effects of the type of solute and their concentration on melting temperature, T_m^*, for RNase at pH = 5.5. T_m^* changed linearly with solute concentration. The similar linear effect of solute concentration on T_m^* was observed for all the proteins tested here.

To analyze the difference among the effect of solutes on the stability of proteins, the reciprocal form of Wyman-Tanford equation [21] described by Eq. (8) was applied. For this purpose, water activity, a_W, should be known as a function of the concentration of sugars. Table 2 summarizes the parameters α, β, and γ in Eq. (19).

Fig. 4 shows the typical reciprocal Wyman-Tanford plot for unfolding of RNase (pH = 5.5), in which the logarithm of K_D of the protein is plotted against the logarithm of a_W of solutions at a fixed temperature of 60 °C. As was expected by Eq. (8), excellent straight lines were obtained in this plot, the slope of which corresponds to the right hand side of Eq. (8). The slope was dependent on the solute coexisting.

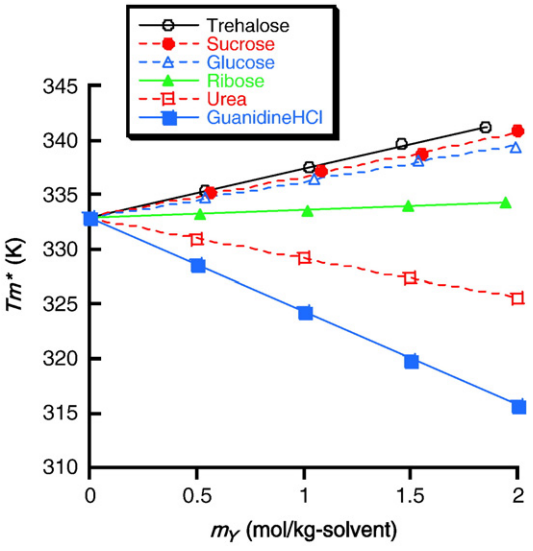

Fig. 3. Effect of denaturant concentration (m_Y) on melting temperature (T_m^*) of RNase at pH = 5.5.

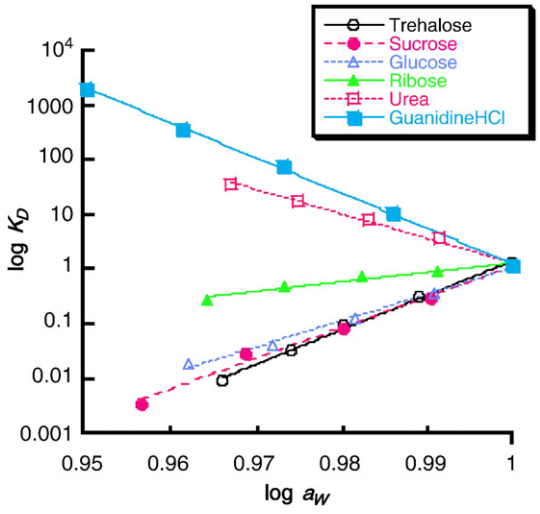

Fig. 4. Effect of water activity on unfolding ratio of RNase in various solutions of pH = 5.5 at 60 °C.

Table 3
Slope of reciprocal Wyman-Tanford plot (Eq. (8)).

Protein	Ph	Guanidine HCl	Urea	Ribose	Glucose	Sucrosese	Treharose
RNase	5.5	−145.2	−101.0	38.0	108.6	125.8	142.0
	4.2	−141.4	−116.2	25.9	88.5	105.9	142.3
	2.8	−178.4	−229.6	30.7	100.8	132.8	145.6
Lyz	5.5	−191.4	−95.9	ND*	147.7	196.2	232.5
CTA	2.0	ND*	−83.6	16.2	94.2	101.3	98.8

*Not measurable because of protein precipitation.

Table 3 summarizes the slope in the reciprocal Wyman-Tanford plot for all the proteins tested here. The positive slope corresponds to the stabilization effect of the solute on the protein while the negative slope corresponds to the destabilization effect. Trehalose showed the highest positive slope while guanidine HCl showed the highest negative slope in the table.

5. Discussions

5.1. Hydration and solute-binding upon protein unfolding

The reciprocal plot of Wyman-Tanford equation (Eq. (8)) gave a well-formed straight line as shown in Fig. 4. The slope of this plot is composed of the hydration term (Δi) and the solute-binding term [$(X_w/X_Y)\Delta j$]. In the literature, the Wyman-Tanford equation have been applied based on the activity of solute as expressed by Eq. (7) to analyze the effect of sugars [3,17–19] and denaturants [20] on the thermal stability of proteins. In the form of Eq. (7), however, both the number of bound-solute molecules (Δj) and the molar fraction ratio of solute to water (X_w/X_Y) are strongly concentration-dependent so that no straight lines have been obtained in the literature [17–19]. Therefore, only the qualitative discussions have been made on the interaction of proteins with solutes and water. Moreover, the hydration term, $((X_Y/X_W)\Delta i)$ was neglected in most cases.

On the contrary in Eq. (8), the hydration number (Δi) and the ratio of bound-solute to its molar fraction ($\Delta j/X_Y$) would not be strongly concentration-dependent. This explains why a good linear relationship was obtained in Fig. 4 based on Eq. (8). As the linearity was proved experimentally, Eq. (8) can be now easily integrated as follows:

$$\Delta \ln K_D = \{\Delta i - (X_w/X_Y)\Delta j\}\Delta \ln a_W \tag{20}$$

When $a_W = 1$, $K_D = K_{D,0}$ so that,

$$\ln K_{D,S} - \ln K_{D,0} = \{\Delta i - (X_w/X_Y)\Delta j\}\ln a_W \tag{21}$$

In the previous paper, Eq. (8) was applied to analyze the hydration number change upon protein unfolding in sugar solutions [22]. The plot of $\ln K_D$ to $\ln a_W$ also gave a good linear relationship and the slope of the plot was positive because sugars stabilize proteins. Among sugars, however, the slopes of the plot were much different between the reducing sugars and the nonreducing sugars. The reducing sugars seemed to interact with proteins through the formation of Schiff-base between the reducing aldehyde terminal of the sugar and the amino group of the protein at high temperature. On the contrary, no such interaction was expected for nonreducing sugars so that the second term for solute-binding in Eq. (8), $(X_w/X_Y)\Delta j$ was negligible for nonreducing sugars. Therefore, the hydration number change upon protein unfolding, Δi, was determined from the average of the slope of the plot based on Eq. (8) for nonreducing sucrose and trehalose in the preceding paper [22].

If the independent binding sites are assumed for hydration and solute-binding, the hydration term and the binding term in Eq. (8) will be separately determined. Although the hydration number change upon protein unfolding has been determined from the average of those for sucrose and trehalose in the preceding paper as described above, trehalose has been reported to bind preferentially to a folded protein than an unfolded one [18,19]. Therefore, the hydration number change, Δi, was determined in the present case from that of sucrose, which was proved to be preferentially excluded from the protein surface and not to bind in the aqueous solution [3]. Therefore, Δj was assumed to be zero for sucrose. The Δi's, thus determined, were 125.8, 105.9, and 132.8 mol/mol-protein for RNase at pH 5.5, 4.2, and 2.8, respectively, 196.2 for Lyz at pH 5.5, and 101.3 for CTA at pH 2.0 (Table 3).

When the change in the hydration number upon protein unfolding, Δi, in Eq. (8) is determined, the binding term $(X_w/X_Y)\Delta j$ is easily determined from the slope in Fig. 4 listed in Table 3 and Δi. From $(X_w/X_Y)\Delta j$, the solute-binding number, Δj, can be easily calculated when the concentration of the solute is fixed. Table 4 summarizes the determined Δj at the solute concentration of 1 mol/kg-solvent. Among the solutes, Δj is highest for guanidine HCl except for RNase at pH2.8. These numbers of Δj are not much different from those in the literature. By using isothermal titration method, Makhatadze and Privalov [8] reported Δj for RNase at the solute concentration of 1 mol/kg-solvent to be 3.9 and 3.0, respectively, for guanidine HCl and urea, and those for Lyz to be 5.1 and 2.9, respectively, for guanidine HCl and urea. This coincidence in Δj of guanidine HCl and urea for RNase and Lyz suggests the effectiveness of the present model in spite of its simplification assumption of independent binding sites for hydration and solute-binding and no interaction of sucrose with protein. Reducing sugars, glucose and ribose, showed small Δj's suggesting that these sugars weakly bind to proteins upon their unfolding.

5.2. Thermodynamic analysis for protein conformational stability

To analyze the conformational stability of proteins in the solution, the free energy difference, $\Delta\Delta G$, is calculated [27,28] by subtracting the free energy for protein unfolding in a solution ($\Delta G_{D,S}$) and in water ($\Delta G_{D,0}$). To this end, Tanford [29] proposed a transfer model as follows:

$$\Delta\Delta G = \Delta G_{D,S} - \Delta G_{D,0} = \sum_k \alpha_k n_k \delta g_{tr,k} \tag{22}$$

where $\delta g_{tr,k}$ is the free energy for transfer of a group of type k from water to a solution, n_k is the total number of groups of type k exposed upon unfolding, and α_k is the average change in the degree of exposure of type k upon unfolding. This transfer model needs a large database of transfer free energy, $\delta g_{tr,k}$, for each amino acid side-chain at each solute concentration.

Greene and Pace [30,31] proposed a linear extrapolation model for $\Delta\Delta G$ as an empirical model:

$$\Delta\Delta G = m[\text{solute}] \tag{23}$$

where m is an empirical parameter called as 'm-value', which is negative for a protein denaturant and positive for a stabilizer. Because of the simplicity and easiness to use, this linear model has been applied to analyze the effect of electrolytes on RNase denaturation [32], unfolding of peptide helices by urea [33] and guanidine HCl [34], urea-induced unfolding of nuclease and its variants [35], RNA folding

Table 4
Number of change in bound-solute molecules upon protein unfolding, Δj, at solute concentration of 1 mol/kg-solvent.

Protein	pH	Guanidine HCl	Urea	Ribose	Glucose	Sucrose	Trehalose
RNase	5.5	5.07	4.35	1.58	0.31	0	−0.29
	4.2	4.45	4.00	1.44	0.31	0	−0.66
	2.8	5.61	6.53	1.84	0.58	0	−0.23
Lyz	5.5	6.98	5.26	ND	0.87	0	−0.65
CTA	2.0	ND	3.33	1.53	0.13	0	0.045

[36], and urea- and guanidine-induced unfolding of RNase and Lyz [37]. Auton and Bolen [38] combined the above two models to obtain *m*-value from the transfer free energy data of amino acid side-chain for unfolding of RNase T1. Schellman [39] analyzed *m*-value as a balance between the excluded volume effect and the association effect of a solute: the former stabilizes and the latter destabilizes proteins.

While neither transfer model nor linear model gives the direct effect of solute concentration on $\Delta\Delta G$, this is easily calculated in the present case by using Eq. (21) as follows:

$$\Delta\Delta G = \Delta G_{D,S} - \Delta G_{D,0} = -RT\left(lnK_{D,S} - lnK_{D,0}\right) = -RT\{\Delta i - (X_w / X_Y)\Delta j\} lna_W \quad (24)$$

This result clearly shows the important role of water activity for protein stability and also shows that the hydration term, Δi, always functions to stabilize proteins because $\ln a_W$ is zero or negative ($a_W \leqq 1$). On the contrary, the solute-binding term, $[(X_w/X_Y)\Delta j]$, always destabilizes proteins unless Δj is negative as in the case of trehalose, which reportedly binds preferentially to a folded protein than an unfolded one [18,19]. The whole stability is determined by a balance between Δi-term and Δj-term. Eqs. (24) and (19) can be applied to obtain the following result.

$$\Delta\Delta G = -RT\{\Delta i - (X_w / X_Y)\Delta j\} ln\{(1 - X_Y) exp(\alpha X_Y + \beta X_Y^2 + \gamma X_Y^3)\} = -RT\{\Delta i - (X_w / X_Y)\Delta j\}\left[ln(1 - X_Y) + \alpha X_Y + \beta X_Y^2 + \gamma X_Y^3\right] \quad (25)$$

This equation directly gives the effect of solute concentration on thermal stability of proteins. Eq. (25) gives a theoretical basis for the linear model when $X_Y \ll 1$ because the higher terms of X_Y are negligible and $\ln(1-X_Y) \fallingdotseq -X_Y$ at a small X_Y. From this equation, $\Delta\Delta G$ was calculated and plotted to solute concentration as shown in Fig. 5. For most solutes, $\Delta\Delta G$ is linearly dependent on concentration showing that the linear model is applicable in this case. For guanidine HCl, however, this is not the case. The strong nonlinearity in $\Delta\Delta G$ for guanidine HCl has also been reported in the literature [40]. As the constancy of $\{\Delta i - (X_w/X_Y)\Delta j\}$ is guaranteed in Fig. 4, it is interesting to point out that this nonlinearity for guanidine HCl solely arises from the nonlinearity in $\ln a_W$ term on solute concentration.

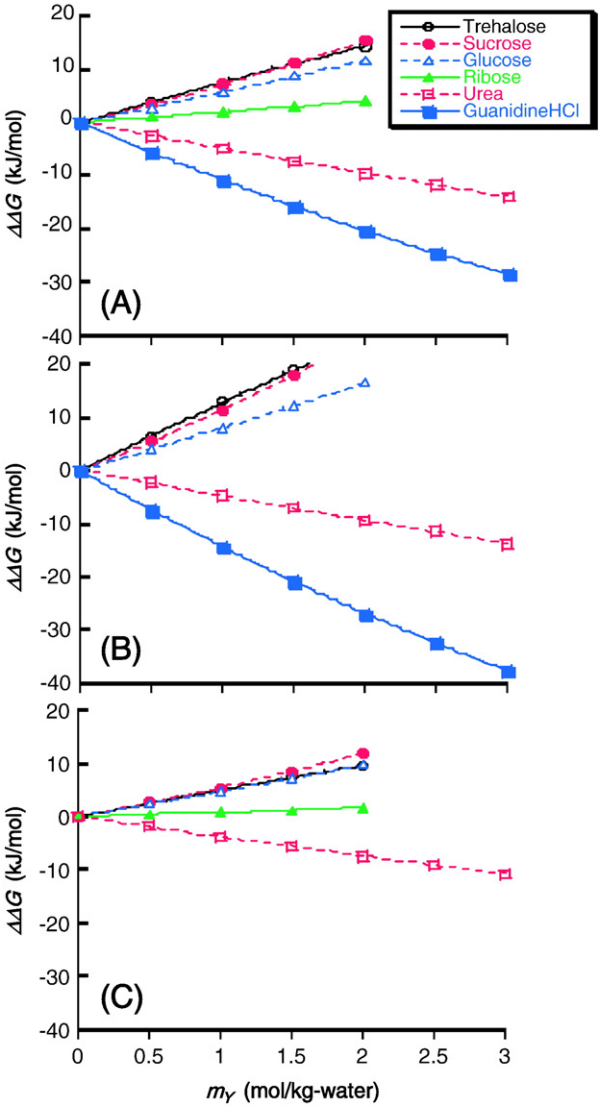

Fig. 5. Calculation of $\Delta\Delta G$ as a function of solute concentration (m_Y; mol/kg-solvent) by Eq. (25) for thermal unfolding of RNase (A) and Lyz (B) at pH 5.5, and CTA at pH 2.0 (C) in various solutions.

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