Journal of Bioscience and Bioengineering
VOL. 122 No. 2, 203–207, 2016

www.elsevier.com/locate/jbiosc

Cooperative hydration effect causes thermal unfolding of proteins and water activity plays a key role in protein stability in solutions

Osato Miyawaki,* Michiko Dozen, and Kaede Hirota

Department of Food Science, Ishikawa Prefectural University, 1-308 Suematsu, Nonoichi, Ishikawa 921-8836, Japan

Received 27 November 2015; accepted 7 January 2016
Available online 16 February 2016

The protein unfolding process observed in a narrow temperature range was clearly explained by evaluating the small difference in the enthalpy of hydrogen-bonding between amino acid residues and the hydration of amino acid residue separately. In aqueous solutions, the effect of cosolute on the protein stability is primarily dependent on water activity, a_w, the role of which has been long neglected in the literature. The effect of a_w on protein stability works as a power law so that a small change in a_w is amplified substantially through the cooperative hydration effect. In the present approach, the role of hydrophobic interaction stands behind. This affects protein stability indirectly through the change in solution structure caused by the existence of cosolute.

[**Keywords:** Protein stability; Thermal unfolding; Unfolding enthalpy; Cooperative hydration; Hydrogen bonding; Water activity; Solution structure; Hydrophobic interaction]

Proteins are known to be marginally stable in aqueous environment with very small stabilization energy because of the almost complete enthalpy–entropy compensation (1). Although there are so many papers to discuss on the mechanism of protein stability, no clear picture has been obtained yet because of the so many small interaction terms of the same size so that it is difficult to determine which mechanism dominates (2).

The unfolding of protein is known to occur rather in a narrow temperature range to bring about a sudden state change, which is described well by the van't Hoff equation by assuming the two state model (3).

$$\ln K = -(\Delta H/R)(1/T - 1/T_m) \quad (1)$$

where ΔH is the change in enthalpy in unfolding and T_m is the melting temperature; mid-point temperature in the unfolding curve.

Major mechanisms for the stabilization of proteins are hydrophobic interaction including van der Waals interaction, hydrogen-bonding between amino acid residues, electrostatic interactions, and entropic effect in conformation (4–7). The first three are stabilizing while the last one is destabilizing. None of these mechanisms by themselves, however, seem to explain the experimental sudden state change from native to unfolded. The only possibility seems to arise by distinguishing the difference in the enthalpy for the hydrogen-bonding between amino acid residues to stabilize protein structure and that for the hydration of amino acid residues to destabilize it. This difference in enthalpy has been almost neglected in the literature (4–8).

In the present paper, a theoretical model is proposed to describe the thermal unfolding of proteins through the cooperative hydration effect. This effect also plays an important role in protein stability in aqueous solutions.

MATERIALS AND METHODS

Materials Ribonuclease A (RNase, bovine pancrease, chromatographically purified), lysozyme (Lyz, chicken egg white, 3× crystallized), α-chymotrypsinogen A (CTA, bovine pancrease, 6× crystallized), ribose, proline, *N,N*-dimethylglycine, *myo*-inositol, 1-butanol, and 2-butanol were purchased from Sigma–Aldrich Japan (Tokyo, Japan). Trehalose, betaine and trimethylamine-*N*-oxide were obtained from Acros Organics (Geel, Belgium). Glycerol was from Merck Japan (Tokyo, Japan). Maltose, glycine, sarcosine, trifluoroethanol, urea, and guanidine HCl were from Nacalai Tesque (Tokyo, Japan). Sucrose, glucose, formamide, methanol, ethanol, 1-propanol, 2-propanol, and *tert*-butanol were from Kanto Kagaku (Tokyo, Japan). These solutes were added to adjust 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.

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

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 (9,10).

$$a_w = (1 - X_Y)\exp\left(\alpha X_Y^2 + \beta X_Y^3\right) \quad (2)$$

The experimental parameters, α and β, have been determined from the freezing point depression and were reported in the literature (10) for sucrose, maltose, glucose, ribose, urea, formamide, methanol, ethanol, 1-propanol, 2-propanol, *tert*-butanol, and glycerol. No data was available for other solutions. In this case, water activity was also measured by the freezing point depression (10).

* Corresponding author. Tel.: +81 76 227 7464; fax: +81 76 227 7410.
E-mail address: osato@ishikawa-pu.ac.jp (O. Miyawaki).

1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2016.01.005

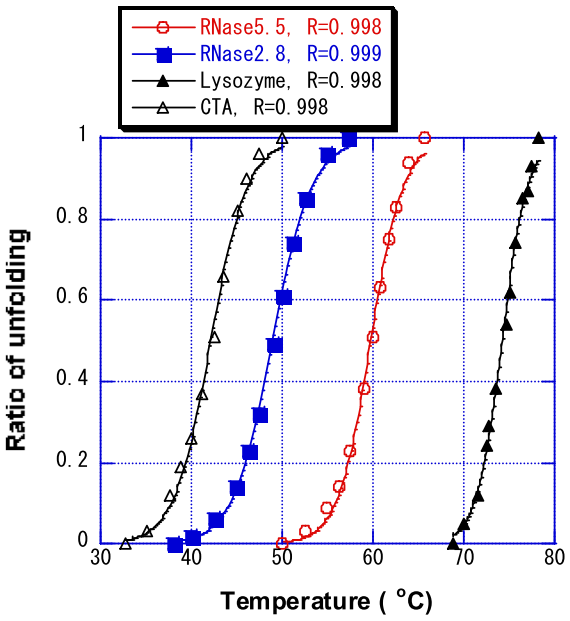

FIG. 1. Thermal unfolding of various proteins in pure water.

RESULTS AND DISCUSSION

Fig. 1 shows thermal unfolding curves of ribonuclease A (RNase) at pH 5.5 and 2.8, lysozyme (Lys) at pH 5.5, and chymotrypsinoge A (CTA) at pH 2.0. This figure shows that the unfolding of protein occurs in a narrow temperature range of 10–20 °C, which shows that the ΔH in Eq. 1 is substantially large.

A free amino acid residue, A, in a protein can form hydrogen-bonding with another amino acid residue or water molecule, W, with the equilibrium constants, K_A and K_W, respectively, as follows.

$$\mathrm{A} + \mathrm{A} \rightleftarrows \mathrm{A} - \mathrm{A} \tag{3}$$

$$\mathrm{A} + \mathrm{W} \rightleftarrows \mathrm{A} - \mathrm{W} \tag{4}$$

$$K_A = [\mathrm{A} - \mathrm{A}] \Big/ [\mathrm{A}]^2 \tag{5}$$

$$K_W = [\mathrm{A} - \mathrm{W}]/[\mathrm{A}]/[\mathrm{W}] \tag{6}$$

These equilibria should be expressed by activity-based equations but the above expressions are chosen for the simplicity. In addition, there are many amino acid residues and their hydrogen-bonding states are not homogeneous. Therefore, these equilibria are considered to be statistical average. The temperature dependence of these equilibria are also expected to be described by the van't Hoff equation.

$$\ln K_A = -\Delta H_A \Big/ RT^2 + \ln K_{A,0} \tag{7}$$

$$\ln K_W = -\Delta H_W \Big/ RT^2 + \ln K_{W,0} \tag{8}$$

where ΔH_A and ΔH_W are the corresponding enthalpy change, T is the absolute temperature, R is the gas constant, and $K_{A,0}$ and $K_{W,0}$ are equilibrium constants at the reference temperature.

In a protein molecule, there will be large number of hydrogen bondings among amino acid residues and some of those (n) will be broken upon unfolding to be hydrated.

$$n\mathrm{A} - \mathrm{A} + 2n\mathrm{W} \rightleftarrows 2n\,\mathrm{A} - \mathrm{W} \tag{9}$$

TABLE 1. Enthalpy relationship in thermal unfolding of proteins in water.

Protein	Mol. weight	pH	T_m[K]	ΔH [kJ/mol]	n	N_{HB}[a]	$\Delta H/n$ [kJ/mol]
RNase	13600	5.5	332.9	516.0	67.0	130	7.70
		4.2	331.1	460.3	62.1		7.41
		2.8	322.1	403.6	69.6		5.80
Lys	14300	5.5	347.4	712.8	100.7	159	7.08
CTA	25670	2.0	315.3	411.7	50.1		8.22

[a] Number of intramolecular hydrogen-bondings determined from the protein structure (12).

The equilibrium constant of this reaction, K, can be described as follows.

$$K = [\mathrm{A} - \mathrm{W}]^{2n} \Big/ [\mathrm{A} - \mathrm{A}]^n \Big/ [\mathrm{W}]^{2n} = K_W^{2n} \Big/ K_A^n \tag{10}$$

The temperature dependence of this reaction is also described by the van't Hoff equation as follows.

$$\ln K = ln\left(K_{W0}^{2n} \Big/ K_{A0}^n\right) - (n/RT)(2\Delta H_W - \Delta H_A) \tag{11}$$

From the comparison between Eqs. 1 and 11, the following equations are obtained.

$$\ln\left(K_{W0}^{2n} \Big/ K_{A0}^n\right) = (\Delta H/R)(1/T_m) \tag{12}$$

$$\Delta H = n(2\Delta H_W - \Delta H_A) \tag{13}$$

This equation shows that the experimentally observed large enthalpy change, ΔH, upon protein unfolding is the accumulation of the small enthalpy difference between the hydrogen bonding among amino acid residues (ΔH_A) and their hydration ($2\Delta H_W$) through the cooperative hydration effect.

Table 1 lists the melting temperature and the observed change in enthalpy upon unfolding for RNase, Lys, and CTA. In the previous paper (11), we estimated the change in the hydration number upon unfolding for these proteins, from which the number of hydrogen-bonding broken upon unfolding, n, was estimated and shown also in Table 1. The n's thus obtained were about 1/2–2/3 of the number of hydrogen-bondings, N_{HB}, determined from the protein structure (12). From these, the change in enthalpy for the breakage of a single hydrogen-bonding between amino acid residues is calculated by $\Delta H/n$, which was as small as 5.80–8.22 kJ/mol as shown in Table 1. This small difference in enthalpy is amplified by the large number of n in unfolding of proteins, which might be called as cooperative hydration effect.

This ΔH in protein unfolding should be compensated by the various entropic effects (1,8), which includes the conformational entropy change of protein structure and the entropy change in water side because of the exposure of hydrophobic amino acid residues.

In aqueous solutions, the thermal unfolding is affected by the coexistence of cosolute. Fig. 2 shows the effect of trehalose on thermal unfolding curve of RNase at pH 5.5. trehalose concentration-dependently stabilized the protein. The unfolding curves could be fitted also by Eq. 1 as shown by the solid lines in Fig. 2. From this fitting, the melting temperature, T_m, and the unfolding enthalpy, ΔH, were obtained. Sugars increased T_m to stabilize protein while urea and formamide decreased T_m to destabilize it (Fig. 3). The ΔH slightly increased with the coexistence of sugars while it decreased by urea and formamide (Fig. 4).

The protein unfolding in aqueous solutions has been described as a multisite reaction with water and cosolute as ligands as follows (13).

$$\mathrm{N} + \Delta i\mathrm{W} + \Delta j\mathrm{Y} \rightleftarrows \mathrm{D} \tag{14}$$