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Extrinsic protein stabilization by the naturally occurring osmolytes β-hydroxyectoine and betaine

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Abstract Thermodynamic aspects of protein stabilization by two widespread naturally occurring osmolytes, β-hydroxyectoine and betaine, were studied using differential scanning calorimetry (DSC) and bovine ribonuclease A (RNase A) as a model protein. The osmolyte β-hydroxyectoine purified from *Marinococcus* was found to be a very efficient stabilizer. At a concentration of 3 M it increased the melting temperature of RNase A (T_m) by more than 12 K and gave rise to a stability increase of 10.6 kJ/mol at room temperature. The heat capacity difference between the folded and unfolded state (ΔC_p) was found to be significantly increased. Betaine stabilized RNase A only at concentrations less than 3 M. Also, here ΔC_p was found to be increased. Calculation of the number of water molecules that additionally bind to unfolded RNase A resulted in surprisingly low numbers for both osmolytes. The significant stabilization of RNase A by β-hydroxyectoine makes this osmolyte an interesting stabilizer in biotechnological processes in which enzymes are applied in the presence of denaturants or at high temperature.

Key words β-Hydroxyectoine · Betaine · RNase A · Differential scanning calorimetry · Enzyme stabilization · Osmolytes

Introduction

Osmolytes were reported initially as small organic molecules that play a role in the adaptation of organisms to high-salt environments. Later on they were also identified as an extrinsic protein stabilizer that enable cells to survive denaturing stresses such as heat, freezing, and high cytoplasmic concentrations of denaturants such as urea (Arakawa and Timasheff 1985; Yancey et al. 1982; Timasheff 1992; Santoro et al. 1992).

Several small molecular compounds such as 2,3-diphosphoglycerate (Hensel and König 1988) and trehalose (Hottiger et al. 1994), as well as di-myo-inositol-1,1′ (3,3′)-phosphate (Martins et al. 1996), have been shown to accumulate to high concentration as a response to environmental stresses. Also ectoines, which belong to the most common solutes found in the cytosol of aerobic heterotrophic bacteria (Galinski 1995), accumulate in cells upon heat stress and are able to increase the thermotolerance of bacteria (Malin and Lapidot 1996).

Osmolytes can be grouped into four major classes: polyols including sugars and sugar derivatives, uncharged amino acids, amino acid derivatives, and methyl ammonium compounds. The presence of osmolytes has been shown to significantly enhance the stability of proteins and preserve their enzymatic activities in an aqueous solvent system. This property makes these compounds not only interesting for basic research but also for biotechnological applications where enzymes may have to function in nonphysiological environments.

As shown by Timasheff and coworkers, the main factor responsible for the stabilization of the native protein is related to the observation that osmolytes are excluded from the protein surface, resulting in a preferential hydration of the protein (Timasheff 1992). The predominant mechanism is hereby an increase in the surface tension of water by the osmolyte that opposes enlargements in surface area. Thus, preferential hydration favors the native state of the protein by making unfolding more unfavorable in the presence of the osmolytes. Because compatible osmolytes act mainly on the denatured state, they leave the native state largely undisturbed. As a result, enzymes show similar catalytic activities in water as well as in osmolyte solutions. Furthermore, it has been demonstrated, by hydrogen exchange experiments, that dynamic aspects of the native state are

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largely unaffected at high sucrose concentrations (Wang et al. 1995).

Here we present a differential scanning calorimetry (DSC) study on the thermal unfolding of bovine ribonuclease A (RNase A) in the presence of the naturally occurring osmolytes betaine and β-hydroxyectoine (2-methyl-4-carboxy-5-hydroxy-3,4,5,6-tetrahydro-pyrimidine), the 5-hydroxy derivative of ectoine. Tetrahydropyrimidine derivatives, first discovered and named after the sulfur bacterium *Ectothiorhodospira halochloris* (Galinski et al. 1985), belong to the most abundant solutes of aerobic heterotrophic bacteria (Galinski 1995). Our study demonstrates that β-hydroxyectoine, which can be isolated from the halophilic bacteria *Marinococcus* (Galinski et al. 1985), is a potent protein stabilizer. We found a linear increase of the melting temperature of RNase A by 4.1 K/mol β-hydroxyectoine as well as a significant increase of the heat capacity change on unfolding in the presence of this compatible osmolyte. At room temperature (298 K) and an osmolyte concentration of 3 M, RNase A was found to be stabilized by 10.6 kJ/mol and at elevated temperature (330 K) even by 13.5 kJ/mol. The thermodynamic data measured on the thermal unfolding of RNase A in the presence of β-hydroxyectoine were compared with data measured under identical conditions using the glycine derivative betaine as a cosolvent.

Materials and methods

Ribonuclease A from bovine pancreas was purchased from Boehringer Mannheim (Germany) (#109169) and was used without further purification. Betaine was purchased from Sigma (St. Louis, MO, USA). β-Hydroxyectoine was purified from *Marinococcus* strain M52 following a method described by Frings et al. (1995).

For differential scanning calorimetry, a concentrated stock solution of bovine RNase A (20 mg/ml) was extensively dialyzed against 1 mM sodium phosphate buffer pH 6.0; 100 µl of this stock solution was used to prepare 2-ml samples for DSC measurements that contained 50 mM phosphate buffer of the desired pH, 200 mM sodium chloride, and the osmolyte used in the individual measurement. The protein concentration of the sample was determined using an extinction coefficient at 278 nm of 9800 l/(mol cm). The pH of the sample was measured before and after the calorimetric measurement and was found to be constant within 0.1 pH unit. Both osmolytes are potent buffers at low pH. To keep the ionic strength constant in all calorimetric experiments, the pH range was restricted by the osmolyte concentration, in the case of 5 M betaine to 4.5–6.5 and to 3.5–6.5 for 3 M β-hydroxyectoine. The concentration of chloride ions was kept constant (200 mM) in all measurements.

Data were measured on a MicroCal MCS calorimeter controlled by the MCS observer program (MicroCal). Samples were routinely degassed for 5 min before they were used for a calorimetric analysis. Buffer baselines were collected under identical conditions and were subtracted from the corresponding data of the protein samples. The scanning rate during the calorimetric measurement was 1 K/min. A second (re-heating) scan was always performed after the DSC experiment to assess the reversibility of the unfolding transition. The reversibility of the unfolding transition was found to be very high (typically 95%), as indicated by the ratio of the areas under the calorimetric endotherm of the first and second thermal scan.

Calorimetric enthalpies (ΔH^{cal}) and van't Hoff enthalpies (ΔH^{vH}) were calculated using the software supplied with the instrument (MicroCal). However, directly determined heat capacity changes of the individual scans scattered significantly. ΔC_p used in all calculations was therefore derived from slopes of $\Delta H^{cal}(T)$ plots, assuming that ΔC_p is constant in the investigated temperature region.

ΔH has been calculated at any temperature according to

$$\Delta H(T) = \Delta H_{Tm} + \Delta C_P(T - T_m) \quad (1)$$

The number of protons exchanged between the protein and the solvent upon denaturation (Δv) was calculated from

$$\Delta v = \frac{1000\Delta H^{cal}}{2.303RT_m^2}\frac{\delta T_m}{\delta pH} \quad (2)$$

The temperature dependence of the Gibbs free energy difference upon denaturation was calculated using

$$\Delta G(T) = \Delta H_m\left(1 - \frac{T}{T_m}\right) - \Delta C_P\left[T_m - T + \left(T\ln\frac{T}{T_m}\right)\right] \quad (3)$$

Subscripts were used according to the Scatchard convention (1 for the solvent [water], 2 for the protein, and 3 for the cosolvent). The change in protein–cosolvent preferential interaction ($\Delta\Phi_{23}$) was calculated at constant pH and at T_m using

$$\Delta\Phi_{23} = \frac{\Delta H_m}{T_m}\left(\frac{\delta T_m}{\delta m_3}\right)_{pH} \quad (4)$$

or was derived using the calculated Gibbs free energy difference upon denaturation:

$$\left(\frac{\delta\Delta G}{\delta m_3}\right)_{T,pH} = \Delta\Phi_{23} \quad (5)$$

were m_3 is the concentration of the osmolyte in mol/kg. For calculations of changes in protein–cosolvent preferential interaction and hydration, the concentration of osmolytes was expressed in moles of osmolytes per kilogram of water rather than in moles per liter, using density measurements of the osmolyte–water mixtures. The resulting values were 1.38 mol/kg and 2.57 mol/kg instead of 1.5 M and 3 M β-hydroxyectoine, respectively. The values for betaine were 1.96, 2.89, 3.73, and 4.59 mol/kg.

Changes in hydration ($\Delta\Gamma_{21}$) between the native and denatured protein were calculated using:

$$\Delta\Gamma_{21} = \frac{\Delta\Phi_{23}}{V_1(\delta\Pi/\delta m_3)_T} \quad (6)$$

Where V_1 is the molar volume of water and $\delta\Pi/\delta m_3$ is the dependence of the osmotic pressure (Π) on the osmolyte concentration (m_3).

Results

Calorimetric measurements of the thermal unfolding transition of bovine RNase A were performed at different osmolyte concentrations in the acidic and neutral pH region. The evaluation of the measurements was based on the assumption that the unfolding transition of RNase A is a reversible two-state process. RNase A is a well-studied protein from a thermodynamic point of view (Makhatadze and Privalov 1993). Also, under the measuring conditions used in this study, the unfolding transition was found to be highly reversible. The reversibility of each measurement was assessed by comparing the calorimetrically measured enthalpy of the first thermal scan to the enthalpy of a second scan of the same sample. The reversibility of the thermal denaturation was typically 95% or better. A representative calorimetric experiment in the presence (3 M) and absence of β-hydroxyectoine is shown in Fig. 1A. The two-state nature of the unfolding transition was confirmed by a perfect fit of the calorimetric endotherm to a two-state model (Fig. 1B) as well as a near unity ratio of the calorimetrically measured enthalpy and van't Hoff enthalpy (Table 1).

Effects of β-hydroxyectoine and betaine on the melting temperature of RNase A

Melting temperatures (T_m) of bovine RNase A were determined at pH 5.5 in 30 mM sodium phosphate buffer in the presence of increasing concentrations of β-hydroxyectoine and betaine (Fig. 1D). As shown in the figure, a significant shift of T_m toward higher temperature was observed at increasing concentrations of β-hydroxyectoine. The T_m of RNase A shows a linear dependence on the β-hydroxyectoine concentration over the entire studied concentration range (0–3 M), which can be described by the equation $T_m = 332 + 4.1T$ (r-value = 0.99), resulting in a T_m shift of more than 12° at a concentration of 3 M. Measurement at even higher osmolyte concentrations were not possible because of the limited solubility of β-hydroxyectoine in water. Betaine, however, showed an apparently linear concentration dependence of T_m only at low concentrations (<2 M) and deviated at higher concentrations significantly from linearity. Finally, the function $T_m(T)$ flattened out at betaine concentrations greater than 3 M. Furthermore, betaine was at all concentrations found to be less efficient as a stabilizer than β-hydroxyectoine. A similar dependence of T_m on betaine concentration has also been described in an earlier study by Santoro et al. (1992).

In a second set of experiments, the pH dependence of the melting temperature of RNase A was studied at several concentrations of β-hydroxyectoine and betaine (Figs. 2, 1C). Both osmolytes are potent buffers in the acidic pH

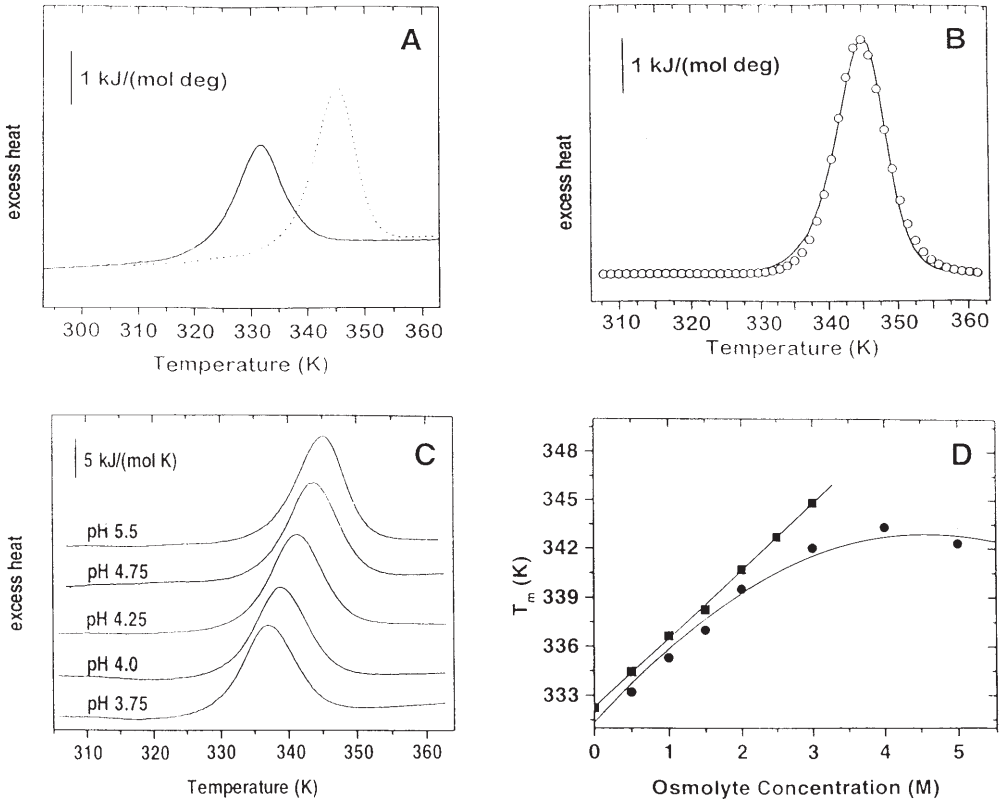

Fig. 1. **A** Effect of β-hydroxyectoine on RNase A unfolding in phosphate buffer, pH 5.5, shown by two calorimetric experiments in the presence of 3 M β-hydroxyectoine (*dotted line*) and without β-hydroxyectoine (*solid line*). The corresponding buffer baselines have been subtracted. **B** Fitting of the data measured in the presence of 3 M β-hydroxyectoine (*solid line*) to a two-state model (*circles*). **C** Representative set of calorimetric experiments measured at constant osmolyte concentration at different pH values shown by calorimetric measurements of the thermal unfolding transition of RNase A in the presence of 3 M β-hydroxyectoine. **D** Dependence of the calorimetrically determined melting temperature (T_m) on the concentration of β-hydroxyectoine (*squares*) and betaine (*circles*). Data measured in 30 mM sodium phosphate buffer at pH 5.5

Table 1. Thermal unfolding of RNase A at pH 5.5 in the presence of β-hydroxyectoine (HE) and betaine (B)

Concen tration (M)	T_m (K)	ΔH_m^a (kJ/mol)	R_H^b	ΔS_m (kJ/(mol K))	ΔC_p (kJ/(mol K))	n^c
β-Hydroxyectoine						
0	333.0	364 ± 14	0.99	1.09 ± 0.05	4.4 ± 0.6	6
1.5	339.4	406 ± 20	0.99	1.22 ± 0.06	5.0 ± 0.5	9
3	345.0	426 ± 23	1.01	1.25 ± 0.07	5.6 ± 0.6	5
Betaine						
0	333.0	364 ± 14	0.99	1.09 ± 0.05	4.4 ± 0.6	6
2	339.5	413 ± 17	0.96	1.22 ± 0.05	4.7 ± 0.3	7
3	341.0	424 ± 13	1.01	1.26 ± 0.04	5.2 ± 0.3	9
4	341.8	427 ± 25	1.00	1.25 ± 0.07	5.1 ± 0.5	10
5	341.1	414 ± 14	1.00	1.23 ± 0.04	5.1 ± 0.3	11

[a] ΔH_m was calculated using the linear fitting functions of ΔH^{cal} versus T_m plots (Fig. 4)

[b] $R_H = \dfrac{\sum_n \Delta H^{cal}}{\sum_n \Delta H^{vH}}$

[c] n, number of measurements

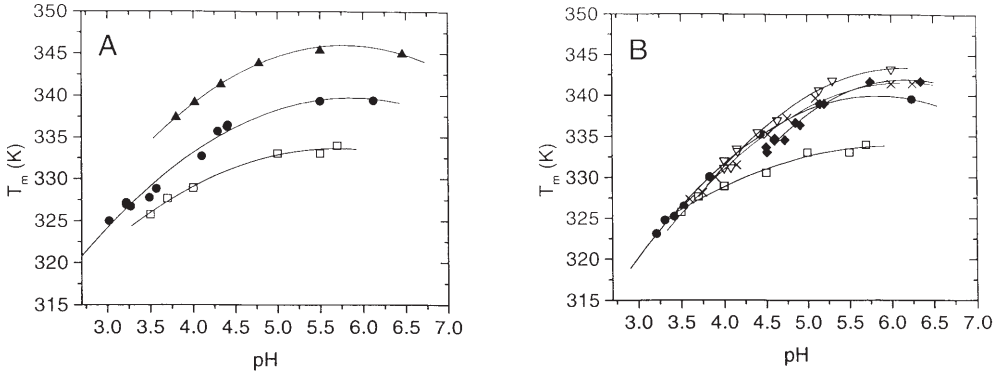

Fig. 2A,B. pH dependence of the melting temperature (T_m) on the osmolyte concentration. **A** Melting temperatures measured at different pH values in the presence of 3 M (*triangles*), 1.5 M (*circles*), and 0 M (*squares*) β-hydroxyectoine. **B** Melting temperatures of RNase A measured at different pH values in 30 mM sodium phosphate buffer and an ionic strength of 200 mM (sodium chloride) in the presence of 5 M (*diamonds*), 4 M (*triangles*), 3 M (*X*), 2 M (*circles*), and 0 M (*squares*) betaine. To guide the eye of the reader, data were fitted to a second-order polynomial function

region. To keep the ionic strength constant (200 mM) the pH range was therefore limited and depended on the concentration of the osmolyte. In the case of β-hydroxyectoine the pH dependence of T_m was studied at a concentration of 1.5 (pH, 3–6) and 3 M (pH, 3.7–6.5). A typical set of experiments at fixed osmolyte concentration and different pH values is shown in Fig. 1C. T_m was unaffected by pH changes in the region pH 5–6 at both concentrations as well as in the absence of osmolyte (Fig. 2). At low pH values, T_m decreased rapidly until this osmolyte lost its ability to stabilize RNase A. A similar behavior of the pH dependence of T_m can be seen in the case of betaine (Fig. 2B), which destabilizes protein even at pH values lower than 3.5.

Effects on the denaturation enthalpy change ΔH_m

As shown in Fig. 3, the calorimetrically measured enthalpy (ΔH^{cal}) determined by integration of the area under the heat absorption peak was in good agreement with the calculated van't Hoff enthalpy, assuming a two-state transition. Principally, in all calorimetric measurements of RNase A unfolding in water–β-hydroxyectoine and water–betaine mixtures, the ratio ($\Delta H^{cal}/\Delta H^{vH}$) did not deviate by more than 5% from unity.

At the melting temperature (T_m), the calorimetric enthalpy (ΔH^{cal}) was always higher in osmolyte–water mixtures than in the absence of the osmolyte for both β-hydroxyectoine and betaine (Fig. 4). At room temperature, however, the calculated ΔH^{298} was significantly lower because of an increase in ΔC_p in the presence of osmolytes (Table 2). Enthalpies measured at different pH values showed a linear temperature dependence (Fig. 4), and linear least squares fit of the measured calorimetric enthalpies plotted versus T_m resulted in the equations $\Delta H_m(T) = -1298 + 5.02T$ ($r = 0.96$) (for 1.5 M β-hydroxyectoine) and $\Delta H(T) = -1487 + 5.55T$ ($r = 0.96$) (for 3 M β-hydroxyectoine); for betaine, $\Delta H(T) = -1167 + 4.67T$ ($r =$

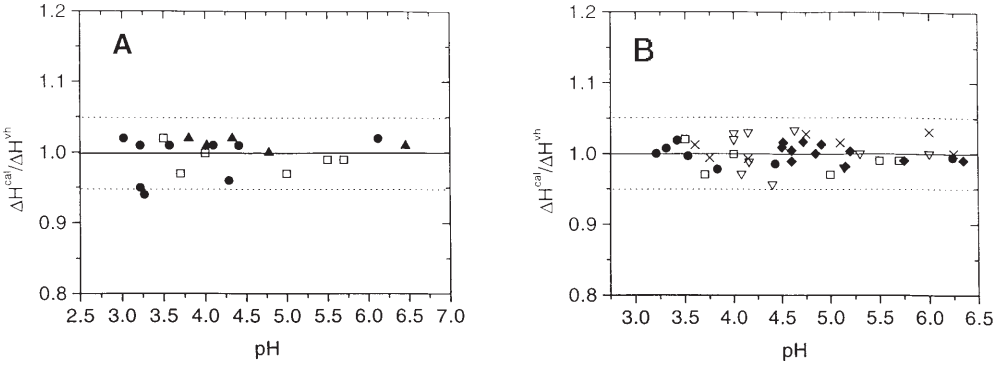

Fig. 3A,B. Ratio of the calorimetric enthalpy (ΔH^{cal}) to van't Hoff enthalpy (ΔH^{vH}) of the thermal denaturation of RNase A; a deviation of 5% is indicated by *dotted lines*. **A** Ratio at different pH values in the presence of 3 M (*triangles*), 1.5 M (*circles*), and 0 M (*squares*) β-hydroxyectoine. **B** Ratio at different pH values in the presence of 5 M (*diamonds*), 4 M (*triangles*), 3 M (*X*), 2 M (*circles*), and 0 M (*squares*) betaine

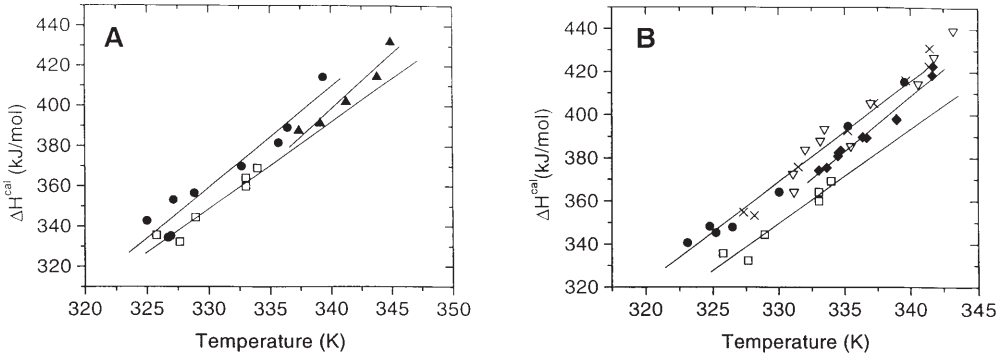

Fig. 4A,B. Calorimetric enthalpy change (ΔH^{cal}) of thermal RNase A unfolding versus temperature (T) shown by data measured at different pH values in the acidic and neutral pH region. **A** Measurements in the presence of 3 M (*triangles*), 1.5 M (*circles*), and 0 M (*squares*) β-hydroxyectoine and the fitted functions (*solid lines*). **B** Measurements carried out in the presence of 5 M (*diamonds*), 4 M (*triangles*), 3 M (*X*), 2 M (*circles*), and 0 M (*squares*) betaine. All data were fitted linearly, but only the fitted functions for 0 M, 2 M, and 5 M betaine are shown. A buffer (30 mM sodium phosphate) of a constant ionic strength of 200 mM (sodium chloride) was always used

0.96) (2 M), $\Delta H(T) = -1366 + 5.25T$ ($r = 0.99$) (3 M), $\Delta H(T) = -1300 + 5.05T$ ($r = 0.96$) (4 M), and $\Delta H(T) = -1326 + 5.10T$ ($r = 0.98$) (5 M). Data measured in the absence of osmolyte resulted in the equation $\Delta H(T) = -1103 + 4.40T$ ($r = 0.96$).

Effects on denaturation heat capacity, ΔC_p

Heat capacity differences between the native and denatured state that were determined directly from individual calorimetric scans did scatter significantly, whereas the plots ΔH_m versus T_m measured at different pH values were essentially linear in the investigated temperature range (Fig. 4).

Such a discrepancy between ΔC_p obtained calorimetrically and assessed from ΔH_m versus T_m plots has been reported earlier (Liu and Sturtevant 1996): ΔC_p, which has been determined directly from individual calorimetric measurements, is known to be very sensitive to inaccuracies in baseline determinations whereas ΔH_m is less prone to such errors. In addition, samples used for calorimetric experiments were not dialyzed against the reference buffer but were mixed from stock solutions because β-hydroxyectoine, purified from the halophilic bacteria *Marinococcus*, was only available in limited amounts. ΔC_p values used in this report were therefore derived from the slope of the linear function $\Delta H_m(T)$.

In the case of β-hydroxyectoine, ΔC_p increased with increasing osmolyte concentration from 4.4 ± 0.6 (kJ/(mol K)) in the absence of this osmolyte to 5.6 ± 0.6 (kJ/mol K)) in the presence of 3 M β-hydroxyectoine. Betaine increased ΔC_p as well to a maximal ΔC_p of 5.2 ± 0.3 (kJ/(mol K)) at a concentration of 3 M betaine. At high concentrations, ΔC_p decreased again slightly, correlating well with the observation that betaine stabilized RNase A most efficiently at concentrations around 3 to 4 M.

Effects on the Gibbs free energy change, ΔG

The change in Gibbs free energy upon denaturation (ΔG) of RNase A at different osmolyte concentrations was calculated as a function of temperature using Eq. 3 (Fig. 5) and at room temperature (298 K) as well as at elevated temperature (330 K) (see Table 2).

At an osmolyte concentration of 2 M (betaine) and 1.5 M (hydroxyectoine), the stability of RNase A was significantly increased. The stability curve ($\Delta G(T)$) was shifted symmetrically upward by a $\Delta\Delta G$ of ≈7 kJ/mol in the entire temperature range. Increasing the concentration of β-

Table 2. Calculated enthalpy and free energy changes

Concentration (M)	ΔH^{298} (kJ/mol)	ΔH^{330} (kJ/mol)	ΔG^{298} (kJ/(mol))	ΔG^{330} (kJ/(mol))
β-Hydroxyectoine				
0	208 ± 10	349 ± 16	29.8	3.2
1.5	198 ± 8	359 ± 15	36.3	10.6
3	167 ± 9	345 ± 16	39.2	16.7
Betaine				
0	208 ± 10	349 ± 16	29.8	3.2
2	224 ± 8	374 ± 14	38.0	10.9
3	199 ± 6	367 ± 11	38.7	12.7
4	205 ± 12	367 ± 22	39.7	13.7
5	194 ± 7	357 ± 11	37.8	12.5

ΔG^{298} and ΔG^{300} were calculated using Eq. 3 and data given in Table 1
Errors for the denaturation enthalpy changes have been estimated from the errors of the linear fitting functions calculated from the ΔH^{cal} versus T_m plots

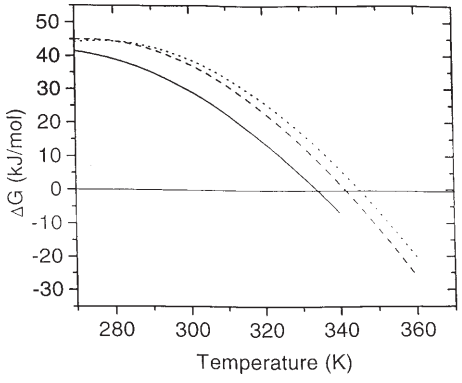

Fig. 5. Gibbs free energy change (ΔG) of the unfolding transition of RNase A as a function of temperature and osmolyte concentration. The curves were calculated according to Eq. 3. Shown are calculated temperature dependencies of ΔG in the absence of osmolyte (*solid line*) and in the presence of 3 M betaine (*dashed line*) and 3 M β-hydroxyectoine (*dotted line*), respectively

hydroxyectoine from 1.5 to 3 M resulted in an additional stabilization of 6.1 kJ/mol at 330 K, thus in a $\Delta\Delta G$ of 13.5 kJ/mol when compared to the protein in buffer only. At 3 M β-hydroxyectoine, the stability curve was significantly shifted upward as well as toward higher temperature. In addition, the curve was also somewhat flattened, indicating that at lower temperatures RNase A was stabilized to a lesser extent than at elevated temperatures. Betaine stabilized RNase A only at concentrations <3 M (Fig. 1D), whereas at higher concentrations this osmolyte apparently lost its ability to stabilize RNase A further. At concentrations of about 3 to 4 M, stabilization by betaine reached a maximum ($\Delta\Delta G$ = 9.9 kJ/mol) and decreased again at even higher concentrations.

Discussion

In this study we were interested in how the osmolytes β-hydroxyectoine and betaine influence the thermodynamic parameters of thermal RNase A unfolding. We found that even at high osmolyte concentration thermal unfolding of RNase A is a highly reversible process that can be described by a two-state transition between the native and denatured protein, offering an ideal model system.

β-Hydroxyectoine was found to stabilize RNase A most efficiently in terms of melting temperature (T_m), resulting in a linear T_m increase of 4.1 K/mol in the entire concentration range studied. Betaine, however, was less efficient in stabilizing RNase A, and the plot T_m versus betaine concentration deviated significantly from linearity at high betaine concentrations. This effect has already been noticed in a study by Santoro et al. (1992) that compared the melting temperatures of RNase A in the presence of glycine and its derivatives sarcosine (*N*-methylglycine), dimethylglycine, and betaine (*N*,*N*,*N*-trimethylglycine). Clearly, an increasing number of methyl groups present in the different glycine derivatives correlated well with its decreasing ability to stabilize RNase A. This observation can be rationalized considering that a hydrophobicity increase of the osmolyte results in an enhanced affinity to hydrophobic areas exposed in the unfolded protein (Arakawa and Timasheff 1983). At low pH values, both β-hydroxyectoine and betaine lose their ability to stabilize RNase A, probably because of the increasingly ionic nature of the osmolyte as well as that of the protein, which results in interaction of the osmolyte to the protein surface rather than in its preferential exclusion. Therefore, osmolytes found in nature are either nonionic or zwitter-ionic compounds (Timasheff 1992).

Some of the stabilizing contributions of β-hydroxyectoine and betaine seem to be enthalpic in nature, because measured enthalpies at the different melting temperatures were always higher than the corresponding values measured in the absence of osmolytes (Fig. 4). The fitted functions $\Delta H_m(T_m)$, calculated using data measured at different pH values and osmolyte concentrations, did not however converge at high temperatures as in a study on the thermal unfolding of RNase A in the presence of sarcosine (Plaza del Pino and Sanchez-Ruiz 1995).

Heat capacity differences between the native and unfolded protein have been reported to increase with in-

creasing osmolyte concentration (Plaza del Pino and Sanchez-Ruiz 1995). This finding was confirmed by the study presented here where ΔC_p increased from 4.4 kJ/(mol K) to 5.6 kJ/(mol K) in the presence of 3 M β-hydroxyectoine. In the case of betaine, ΔC_p increased initially up to a concentration of 3 M and remained constant at higher concentrations. In general, ΔC_p can be calculated from changes in nonpolar (ΔASA_{np}) and polar-accessible surface areas (ΔASA_{pol}) upon denaturation, assuming an extended conformation as a model for the unfolded protein and using data measured on small molecular model compounds (Murphy et al. 1990):

$$\Delta C_p = \Delta C_{np}\, \Delta ASA_{np} + \Delta C_{pol}\, \Delta ASA_{pol} \quad (7)$$

Nonpolar and polar groups contribute to the hydration heat capacity increment with opposite signs, which is positive for nonpolar groups and negative for polar groups (Makhatadze and Privalov 1990, 1994, 1995; Murphey and Gill 1991). Because in the presence of osmolytes ΔC_{np} and ΔC_{pol} as well as ΔASA may vary, we can at this point only speculate about the molecular mechanisms of the measured ΔC_p increase. However, the comparison of the concentration dependence of ΔC_p for sarcosine (Plaza del Pino and Sanchez-Ruiz 1995) and betaine suggests a mechanism for the effect that ΔC_p did not increase further at high betaine concentrations whereas a strong increase was still observed at very high sarcosine concentrations. Because these two osmolytes differ only in two methyl groups, it is reasonable to assume that the additional hydrophobic groups in betaine promote binding to nonpolar surfaces exposed upon denaturation and reduce the denaturational differences in nonpolar-accessible surface area (ΔASA_{np}). Such a mechanism leading to a decrease in ΔC_p has been described for other solvent systems with higher solvent hydrophobicity such as alcohols (Velicelebi and Sturtevant 1979; Fu and Freire 1992).

The function $\Delta G(T)$ defines the thermodynamic stability of a protein (Becktel and Schellman 1987). As shown in Fig. 5 and Table 2, the presence of β-hydroxyectoine and betaine gives rise to significant increases in ΔG and $\Delta\Delta G$ and demonstrates that osmolytes may stabilize proteins to an extent comparable or even better than site-directed mutagenesis approaches. Equilibrium dialysis and transfer free energy measurements have shown that the stabilization of proteins by osmolytes cannot be accounted for by the stabilization of the native state of proteins in water osmolyte mixtures (Gekko and Timasheff 1981; Lee and Timasheff 1981; Timasheff 1992; Liu and Bolen 1995). In fact, the transfer of the native protein into an osmolyte solution is even unfavorable. Stabilization results from the extremely unfavorable transfer of the unfolded state from water into osmolyte solution, leading ultimately to the observed stabilization effect.

Surprisingly, the very large destabilization of the denatured state in osmolyte solutions originates not from the unfavorable transfer of exposed hydrophobic side chains, but from the very unfavorable interaction of the peptide backbone with osmolyte solutions (Liu and Bolen 1995). Transfer free energy measurements estimated a stabilization of RNase A in 2 M and 4 M solutions of sarcosine of about 6.8 and 20 kJ/mol, respectively. These values are in good agreement with $\Delta\Delta G$ values measured here in water–β-hydroxyectoine mixtures.

One of the interesting aspects of thermodynamic data measured in the presence of different concentrations of osmolytes is that denaturational changes in hydration ($\Delta\Gamma_{21}$) can be estimated. In the case of RNase unfolding in the presence of sarcosine, such an estimation resulted in a surprisingly low number of water molecules (<100) that bind to RNase A upon unfolding. By assuming RNase A as a fully solvated extended chain in the unfolded state, at least 600 bound water molecules have been predicted (Plaza del Pino and Sanchez-Ruiz 1995).

For betaine, a concentration dependence of the osmotic pressure ($\delta\Pi/\delta m_3$) of 32.1 atm kg mol^{-1} has been reported (Timasheff 1992). Using Eq. 4 and data from Table 1, the change in cosolvent–protein preferential interaction ($\Delta\Phi_{23}$) is 4 kJ kg mol^{-2}, which gives rise to only 68 water molecules that additionally bind to RNase A upon unfolding (Eq. 6).

The effect of β-hydroxyectoine on the osmotic pressure ($\delta\Pi/\delta m_3$) is to date not known. However, using an ideal value of 23.5 atm kg mol^{-1} ($RT/V_1/(55.56 + m_3)$) for 1.5 M (1.38 mol/kg) β-hydroxyectoine or, alternatively, a value of 34.8 atm kg mol^{-1} calculated for a hypothetical superosmolyte (Timasheff 1992), $\Delta\Gamma_{21}$ results also here in only 128 and 87 water molecules, respectively. The estimation of $\Delta\Gamma_{21}$ is based on a number of assumptions including that $\delta\Pi/\delta m_3$ and $\Delta\Phi_{23}$ are not strongly temperature dependent. However, at low osmolyte concentration it is reasonable to assume $\Delta\Phi_{23}$ to be largely temperature independent because $\delta\Delta G/\delta m_3$ (Eq. 5) shows no strong temperature dependence. Despite those approximations, it is safe to conclude that the number of water molecules which bind the RNase A upon denaturation is significantly lower than expected for a fully hydrated extended chain. In accordance with the observed low $\Delta\Gamma_{21}$ values, several biophysical studies using a variety of independent methods have shown that the denatured state of proteins is indeed rather compact (Sosnick and Trewhella 1992; Neri et al. 1992; Wang and Shortle 1995).

During the last few years osmotic stress strategies have been successfully used to measure differences in macromolecular hydration of different conformational states during enzyme catalysis. These measurements have revealed a surprisingly large influence of water activity on macromolecular reactions and conformational transitions. In a large number of studies, the role of water during protein folding and its role in protein stability have often been neglected. It will be an interesting challenge to apply the osmotic stress strategy to understand more about the influence of water in protein folding and the extrinsic mechanism used in the cell to maintain the functional native conformation of macromolecules during stress situations. Results from such studies will not only answer fundamental biological questions such as how thermophiles protect proteins at extreme temperatures but will also promote our understanding of how enzymatic activity can be conserved in industrial processes.

References

Arakawa T, Timasheff SN (1983) Preferential interactions of proteins with solvent compounds in aqueous amino acid solutions. Arch Biochem Biophys 244:169–177

Arakawa T, Timasheff SN (1985) The stabilization of proteins by osmolytes. Biophys J 47:411–414

Becktel WJ, Schellman JA (1987) Protein stability curves. Biopolymers 26:1859–1877

Frings E, Sauer T, Galinski EA (1995) Production of hydroxyectoine: high cell-density cultivation and osmotic downshock of *Marinococcus* strain M52. J Biotechnol 43:53–61

Fu L, Freire E (1992) On the origin of the enthalpy and entropy convergence temperatures in protein folding. Proc Natl Acad Sci USA 89:9335–9338

Galinski EA (1995) Osmoadaptation in bacteria. Adv Microb Phys 37:273–328

Galinski EA, Pfeiffer HP, Trüper HG (1985) 1,4,5,6-Tetrahydro-2-methyl-4 pyrimidinecarboxylic acid: a novel cyclic amino acid from halophilic phototrophic bacteria of the genus *Ectothiorhodospira halochloris*. Eur J Biochem 149:135–139

Gekko K, Timasheff SN (1981) Mechanism of protein stabilization by glycerol: preferential hydration in glycerol water mixtures. Biochemistry 20:4667–4676

Hensel R, König H (1988) Thermoadaptation of methanogenic bacteria by intracellular ion concentration. FEMS Microbiol Lett 49:75–79

Hottiger T, De Virgilio C, Hall MN, Boller T, Wiemken A (1994) The role of trehalose synthesis for the acquisition of thermotolerance in yeast. Physiological concentration of trehalose increases the thermal stability of proteins in vitro. Eur J Biochem 219:187–193

Lee JC, Timasheff SN (1981) The stabilization of proteins by sucrose. J Biol Chem 256:7193–7201

Liu Y, Bolen DW (1995) The peptide backbone plays a dominant role in protein stabilization by naturally occurring osmolytes. Biochemistry 34:12884–12891

Liu Y, Sturtevant JM (1996) The observed change in heat capacity accompanying the thermal unfolding of proteins depends on the composition of the solution and on the method employed to change the temperature of unfolding. Biochemistry 35:3059–3062

Makhatadze GI, Privalov PL (1990) Heat capacity of proteins. I. Partial molar heat capacity of individual amino acid residues in aqueous solution: hydration effect. J Mol Biol 213:375–384

Makhatadze GI, Privalov PL (1993) Contribution of hydration to protein folding thermodynamics. I. The enthalpy of hydration. J Mol Biol 232:639–659

Makhatadze GI, Privalov PL (1994) Hydration effects in protein unfolding. Biophys Chem 51:291–309

Makhatadze GI, Privalov PL (1995) Energetics of protein structure. Adv Prot Chem 47:307–425

Malin G, Lapidot A (1996) Induction of synthesis of tetrahydropyrimidine derivatives in *Streptomyces* strains and their effect on *Escherichia coli* in response to osmotic and heat stress. J Bacteriol 178:385–395

Martins LO, Carreto LS, Da Costa MS, Santos H (1996) New compatible solutes related to di-myo-inositol-phosphate in members of the order *Thermotogales*. J Bacteriol 178:5644–5651

Murphy KP, Privalov PL, Gill SJ (1990) Common features of protein unfolding and dissolution of hydrophobic compounds. Science 247:559–561

Neri D, Billeter M, Wider G, Wuthrich K (1992) NMR determination of residual structure in a urea-denatured protein, the 434 repressor. Science 257:1559–1563

Plaza del Pino IM, Sanchez-Ruiz JM (1995) An osmolyte effect on the heat capacity change for protein folding. Biochemistry 34:8621–8630

Santoro MM, Liu Y, Khan SMA, Hou L-X, Bolen DW (1992) Increased thermal stability of proteins in the presence of naturally occurring osmolytes. Biochemistry 31:5278–5283

Sosnick TR, Trewhella J (1992) Denatured states of ribonuclease A have compact dimensions and residual secondary structure. Biochemistry 32:10359–10370

Timasheff SN (1992) A physicochemical basis for the selection of osmolytes by nature. In: Somero GN (ed) Water and life. Springer-Verlag, New York, pp. 70–84

Velicelebi G, Sturtevant JM (1979) Thermodynamics of the denaturation of lysozyme in alcohol water mixtures. Biochemistry 18:1180–1186

Wang Y, Shortle D (1995) The equilibrium folding pathway of staphylococcal nuclease: identification of the most stable chain-chain interactions by NMR and CD spectroscopy. Biochemistry 34:15895–15905

Wang A, Robertson AD, Bolen DW (1995) Effects of a naturally occurring compatible osmolyte on the internal dynamics of ribonuclease A. Biochemistry 34:15096–15104

Yancey PH, Clark ME, Hand SC, Bowlus RD, Somero GN (1982) Living with water stress: evolution of osmolyte systems. Science 217:1214–1222