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Thermodynamic analysis of osmolyte effect on thermal stability of ribonuclease A in terms of water activity

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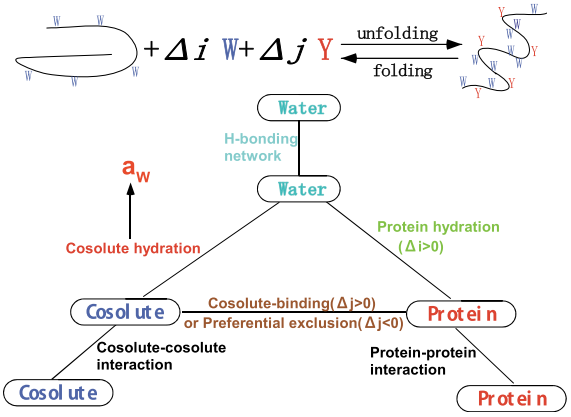
HIGHLIGHTS

- The thermal unfolding of ribonuclease A (RNase) was analyzed in various osmolyte solutions.
- The Wyman-Tanford equation, correlating the unfolded-to-folded protein ratio with water activity, gave a linear plot.
- From this experimental linearity, the Wyman-Tanford plot was integrated to obtain protein stabilization free energy (ΔΔG).
- The ΔΔG was proved to be described by protein hydration, cosolute-binding, preferential exclusion, and water activity.
- The present approach established the role of water activity on thermal stability of proteins quantitatively.

GRAPHICAL ABSTRACT



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ABSTRACT

Thermal unfolding of ribonuclease A (RNase) was analyzed in various osmolyte solutions of glycine, proline, sarcosine, *N,N*-dimethylglycine, betaine, *myo*-inositol, taurine, and trimethylamine-*N*-oxide (TMAO). All the osmolytes tested stabilized the protein. The thermal unfolding curve was described well by the van't Hoff equation and the melting temperature and the enthalpy of protein unfolding were obtained. The Wyman–Tanford equation, which describes the unfolded-to-folded protein ratio as a function of water activity, was successfully applied to obtain a linear plot. In consideration of this experimentally obtained linearity, the Wyman–Tanford plot could be integrated to calculate the stabilization free energy of the protein (ΔΔ*G*) in the solution. The ΔΔ*G* was proved to be described by the property of the microstructure around the protein surface, which is composed of the protein hydration, the cosolute-binding, and the preferential exclusion, and the property of the bulk solution; water activity. The *m*-values of osmolytes for protein unfolding were obtained from ΔΔ*G* calculated. Among the osmolytes tested, *myo*-inositol showed the highest *m*-value.

1. Introduction

Osmolytes are organic small cytoprotectant solutes accumulated in cells under water stressed conditions [1,2]. As the protective effect of osmolytes involves stabilization of biomacromolecules, effects of osmolytes on protein stability have been extensively investigated [3–9]. As for the effect of cosolute on protein stability in aqueous solutions, Tanford [10] proposed a stoichiometric approach based on the Wyman's linkage analysis [11] as follows:

$$N + \Delta i W + \Delta j Y \leftrightarrows D \quad (1)$$

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where N and D are native and unfolded proteins, respectively, W is water, Y is cosolute as a ligand, Δi is the change in the hydration number per protein molecule upon unfolding, and Δj is the change in the bound-cosolute molecules per protein molecule. Then, the equilibrium between the unfolded to native protein $K_{D,S}$ (=[D]/[N]) is described as a function of the activity of cosolute, a_Y, as follows.

$$\mathrm{dln}K_{D,S}/\mathrm{dln}a_Y = \Delta j - (m_Y/m_W)\Delta i \tag{2}$$

where m_W and m_Y are the molality of each component in the solution.

As for the direct interaction parameter between a protein and a cosolute in a solution, Timasheff [12,13] introduced preferential interaction parameter between the protein (P) and cosolute, $\delta m_Y/\delta m_P$, which could be determined by a dialysis equilibrium experiment. Upon unfolding of protein, this preferential interaction parameter changes as follows:

$$\mathrm{dln}K_{D,S}/\mathrm{dln}a_Y = \Delta(\delta m_Y/\delta m_P). \tag{3}$$

From Eqs. (2) and (3), the following equation is obtained.

$$\Delta(\delta m_Y/\delta m_P) = \Delta j - (m_Y/m_W)\Delta i \tag{4}$$

Timasheff said that the left-hand side of Eq. (4) is a measurable quantity but in the right-hand side of the two terms, the hydration Δi term and the Δj term of direct interaction term, are not separable so this is only phenomenological [13,14].

Recently, the Kirkwood–Buff theory [15,16] has been introduced to analyze the two terms in the right-hand side of Eq. (4) separately [17–23]. In this approach, the molar-based preferential interaction parameter, ν_P, is described as follows:

$$\nu_P = -\delta\mu_P/\delta\mu_Y = \delta n_Y/\delta n_P = N_{PY} - (n_Y/n_W)N_{PW} \tag{5}$$

where n_i is the molarity of component i and N_{Pi} is the excess number of water or cosolute around the protein defined by

$$N_{Pi} = n_i N_A \int \mathrm{d}\mathbf{r}[g_{Pi}(\mathbf{r}) - 1]\mathrm{d}\mathbf{r} \tag{6}$$

where g_{Pi} ($\mathbf{r}$) is the correlation function between the protein and the component i when they are separated by the distance $\mathbf{r}$ and N_A is Avogadro's number. Upon unfolding of the protein, Eq. (5) will be

$$\Delta(\delta n_Y/\delta n_P) = \Delta N_{PY} - (n_Y/n_W)\Delta N_{PW}. \tag{7}$$

In this equation, ΔN_{PY} and ΔN_{PW}, in principle, correspond to Δj and Δi in Eq. (4), respectively. By applying the Kirkwood–Buff theory, an additional equation is obtained for ΔN_{PY} and ΔN_{PW} so that it became possible to discuss these two parameters separately [17–23]. For this analysis, a good estimation of the change in partial molar volume of protein is necessary.

By applying the Gibbs–Duhem equation, Eq. (2) can be converted to the following form.

$$\mathrm{dln}K_{D,S}/\mathrm{dln}a_W = \Delta i - (m_W/m_Y)\Delta j \tag{8}$$

where a_W is water activity. This water activity-based equation was also introduced by Tanford [10] himself but he did not recommend this form to use because he assumed the effect of water activity almost negligible in the biological process like protein unfolding. In this connection, the osmotic stress analysis has been proposed to analyze biological processes based on the osmotic pressure of the solution [24,25]. This approach is physicochemically the same with Eq. (8) because the osmotic pressure theoretically is directly related to water activity but the direct interaction term, Δj, was neglected in this method.

Eq. (8) has been applied to analyze protein solubility in solutions [26]. In the previous papers, we used this equation to analyze the change in hydration in protein unfolding [27], to analyze the effects of sugars and denaturant on the stability of proteins in solutions [28], and to analyze the effect of alcohols on protein stability [29]. In all the case, the plot between $\ln K_{D,S}$ and $\ln a_W$ gave the good-correlated linear line so that Eq. (8) could be directly integrated to obtain the free energy difference ($\Delta\Delta G$) for the protein stability between a solution and pure water. In the present paper, effects of osmolytes on thermal stability of ribonuclease A (RNase) are analyzed in terms of water activity.

2. Materials and method

2.1. Materials

RNase (bovine pancrease, chromatographically purified), proline, *N,N*-dimethylglycine, *myo*-inositol, and taurine were purchased from Sigma-Aldrich (Tokyo, Japan). Betaine and trimethylamine-*N*-oxide were obtained from Acros Organics (Geel, Belgium). Glycine and sarcosine were from Nacalai Tesque (Tokyo, Japan). These osmolytes were added in RNase solutions, pH of which were adjusted at 5.5 or 4.2 (0.04 M acetate buffer).

2.2. Measurement of protein unfolding

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

2.3. Measurement of water activity

Water activity, a_W, of a protein solution with a cosolute Y is described, by neglecting the concentration of protein, as a function of the molar fraction of Y, X_Y, by the following equation [30,31].

$$a_W = (1 - X_Y)\exp\left(\alpha {X_Y}^2 + \beta {X_Y}^3\right) \tag{9}$$

The experimental parameters, α and β, have been determined from the freezing point depression [31].

3. Results and discussions

Fig. 1 shows the typical thermal unfolding curve of RNAse at pH = 5.5 in betaine solution with varied concentrations. RNAse was stabilized by betaine and the extent of stabilization increased with an increase in betaine concentration. In the thermal unfolding process of proteins, the van't Hoff equation [32] has been applied.

$$\mathrm{dln}K_{D,S}/\mathrm{d}T = \Delta H^*/RT^2 \tag{10}$$

where $K_{D,S}$ (=[D]/[N]) is the concentration ratio of unfolded protein (D) to native protein (N) in the solution, ΔH^* is the enthalpy of protein unfolding, and R is the 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 [33]. By the integration of Eq. (10), the following equation is obtained.

$$K_{D,S} = [\mathrm{D}]/[\mathrm{N}] = \exp\left[-\left(\Delta H^*/R\right)\left(1/T - 1/{T_m}^*\right)\right] \tag{11}$$

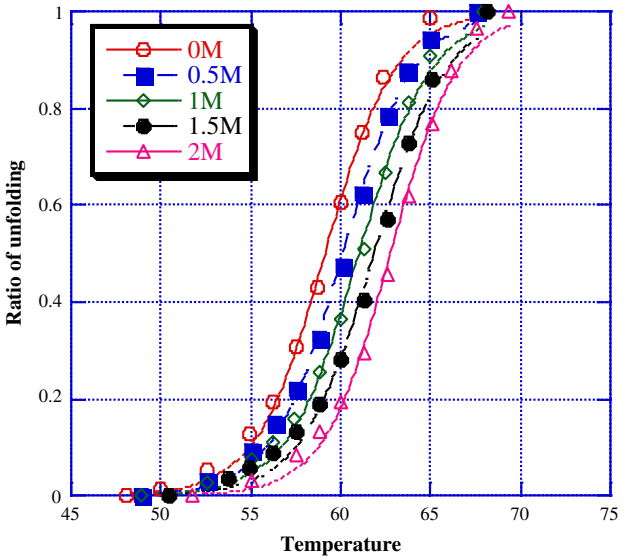

Fig. 1. Thermal unfolding of RNase A in betaine solutions with varied concentrations at pH = 5.5.

where T_m^* is the melting temperature of protein. From this equation, unfolding ratio of protein, x_{unf}, which corresponds to the thermal unfolding curve in Fig. 1, is obtained as follows:

$$x_{unf} = [\mathrm{D}]/\{[\mathrm{D}] + [\mathrm{N}]\} = 1/\left[1 + \exp\left\{-\left(\Delta H^*/R\right)\left(1/T - 1/T_m^*\right)\right\}\right]. \quad (12)$$

Solid lines in Fig. 1 are the best-fitted curve of unfolding described by Eq. (12), which agreed well with the experimental results. The best-fit parameters in Eq. (12) were determined for RNase at pH 5.5 and 4.2 in osmolyte solutions at various concentrations.

Table 1 summarizes the best-fit parameters in Eq. (12) for RNase at pH 5.5 and 4.2 in various osmolyte solutions with varied concentrations. Fig. 2 shows the effects of the type of osmolytes and their concentration on melting temperature, T_m^*, which changed linearly with osmolyte concentration. The similar linear effect of cosolute concentration on T_m^* was observed for all the osmolytes tested here. As for the effect of pH, T_m^* decreased with a decrease in pH showing that the acidic pH facilitates protein unfolding.

To analyze the unfolding process of the protein in solutions, Eq. (8) was applied. For this purpose, water activity, a_W, should be known as a function of cosolute concentration. Table 2 summarizes the parameters α and β in Eq. (9) to describe a_W.

Fig. 3 shows the effect of water activity on the unfolding ratio of RNAse (pH = 5.5) based on Eq. (8) at a fixed temperature of 60 °C. In this plot, well-correlated straight lines were obtained, the slope of which corresponds to the right hand side of Eq. (8). This slope was dependent on the type of osmolyte.

The slope in Fig. 3 based on Eq. (8) is composed of the hydration term (Δi) and the cosolute-binding term $[(m_W/m_Y)\Delta j]$. In the literature, Wyman–Tanford equation has been applied based on the activity of solute as expressed by Eq. (2) to analyze the effect of sugars [34–37] and denaturants [38] on the thermal stability of proteins. In this form, however, both of the number of bound-cosolute molecules (Δj) and the molar ratio of cosolute to water (m_Y/m_W) are strongly dependent on the concentration of cosolute so that no straight lines have been obtained in the literature [35–37]. Moreover, the hydration term, $((m_Y/m_W)\Delta i)$ has been neglected in most cases.

On the contrary in Eq. (8), the hydration number (Δi) and the cosolute-binding term $[(m_W/m_Y)\Delta j]$ would be not strongly dependent on the cosolute concentration because the changes in Δi and m_W by the change in cosolute concentration are small and the changes in m_Y

Table 1
Parameters in van't Hoff equation (Eq.(12)) for thermal unfolding of RNase A in osmolye solutions.

pH	Solute	m_Y (mol/kg-solvent)	T_m^* (K)	ΔH^* (kJ/mol)	r^a
5.5	Glycine	0	332.5	593.1	0.9988
		0.449	334.4	582.9	0.9988
		0.93	335.9	584.9	0.9983
		1.446	337.8	601.7	0.9986
		2.0	339.7	611.6	0.9984
	Sarcosine	0	332.0	549.4	0.9984
		0.441	333.3	520.0	0.9977
		0.918	335.0	531.8	0.9982
		1.436	336.6	540.6	0.9971
		2.0	338.4	549.5	0.9979
	N,N-dimethylglycine	0	332.0	508.4	0.9977
		0.349	333.0	524.1	0.9985
		0.725	333.8	452.7	0.9950
		1.13	335.6	546.8	0.9977
		1.569	336.9	615.3	0.9989
	Betaine	0	332.2	573.3	0.9988
		0.5	333.2	567.6	0.9988
		1.0	334.1	558.1	0.9982
		1.5	335.0	559.3	0.9974
		2.0	335.9	608.2	0.9981
	Proline	0	332.5	594.5	0.9990
		0.426	333.2	619.7	0.9988
		0.896	333.5	598.8	0.9988
		1.418	334.5	565.0	0.9996
		2.0	334.8	593.4	0.9990
	myo-Inositol	0	332.7	586.5	0.9990
		0.15	333.3	590.6	0.9994
		0.35	334.3	597.7	0.9993
		0.55	335.4	621.5	0.9991
		0.75	336.8	612.4	0.9993
	Taurine	0	332.7	591.9	0.9993
		0.142	332.7	632.6	0.9996
		0.289	333.1	602.0	0.9993
		0.442	333.4	614.4	0.9997
		0.6	333.6	587.5	0.9990
	TMAO[b]	0	332.6	573.3	0.9993
		0.339	333.3	584.5	0.9988
		0.678	334.1	589.5	0.9978
		1.016	335.0	640.1	0.9985
		1.355	335.6	603.7	0.9984
4.2	Glycine	0	330.2	605.6	0.9996
		0.449	331.3	674.9	0.9998
		0.93	332.4	571.5	0.9995
		1.446	333.7	570.4	0.9990
		2.0	335.7	578.2	0.9986
	Sarcosine	0	329.1	499.2	0.9969
		0.441	329.8	514.7	0.9983
		0.918	331.2	527.6	0.9984
		1.436	332.6	565.3	0.9985
		2.0	334.4	567.7	0.9988
	Betaine	0	328.9	510.7	0.9988
		0.395	329.6	517.5	0.9986
		0.832	330.5	538.4	0.9989
		1.315	331.3	559.5	0.9990
		1.853	332.2	544.4	0.9985
	Proline	0	329.8	596.0	0.9995
		0.426	329.4	563.7	0.9993
		0.896	329.7	607.5	0.9995
		1.418	329.9	589.1	0.9997
		2.0	330.0	599.7	0.9994
	myo-Inositol	0	329.5	526.3	0.9989
		0.15	329.8	530.0	0.9991
		0.35	330.8	519.5	0.9984
		0.55	331.7	526.7	0.9987
		0.75	332.9	514.7	0.9985
	Taurine	0	329.1	480.9	0.9989
		0.142	329.1	505.0	0.9987
		0.289	329.0	525.6	0.9989
		0.442	329.1	512.3	0.9988
		0.6	329.3	510.0	0.9987

[a] Correlation coefficient.
[b] Trimethylamine-*N*-oxide.

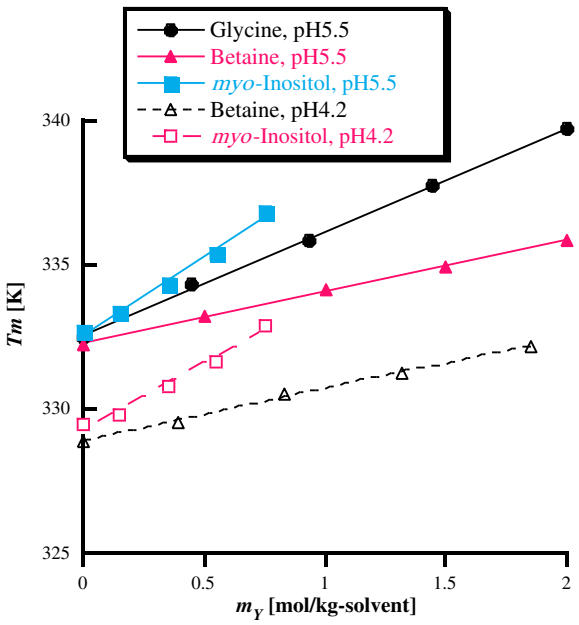

Fig. 2. Effect of osmolyte concentration (m_Y; mol/kg-solvent) on melting temperature (T_m) of RNase A.

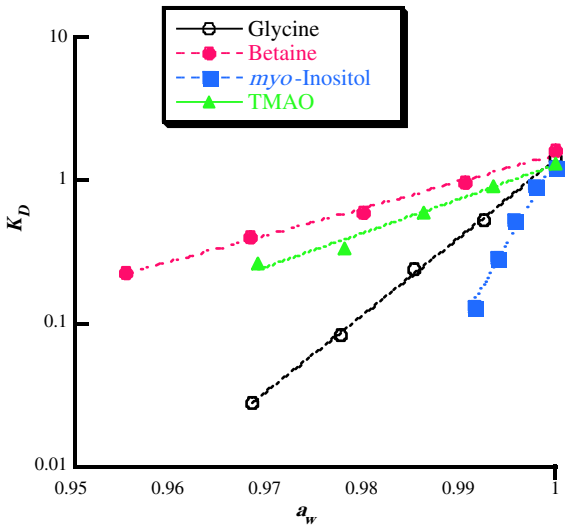

Fig. 3. Effect of water activity on unfolding ratio of RNAse A in osmolyte solutions of pH = 5.5 at 60 °C.

and Δj cancel the change in $\Delta j/m_Y$ with each other. This explains why a good linear relationship was obtained in Fig. 3. Table 3 summarizes the slope of this plot. The positive slope corresponds to the stabilization effect of osmolyte on the protein. Among the osmolytes tested here, *myo*-inositol showed the highest stabilization effect on RNase.

To analyze the protein stability in aqueous solutions, the free energy difference, $\Delta\Delta G$, is calculated [38–40] by subtracting the free energy for protein unfolding in a solution ($\Delta G_{D,S}$) and in pure water ($\Delta G_{D,0}$).

$$\Delta\Delta G = \Delta G_{D,S} - \Delta G_{D,0} \tag{13}$$

Protein stabilizers, such as sugars and polyols, give positive $\Delta\Delta G$ while protein destabilizers, such as urea and guanidine HCl, give negative $\Delta\Delta G$.

Greene and Pace [41,42] proposed a linear extrapolation model (LEM model) for $\Delta\Delta G$ as an empirical model:

$$\Delta\Delta G = m[\text{solute}] \tag{14}$$

where m is an experimental 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 cosolutes on various biological phenomena [43–49]. Auton and Bolen [8] calculated m-value from the transfer free energy of amino acid side-chain for the unfolding of RNAse T1. Schellman [50] described m-value as a balance between the entropic contribution of excluded volume to the stabilization of native protein and the enthalpic contribution of the contact of cosolute to protein destabilization.While LEM model is an empirical model without theoretical basis, this is easily established by the present approach with Eq. (8), which could be integrated in consideration of the experimentally-proved linear correlation between $\ln K_D$ and $\ln a_W$ shown in Fig. 3 as follows:

$$\Delta \ln K_D = \{\Delta i - (m_W/m_Y)\Delta j\}\Delta \ln a_W. \tag{15}$$

When $a_W = 1$, $K_D = K_{D,0}$ (=[D]/[N] in water) so that,

$$\Delta\Delta G = -RT\left(\ln K_{D,S} - \ln K_{D,0}\right) = -RT\{\Delta i - (m_W/m_Y)\Delta j\}\ln a_W. \tag{16}$$

To this equation, Eq. (9) can be applied to obtain the following result.

$$\Delta\Delta G = -RT\{\Delta i - (m_W/m_Y)\Delta j\}\left[\ln(1-X_Y) + \alpha {X_Y}^2 + \beta {X_Y}^3\right] \tag{17}$$

This equation directly gives the effect of cosolute concentration on thermal stability of proteins. From Eq. (17), $\Delta\Delta G$ was calculated and plotted to cosolute concentration as shown in Fig. 4. In the present case, $\Delta\Delta G$ was linearly dependent on cosolute concentration when $m_Y < 1$ showing that the LEM model is applicable to the dilute solutions. From this plot, m-value at $m_Y = 1$ could be easily calculated and listed in Table 4, which also includes m-values of sucrose, urea, and guanidine HCl calculated from the data in the previous paper [28].

Table 2
Parameters in Eq. (9) to describe water activity of osmolyte solutions.

Solute	α	β	X_{Max}[a]	r[b]
Glycine	8.989	−185.6	0.027	0.9999
Sarcosine	−2.743	–	0.035	0.9998
N,N-Dimethylglycine	−16.81	127.3	0.028	0.9999
Betaine	−8.592	–	0.033	0.9999
Proline	−3.232	–	0.035	0.9998
myo-Inositol	66.49	−2955.2	0.014	0.9968
Taurine	56.23	−4240	0.011	0.9996
TMAO[c]	−13.42	–	0.05	0.9999

[a] Maximal molar fraction of applicability of Eq. (9).
[b] Correlation coefficient.
[c] Trimethylamine-*N*-oxide.

Table 3
Slope of the plot between unfolding ratio and water activity based on Eq. (8) for thermal unfolding of RNase A in osmolyte solutions.

Osmolytes	RNase (pH 5.5)	RNase (pH 4.2)
Glycine	120.8	91.4
Sarcosine	81.9	64.9
N,N-Dimethylglycine	67.6	ND[a]
Betaine	42.6	35.8
Proline	31.5	3.47
myo-Inositol	264.8	199
Taurine	59.3	−1.3
TMAO[b]	53	ND[a]

[a] Not measurable because of protein precipitation.
[b] Trimethylamine-*N*-oxide.

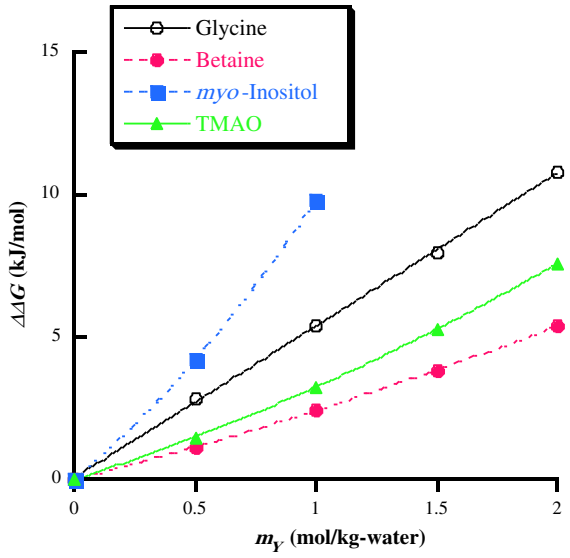

Fig. 4. Calculation of $\Delta\Delta G$ as a function of solute concentration (m_Y; mol/kg-solvent) by Eq. (17) for thermal unfolding of RNase A at pH 5.5 in osmolyte solutions.

Table 4
m-Value of osmolytes for unfolding of RNase A.

Osmolytes	RNase (pH 5.5)	RNase (pH 4.2)
Glycine	5.38 kJ/mol/m	4.07 kJ/mol/m
Sarcosine	4.25	3.36
N,N-Dimethylglycine	4.20	ND[a]
Betaine	2.42	2.04
Proline	1.65	0.18
myo-Inositol	9.84	7.39
Taurine	3.90	0.086
TMAO[b]	3.24	ND[a]
Sucrose[c]	7.03	5.92
Urea[c]	−4.92	−5.66
Guanidine-HCl[c]	−11.00	−10.71

[a] Not measurable because of protein precipitation.
[b] Trimethylamine-*N*-oxide.
[c] From Ref. [28].

m-Values reported here are comparable with those in the literature [8,43,46,48]. Although all the plot of $\Delta\Delta G$ to m_Y for osmolytes showed linearity when the solute concentration was not so high, this is not guaranteed in the wide concentration range [28,51]. Among the osmolytes tested, *myo*-inositol showed the highest *m*-value. Glycine, sarcosine, *N,N*-dimethylglycine, and betaine are the series of substances with an increasing number of the substitution of *N*-bound hydrogen in glycine molecule with methyl group. The *m*-value observed decreased with this order of the substitution.

Eq. (16) theoretically relates $\Delta\Delta G$ to the slope of the plot by Eq. (8) and water activity. From Eq. (16), it became clear that the protein stability in an aqueous solution is determined by a balance between Δi-term and Δj-term. 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, $[(m_W/m_Y)\Delta j]$, seems to destabilize proteins, apparently, as long as Δj is positive.

For the mechanism of protein stabilization in solutions, the preferential exclusion effect of cosolutes from protein surfaces has been proposed [18,40,52]. In this case, the volumetric exclusion effect from a protein surface by inert molecules, such as crowders, causes the stabilization of proteins. This entropic effect could be interpreted as a case with a negative Δj in Eq. (16). In this case, the stoichiometric equation would be expressed by the following equation instead of Eq. (1) because the cosolute Y prefers the native state to the unfolded state of protein as a whole. The stoichiometric equation is phenomenological so that it has to reflect the actual situation.

$$\mathrm{N} + \Delta i\,\mathrm{W} \leftrightarrows \mathrm{D} + \Delta j\,\mathrm{Y}. \tag{18}$$

In this way, the negative Δj value is allowed in Eq. (16). The Δj reflects both the direct-binding effect and the preferential exclusion effect.

Thus, the protein stability in aqueous solution is determined by a balance among the protein hydration effect, the binding effect of cosolute to the protein, and the preferential exclusion effect for cosolute from the protein (preferential hydration). It must be kept in mind that the $\Delta\Delta G$ is also affected by the a_W term as shown in Eq. (16). In an aqueous solution with a protein and a cosolute, the six intermolecular interactions should be considered: protein–water, protein–cosolute, cosolute–water (cosolute hydration), water–water (hydrogen-bonding network), cosolute–cosolute, and protein–protein (Fig. 5). The former two are relevant to the microstructure around the protein surface and the cosolute hydration by cosolute–water interaction determines a_W; a property of the bulk solution. The change in a_W by cosolute hydration determines the direction and the extent of the change in $\Delta\Delta G$. Thus, $\Delta\Delta G$ reflects the property of the microstructure around protein surface represented by $\{\Delta i - (m_w/m_Y)\Delta j\}$ and the bulk property represented by a_W, the reduction of which through the cosolute hydration would

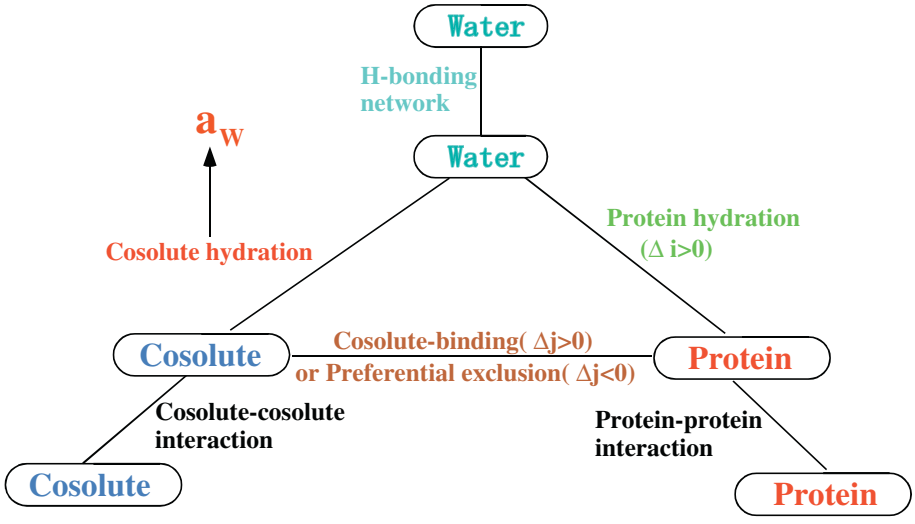

Fig. 5. Various intermolecular interactions in an aqueous solution with a protein and a cosolute.

cause the dehydration of protein as a whole even in the case with the preferential hydration around protein surface.

In conclusion, the thermal unfolding of RNase was analyzed in various osmolyte solutions. All the osmolytes tested stabilized the protein. The Wyman–Tanford plot based on water activity was successfully applied. From the slope of this plot and water activity, the stabilization free energy of the protein in a solution was calculated and *m*-values of osmolytes for RNase unfolding were obtained in consequence. The stabilization effect of osmolytes expressed in free energy was proved to be described by the property of the microstructure around the protein surface and the bulk solution property represented by water activity.

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