*Acta Chim. Slov.* **1999,** *46(3),* pp. 315-322

# THERMODYNAMIC STABILITY OF RIBONUCLEASE A AT 25 °C IN AQUEOUS SOLUTIONS OF GUANIDINE HYDROCHLORIDE, UREA AND ALKYLUREAS[†]

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(Received 6.1.1999)

**Abstract:** The previously published results of the effect of pH, guanidinium hydrochloride (GuHCl), urea, methylurea, N,N'-dimethylurea, ethylurea and butylurea on the thermal stability of ribonuclease A (RNase A) studied by differential scanning calorimetry (DSC) (N. Poklar, N. Petrovèiè, M. Oblak, G. Vesnaver, *Protein Sci.* **1999**, *8, 832-840)* were used to calculate the RNase A thermodynamic stability at 25°C. It has been shown that thermodynamic stability of RNase A at 25 °C decreases with increasing concentration of denaturants and the size of the hydrophobic group substituted on the urea molecule.

## INTRODUCTION

In the previously published articles [1, 2] the combination of calorimetric (differential scanning calorimetry (DSC)) and spectroscopic techniques (UV-spectroscopy and CD-spectropolarimetry) was used to follow the changes in thermodynamic quantities accompanying the thermal denaturation of ribonuclease A

[†] Dedicated to the memory of Jože Šiftar.

(RNase A) at different concentrations of guanidinium hydrochloride (GuHCl), urea and several alkylureas and in aqueous solutions of different pH [1]. Furthermore, the influence of GuHCl, urea and alkylureas on the intrinsic fluorescence properties of RNase A in comparison with the intrinsic fluorescence of the model dipeptides was studied by the emission fluorescence spectroscopy at 25 °C [2]. The major conclusions based on our previously published results are that the thermal stability of ribonuclease A decreases with increasing concentration of denaturants and the size of hydrophobic group substituted on the urea molecule and that the effect of butylurea on the thermal stability of RNase A is more pronounced than that of GuHCl. This second conclusion implies that the ability of butylurea to change the properties of the solvent (water) is at least as important as the ability of GuHCl to form hydrogen bonds. Furthermore, it has been observed that the tertiary structure of RNase A melts at lower temperatures than its secondary structure indicating a hierarchy in structural building blocks of RNase A native state even at conditions at which a two-state approximation of the unfolding process is valid. The far-UV CD spectra have shown that the denatured states of RNase A in the presence of different denaturants at higher temperatures are very similar but differ from the denatured states that exist at higher concentrations of urea or GuHCl at 25 °C [1].

In this article we present calculations of the standard enthalpies, $\Delta H^{o}_{T}$, entropies, $\Delta S^{o}_{T}$, and free energies, $\Delta G^{o}_{T}$, of denaturation of RNase A at 25 °C in solutions of different pH and in the presence of different concentrations of GuHCl, urea and alkylureas, based on our previously published DSC data [1].

## RESULTS AND DISCUSSION

Using the experimentally determined enthalpies of denaturation, $(\Delta H_{Td})_{DSC}$, obtained from DSC thermograms [1] at temperatures of half transition, $T_d^{DSC}$, the standard enthalpy, $\Delta H^{o}_{T}$, entropy, $\Delta S^{o}_{T}$, and free energy, $\Delta G^{o}_{T}$, of denaturation of RNase A can be calculated at T = 25 °C as:

$$\Delta H_T^o = (\Delta H_{T_d})_{DSC} - \Delta C_P^o (T - T_d^{DSC}); \quad (\Delta H_{T_d})_{DSC} = (\Delta H_{T_d}^o)_{DSC} \qquad (1)$$

$$\Delta S_T^o = \frac{(\Delta H_{T_d})_{DSC}}{T_d^{DSC}} + \Delta C_P^o \ln \frac{T}{T_d^{DSC}} \qquad (2)$$

$$\Delta G_T^o = \Delta H_T^o - T\Delta S_T^o \qquad (3)$$

These calculations are based on the assumption that the measured difference between the specific heat capacity of the protein in the denatured and native state, $\Delta C_p$, is not a function of concentration ($\Delta C_p = \Delta C_p^o$) and not a function of temperature and that the enthalpy of denaturation does not depend on the protein concentration (Table 1). Several accurate DSC measurements performed over broad temperature intervals have indicated that the heat capacity change accompanying denaturation of globular proteins usually depends on temperature. At high temperatures it decreases with temperature to become zero well above 100 °C [3-5]. However, these studies have also shown that no serious error is introduced if $\Delta C_p$ is taken to be constant within the temperature range from about 20 to about 80 °C [3, 6]. The comparatively narrow range of measured $T_d^{DSC}$ values, together with the experimental error of the $(\Delta H_{Td})_{DSC}$ values makes it very unlikely that the small temperature dependence of $\Delta C_p$ can be detected in a $(\Delta H_{Td})_{DSC}$ *versus* $T_d^{DSC}$ plot [7]. By applying the temperature-independent heat capacity approximation, the slope of the plot $(\Delta H_{Td})_{DSC}$ *versus* $T_d^{DSC}$ measured at different conditions of denaturation gave us the corresponding $\Delta C_p$ values that vary substantially with the nature of the denaturant (Table 2). These $\Delta C_p$ values are average values over the measured concentration range obtained for each denaturant from corresponding $(\Delta H_{Td})_{DSC}$ *versus* $T_d^{DSC}$ plot. Due to large scattering of individual $(\Delta H_{Td})_{DSC}$ and $T_d^{DSC}$ points obtained directly from the DSC thermograms the uncertainties in $\Delta C_p$ are rather high. In spite of that, they agree well with the RNase A literature $\Delta C_p$ values that vary from 4.2 kJ/molK [8] to 9.2 kJ/molK [9] depending on the conditions of denaturation. Table 1 lists the thermodynamic quantities of conformational transition of RNase A in different denaturant solutions at 25 °C obtained from eqs. 1-3. It is to be noted that the accuracy of the values $\Delta H^o_{298}$, $\Delta S^o_{298}$,

**Table 1**. Thermodynamic Stabilization Parameters of RNase A Denaturation at 25 °C at Different pH and Different Concentrations of GuHCl, Urea and Alkylurea Solutions.

| | $\Delta H^{o}_{298}$ (kJ/mol) | $T\Delta S^{o}_{298}$ (kJ/mol) | $\Delta G^{o}_{298}$ (kJ/mol) |
|---|---|---|---|
| pH | | | |
| 1.1 | 152 | 150 | 2 |
| 1.5 | 132 | 129 | 3 |
| 2.0 | 173 | 169 | 4 |
| 3.0 | 168 | 146 | 22 |
| 3.5 | 142 | 111 | 31 |
| 7.0-7.4 (distilled water) | 153 | 114 | 39 |
| $C_{GuHCl}$ (M) | | | |
| 1.0 | 113 | 90 | 23 |
| 1.5 | 88 | 73 | 15 |
| 2.0 | 79 | 69 | 10 |
| 2.5 | 89 | 83 | 6 |
| 3.0 | 113 | 110 | 3 |
| $C_{urea}$ (M) | | | |
| 4 | 136 | 117 | 19 |
| 5 | 109 | 96 | 13 |
| 6 | 94 | 85 | 8 |
| 7 | 86 | 81 | 5 |
| 8 | 23 | 21 | 2 |
| $C_{methylurea}$ (M) | | | |
| 2 | 254 | 221 | 33 |
| 3 | 257 | 228 | 29 |
| 4 | 247 | 223 | 24 |
| 5 | 224 | 206 | 18 |
| 6 | 239 | 223 | 16 |
| 7 | 251 | 239 | 13 |
| 8 | 259 | 249 | 10 |
| $C_{N,N'\text{-}Dimethylurea}$(M) | | | |
| 1 | 308 | 269 | 39 |
| 2 | 329 | 294 | 35 |
| 3 | 350 | 319 | 31 |
| 4 | 349 | 322 | 27 |
| 5 | 327 | 305 | 22 |
| 6 | 351 | 330 | 21 |
| 7 | 345 | 327 | 18 |
| 8 | 344 | 330 | 14 |
| $C_{ethylurea}$ (M) | | | |
| 1 | 293 | 256 | 37 |
| 2 | 267 | 240 | 27 |
| 3 | 284 | 261 | 23 |
| 4 | 297 | 278 | 20 |
| 5 | 306 | 292 | 14 |
| 6 | 293 | 283 | 10 |
| 7 | 264 | 259 | 5 |
| $C_{butylurea}$ (M) | | | |
| 0.2 | 127 | 95 | 32 |
| 0.4 | 125 | 97 | 27 |
| 0.6 | 156 | 132 | 24 |
| 0.8 | 126 | 107 | 19 |

The relative error in all reported thermodynamic quantities at 25°C is estimated to be ±20%.

and $\Delta G^{o}_{298}$ suffers from the already mentioned uncertainties in $\Delta C_p$. The $\Delta G^{o}_{298}$ value obtained for denaturation of RNase A in triple distilled water at 25°C is (39 ± 7) kJ/mol and it decreases with decreasing pH. In the presence of GuHCl and urea with all of its derivatives the $\Delta G^{o}_{298}$ of RNase A also decreases with increasing denaturant concentration. For each denaturant a characteristic concentration of a half transition, $c_{1/2}$, can be determined at which the equilibrium constant, $K_{app}$, describing the two-state approximation of the protein folded-unfolded state equilibrium at 25 °C equals to one and the corresponding $\Delta G^{o}_{298}$ equals to zero (Table 2). A number of studies on urea, alkylureas and GuHCl denaturation of proteins have shown that over the concentration range in which the denaturation process can be followed, $\Delta G^{o}_{298}$ varies linearly with the denaturant concentration following the empirical relation [10-12]:

$$\Delta G^{o}_{298} = \Delta G^{o}_{298}(H_2O) - m \cdot c_{den} \qquad (4)$$

in which m is the rate of change of $\Delta G^{o}_{298}$ with denaturant concentration, $c_{den}$, and $\Delta G^{o}_{298}$ ($H_2O$) is the standard Gibbs free energy of denaturation in the absence of denaturant. The physical significance of the factor m is not completely clear, although at least one model suggests that it is related to differences in the amount of the denaturant interacting with the native and denatured states of the polypeptide chain, respectively [13-15]. Thus, m appears to reflect the difference between the accessibility of surface areas of these two states for a given denaturant [13-16] and is therefore believed to be a measure of the compactness of the denatured states.

Inspection of Table 2 shows that for RNase A in all denaturant solutions the $\Delta G^{o}_{298}$ values obtained from DSC data employing eq. 4 decrease linearly with the increasing denaturant concentration. The corresponding characteristic values of $\Delta G^{o}_{298}(H_2O)$, m and $c_{1/2}$ obtained from $\Delta G^{o}_{298}$ *vs.* $c_{den}$ plots are presented in Table 2. Since the high m and low $c_{1/2}$ values indicate high effectiveness of a given denaturant the results presented in Table 2 show that RNase A is effectively denatured only by butylurea and GuHCl. For other denaturants the corresponding $c_{1/2}$ values are very similar and except for urea so high that they exceed their solubility. Similar behavior

has been observed with another globular protein, α-chymotrypsinogen, when its thermal denaturation has been studied in the presence of the same denaturants [12].

**Table 2.** Thermodynamic Characteristics of GuHCl, Urea and Alkylureas Denaturation of RNase A at 25 °C Calculated from DSC Data.

| | $\Delta C_p$ (kJ/mol·K) | $\Delta G^o_{298}(H_2O)$ (kJ/mol) | m (kJ·L/mol$^2$) | $c_{1/2}$ (mol/L) |
|---|---|---|---|---|
| GuHCl | 10.6 | 26.5 | 8.0 | 3.3 |
| Urea | 9.6 | 34.2 | 4.1 | 8.3 |
| Methylurea | 7.6 | 40.0 | 3.9 | 10.3 |
| N,N'-Dimethylurea | 4.8 | 41.7 | 3.5 | 11.9 |
| Ethylurea | 5.0 | 36.4 | 4.4 | 8.3 |
| Butylurea | 10.2 | 36.0 | 21 | 1.8 |

The relative error of all thermodynamic quantities is estimated to be ± 20%.

Inspection of Table 2 shows that the extrapolated $\Delta G^o_{298}(H_2O)$ values determined for RNase A in all the measured denaturants are surprisingly close regarding the large uncertainties in $\Delta G^o_{298}$ values calculated from eqs. 1-3. Interestingly, the value of m obtained from eq. 4 are very close for urea, methyl-, ethyl- and N,N'-dimethylurea indicating that the accessible surface of the unfolded RNase A is similar in solutions of all the measured denaturants except of GuHCl and butylurea. The $\Delta G^o_{298}(H_2O)$ values obtained in GuHCl and urea solutions by linear extrapolation of $\Delta G^o_{298}$ values to zero denaturant concentration are 26.5 and 34.2 kJ/mol, respectively, and are in reasonable agreement with the corresponding values obtained from intrinsic fluorescence intensity measurements at 303 nm [2].

Several studies on protein conformational transitions have shown that the increasing of the heat capacity increment, $\Delta C_p$, and the accompanying increasing of the m value are proportional to the increase in the protein accessible surface area, $\Delta$ASA, that results from the unfolding of the protein [4-5, 17]. The observed $\Delta C_p$ values are believed to be due primarily to the increased hydration of the nonpolar groups that

become exposed to the solvent during the process of the protein denaturation [18]. A recent survey of data describing unfolding of various proteins has shown a strong correlation of ΔASA with m obtained from solvent denaturation with GuHCl and urea and with the corresponding $\Delta C_p$ obtained from thermal denaturation [19]. Another recently published work on the thermal and solvent denaturation of iso-1-cytochrome c and its mutants has shown, however, just the opposite, a lack of correlation of m with $\Delta C_p$ values [20]. This result implies that with iso-1-cytochrome c and its mutants there exists a basic difference in the mechanism of their solvent and thermal denaturation.

## ACKNOWLEDGMENTS

This work was supported by the Ministry of Science and Technology of the Republic of Slovenia.

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## Povzetek

Že objavljene rezultate o vplivu pH, gvanidinijevega hidroklorida (GuHCl), sečnine, metilsečnine, N,N'-dimetilsečnine, etilsečnine in butilsečnine na termično stabilnost ribonukleaze A (RNase A) dobljene z uporabo diferenčne dinamične kalorimetrije (DSC) (N. Poklar, N. Petrovčič, M. Oblak, G. Vesnaver, *Protein Sci.* **1999***8, 832-840*) smo uporabili za izračun termodinamične stabilnosti RNase A pri 25 °C. Ugotovljeno je bilo, da termodinamična stabilnost RNase A pri 25 °C pada z naraščanjem koncentracije denaturanta kot tudi z naraščanjem velikosti hidrofobne skupine substituirane na molekuli sečnine.