

Thermodynamic stability of ribonuclease A in alkylurea solutions and preferential solvation changes accompanying its thermal denaturation: A calorimetric and spectroscopic study¹

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Abstract

The effect of methylurea, *N,N'*-dimethylurea, ethylurea, and butylurea as well as guanidine hydrochloride (GuHCl), urea and pH on the thermal stability, structural properties, and preferential solvation changes accompanying the thermal unfolding of ribonuclease A (RNase A) has been investigated by differential scanning calorimetry (DSC), UV, and circular dichroism (CD) spectroscopy. The results show that the thermal stability of RNase A decreases with increasing concentration of denaturants and the size of the hydrophobic group substituted on the urea molecule. From CD measurements in the near- and far-UV range, it has been observed that the tertiary structure of RNase A melts at about 3 °C lower temperature than its secondary structure, which means that the hierarchy in structural building blocks exists for RNase A even at conditions at which according to DSC and UV measurements the RNase A unfolding can be interpreted in terms of a two-state approximation. The far-UV CD spectra also show that the final denatured states of RNase A at high temperatures in the presence of different denaturants including 4.5 M GuHCl are similar to each other but different from the one obtained in 4.5 M GuHCl at 25 °C. The concentration dependence of the preferential solvation change $\Delta\Gamma_{23}$, expressed as the number of cosolvent molecules entering or leaving the solvation shell of the protein upon denaturation and calculated from DSC data, shows the same relative denaturation efficiency of alkylureas as other methods.

Keywords: CD spectropolarimetry; denatured states; differential scanning calorimetry; preferential solvation changes; ribonuclease A; thermal and solvent denaturation; UV spectroscopy

It is well known that various solvent additives, such as guanidine hydrochloride, urea, and alcohols, affect proteins in different ways, acting as effective probes of their conformations in solutions. Investigations of such conformational changes have provided valuable information about the role of the solvent in the maintaining the native, intermediate, or denatured state of a given protein. Recently, numerous papers studying the influence of different solvents especially alcohols, on the formation of α -helical intermediates have been published with the common goal of better understanding the protein folding pathways (Bychkova et al., 1996; Jayaraman et al., 1996; Kamatari et al., 1996; Hirota et al., 1997). It has been shown by CD spectroscopy at

isothermal conditions that alkylureas similarly to alcohols (Hirota et al., 1997) induce a formation of α -helical secondary structure in β -lactoglobulin A (β -lg) (Lapanje & Kranjc, 1982) known to be a predominantly β -sheet protein and in α -chymotrypsinogen A (α -ctg A) (Lapanje et al., 1981), a protein whose antiparallel pleated β -sheets are significantly distorted (Freer et al., 1970). The influence of alkylureas on the thermodynamic stability of β -lg and α -ctg A has been studied extensively by Poklar and Lapanje (1992) and Poklar et al. (1993, 1995, 1996). It has been found that the thermal and thermodynamic stability of α -ctg A, expressed in terms of the standard free energy of denaturation ΔG_{298}° , decreases with the increasing size of the hydrophobic group substituted on the urea molecules. This result has been interpreted in terms of hydrophobic interactions between the solvent and the nonpolar groups on the protein that increase upon the unfolding of protein molecules. Apparently, depending on their hydrophobicity and concentration denaturants destabilize the protein tertiary structure and in some cases also cause rearrangements and/or distortion of protein secondary structure.

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¹Dr. Nataša Poklar dedicates this paper to the memory of Prof. Dr. Savo Lapanje (1925–1997).

Abbreviations: RNase A, ribonuclease A; GuHCl, guanidine hydrochloride; β -lg, β -lactoglobulin; α -ctg A, α -chymotrypsinogen A; DSC, differential scanning calorimetry; CD, circular dichroism.

In the present work we used the combination of calorimetric (DSC) and spectroscopic techniques (UV spectroscopy and CD spectropolarimetry) to follow the changes in thermodynamic quantities accompanying the thermal denaturation of RNase A at different concentrations of several alkylureas (methylurea, *N,N'*-dimethylurea, ethylurea, and butylurea). The main goal was to determine whether these alkylureas at neutral pH have similar effect on the structural rearrangement of RNase A as they have on β -lg and α -ctg A. For better description of the denaturation efficiency of alkylureas, the pH-, GuHCl-, and urea-induced denaturations of RNase A were included in our investigation, although a number of excellent studies on pH-, GuHCl-, and urea-induced denaturation of RNase A can be found in the literature (Ginsburg & Carroll, 1965; Kugimiya & Bigelow, 1973; Green & Pace, 1974; Makhatadze & Privalov, 1992; Liu & Sturtevant, 1996).

Results and discussion

Thermal denaturation of RNase A followed by DSC and UV spectroscopy

It is well known that calorimetric measurements provide a direct, model independent determination of transition enthalpies. Assuming that the calorimetrically determined enthalpy of denaturation $(\Delta H_{T_d})_{DSC}$ at the temperature of the half transition T_d^{DSC} does not depend on the protein concentration the measured $(\Delta H_{T_d})_{DSC}$ equals the standard enthalpy of denaturation $(\Delta H_{T_d}^\circ)_{DSC}$. Further analysis of DSC thermograms also leads to a model-dependent van't Hoff enthalpy of conformational transition $(\Delta H_{vH}^\circ)_{DSC}$, expressed for a two-state transition as (Privalov & Khechinashvili, 1974):

$$(\Delta H_{vH}^\circ)_{DSC} = 2 \cdot T_d^{DSC} \cdot (RM_2 \Delta C_P)^{1/2} \quad (1)$$

where M_2 is the protein molecular weight and ΔC_P is the difference between the measured specific heat capacity of the protein at T_d^{DSC} and the average value of the specific heat capacities of the native and denatured protein extrapolated to the T_d^{DSC} .

The data obtained from DSC scans of RNase A at different pH and in the presence of different concentrations of various denaturants (Fig. 1) are summarized in Table 1. In the presence of GuHCl, the temperature T_d^{DSC} and the enthalpy of denaturation $(\Delta H_{T_d})_{DSC}$ of RNase A change very fast with increasing denaturant concentration c . The average rate of these changes was $\partial T_d^{DSC} / \partial c = -11.8^\circ\text{C}/\text{M}$ and $\partial(\Delta H_{T_d})_{DSC} / \partial c = -125 \text{ kJ/mol M}$. In 4 M aqueous GuHCl solution RNase A appears to be completely denatured. In urea solutions T_d^{DSC} and $(\Delta H_{T_d})_{DSC}$ of RNase A do not change so fast with the increasing denaturant concentration as they do in GuHCl ($\partial T_d^{DSC} / \partial c = -4.3^\circ\text{C}/\text{M}$ and $\partial(\Delta H_{T_d})_{DSC} / \partial c = -62.1 \text{ kJ/mol M}$). Furthermore, even in 8 M aqueous urea solution, RNase A appears not to be completely denatured. For other denaturants the corresponding average rates of changing of T_d^{DSC} and $(\Delta H_{T_d})_{DSC}$ with denaturant concentration were: for methylurea $\partial T_d^{DSC} / \partial c = -3.3^\circ\text{C}/\text{M}$ and $\partial(\Delta H_{T_d})_{DSC} / \partial c = -25.4 \text{ kJ/mol M}$; for *N,N'*-dimethylurea $\partial T_d^{DSC} / \partial c = -2.9^\circ\text{C}/\text{M}$ and $\partial(\Delta H_{T_d})_{DSC} / \partial c = -9.7 \text{ kJ/mol M}$; for ethylurea $\partial T_d^{DSC} / \partial c = -4.7^\circ\text{C}/\text{M}$ and $\partial(\Delta H_{T_d})_{DSC} / \partial c = -25.5 \text{ kJ/mol M}$ and for butylurea $\partial T_d^{DSC} / \partial c = -17.9^\circ\text{C}/\text{M}$ and $\partial(\Delta H_{T_d})_{DSC} / \partial c = -167.5 \text{ kJ/mol M}$. From these results one can conclude that denaturation effectiveness of alkylureas fol-

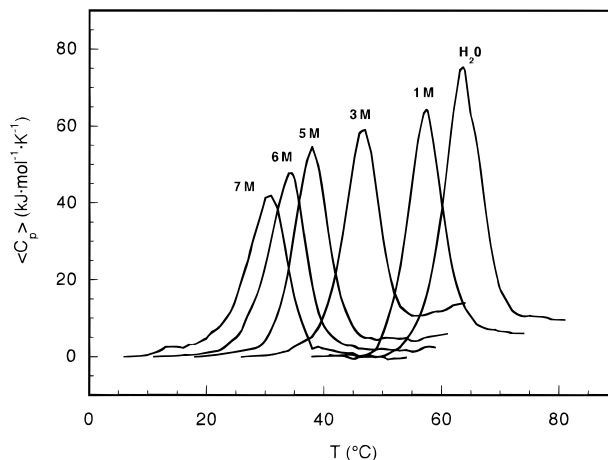


Fig. 1. DSC thermograms (baseline subtracted) of RNase A in triple distilled water at pH 7.0–7.4 (H_2O) and in the presence of different concentrations of aqueous solutions of ethylurea (from 1 to 7 M). Protein concentration was $4 \text{ mg/g}_{\text{solution}}$.

lows the hierarchy: butylurea > ethylurea > methylurea > *N,N'*-dimethylurea and that butylurea is stronger denaturant than GuHCl or urea. Our observations agree well with the results of denaturation studies of RNase A by alkylalcohols, alkylammonium salts, and salts of simple organic acids and bases according to which denaturation effectiveness of denaturants increases with the increasing size of their hydrocarbon groups (Schrier et al., 1965; von Hippel & Wong, 1965; Schrier & Mackey, 1968). pH-Induced denaturation of RNase A shows that decreasing of pH from 7.4 to 1.1 causes its thermal (lowering of T_d^{DSC}) and enthalpic (lowering of $(\Delta H_{T_d})_{DSC}$) destabilization. Acidic pH, however, does not appear to be a very effective denaturant since at the lowest measured pH of 1.1 the measured $(\Delta H_{T_d})_{DSC}$ still retains about 35% of its value in water. Inspection of Table 1 also shows that the measured pH dependence of the heat of denaturation of RNase A is in good agreement with the literature data (Tsong et al., 1970; Fujita & Noda, 1991; Liu & Sturtevant, 1996).

Table 1 lists the values of van't Hoff enthalpies of denaturation $(\Delta H_{vH}^\circ)_{DSC}$ obtained from DSC measurements by applying Equation 1 and the corresponding $(\Delta H_{vH}^\circ)_{UV}$ values obtained from UV-melting curves (Fig. 2). These were determined directly from the slope of the absorbance vs. temperature curve at the temperature of the half-transition T_d^{UV} , using the relation (Marky & Breslauer, 1987):

$$(\Delta H_{vH}^\circ)_{UV} = 4 \cdot R \cdot (T_d^{UV})^2 \left(\frac{\partial f_D}{\partial T} \right)_{T=T_d^{UV}} \quad (2)$$

The degree of the protein denaturation at given temperature f_D was obtained from the measured absorbance A as

$$f_D = \frac{A(T) - A_N(T)}{A_D(T) - A_N(T)} \quad (3)$$

where N and D refer to the native and denatured state, respectively.

Table 1. Thermodynamic parameters of RNase A denaturation at transition temperature at different pH, and at different concentrations of aqueous GuHCl, urea, and alkylurea solutions^a

	$T_d^{\text{DSC b}}$ (°C)	$T_d^{\text{UV b}}$ (°C)	$(\Delta H_{T_d})_{\text{DSC c}}$ (kJ/mol)	$(\Delta H_{\text{vH}}^{\circ})_{\text{DSC c}}$ (kJ/mol)	$(\Delta H_{\text{vH}}^{\circ})_{\text{UV c}}$ (kJ/mol)	$\left(\frac{\Delta H_{T_d}}{\Delta H_{\text{vH}}^{\circ}}\right)_{\text{DSC}}$	Rev. ^{DSC d} (%)
pH							
1.1	28.2	25.9	181	233	258	0.77	77
1.5	32.3	28.7	199	253	257	0.78	99
2.0	37.0	33.0	288	277	274	1.04	80
3.0	50.2	46.1	400	389	364	1.03	80
3.5	59.4	52.7	458	443	394	1.04	73
7.0–7.4 (distilled water)	64.4	62.8	515	486	452	1.06	26
C_{GuHCl} (M)							
1.0	54.3	51.6	424	440	407	0.96	57
1.5	48.4	46.7	336	381	365	0.88	60
2.0	43.0	41.7	270	324	332	0.83	73
2.5	37.1	33.4	217	265	268	0.82	84
3.0	30.5	—	171	227	—	0.75	57
C_{urea} (M)							
2	57.4	55.4	—	—	401	—	—
3	53.6	51.8	364	389	351	0.94	63
4	49.6	47.4	372	369	319	1.01	82
5	45.6	42.7	307	320	306	0.96	84
6	41.2	37.6	250	282	278	0.88	100
7	35.2	32.6	184	239	—	0.77	97
8	32.6	28.6	96	179	—	0.55	—
$C_{\text{methylurea}}$ (M)							
2	55.1	53.2	483	462	473	1.04	73
3	51.1	49.5	455	440	381	1.03	86
4	47.8	45.5	420	421	389	1.00	93
5	44.5	41.8	372	399	357	0.93	97
6	41.5	38.2	364	383	355	0.95	95
7	37.9	34.6	349	366	348	0.95	95
8	35.0	—	335	348	—	0.96	99
$C_{\text{N,N'-dimethylurea}}$ (M)							
1	58.0	56.1	466	466	423	1.00	26
2	53.4	51.3	465	467	421	1.00	31
3	49.8	48.3	469	458	420	1.02	46
4	46.8	44.7	454	453	419	1.00	53
5	44.3	42.2	420	441	427	0.95	68
6	41.7	39.2	431	437	410	0.99	83
7	39.6	36.0	415	416	420	1.00	89
8	36.5	32.8	399	380	405	1.03	86
$C_{\text{ethylurea}}$ (M)							
1	57.3	55.7	454	458	422	0.99	18
2	51.8	49.7	401	428	410	0.94	40
3	46.6	44.7	392	414	408	0.95	45
4	42.1	39.3	382	408	415	0.94	53
5	37.8	34.6	370	395	369	0.94	63
6	33.9	30.5	337	355	360	0.95	68
7	30.6	27.6	292	331	343	0.88	89
$C_{\text{butylurea}}$ (M)							
0.2	60.4	58.9	488	481	414	1.01	—
0.4	56.8	55.2	449	460	418	0.98	—
0.6	53.2	52.1	444	414	423	1.07	35
0.8	49.7	48.3	378	388	376	0.97	48

^aProtein concentration was 4 and 0.5 mg/mL for DSC and UV spectroscopy measurements, respectively. T_d is the transition temperature; ΔH_{T_d} is the enthalpy of transition at the transition temperature; $\Delta H_{\text{vH}}^{\circ}$ is van't Hoff enthalpy. Indexes DSC and UV refer to the data obtained from calorimetry and UV spectroscopy, respectively.

^bEstimated error is ± 0.3 °C.

^cEstimated relative error is $\pm 10\%$.

^dRev.^{DSC} is the reversibility expressed in % (second scan is compared with the first one).

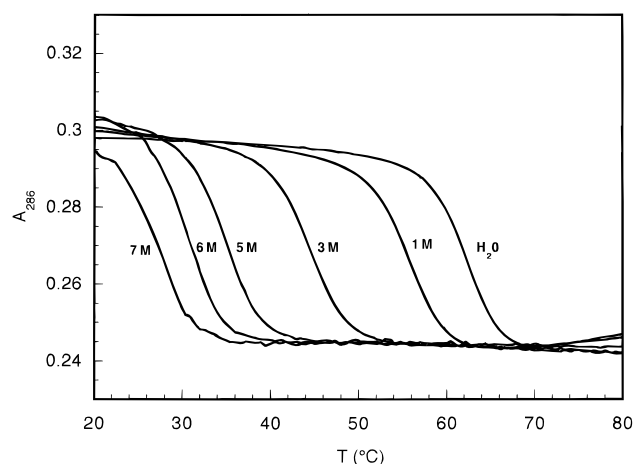


Fig. 2. Absorbance of RNase A at 286 nm, A_{286} , as a function of temperature in triple distilled water at pH 7.0–7.4 (H_2O) and in the presence of different concentrations of aqueous solutions of ethylurea (from 1 to 7 M). Protein concentration was 0.5 mg/g_{solution}.

The van't Hoff enthalpies calculated from the UV-melting curves and DSC thermograms of RNase A at different pH and different concentrations of GuHCl, urea, and alkylureas are in good agreement with the corresponding $(\Delta H_{T_d})_{DSC}$ values obtained from the areas under the measured DSC thermograms. Comparison of the model dependent van't Hoff enthalpy of denaturation ΔH_{VH}^o with the corresponding model independent calorimetric value $(\Delta H_{T_d})_{DSC}$ enables us to decide whether the process of denaturation may be considered as a two-state process or not. Our results show that in aqueous solutions at pH between 2 and 7, in solutions of methyl-, *N,N'*-dimethyl-, ethyl-, and butylurea at all measured denaturant concentrations and in solutions of GuHCl and urea at low denaturant concentration the thermal transitions of RNase A are characterized by $(\Delta H_{T_d})_{DSC} \approx \Delta H_{VH}^o$ and may be therefore considered as two-state transitions. Noticeable deviations from the two-state process, expressed as $(\Delta H_{T_d})_{DSC} < \Delta H_{VH}^o$ were observed in aqueous solutions at pH < 2.0, in GuHCl solutions above 1.5 M and in urea solutions above 6 M. These deviations from a two-state transition appear in all cases at conditions at which the highest reversibility of denaturation process was observed. This means that the failure of the two-state process to describe the denaturation of RNase A at the mentioned specific conditions can be ascribed to the partial denaturation of the protein initial states rather than to the aggregation of the protein molecules in their final denatured states (Haynie & Freire, 1994; Poklar et al., 1997).

Gordon and Jencks (1963) defined an effective denaturant as a noncovalent cross-linking agent, whose effectiveness is based on its ability to form more than one hydrogen bond. Such molecules are perfect for forming hydrogen bonds with different parts of protein molecule and thus for destabilizing its native state. This definition of effective denaturants applies quite well for denaturation of RNase A with GuHCl, urea, methylurea, and *N,N'*-dimethylurea, but according to the observed denaturation effectiveness (Table 1), it does not work for ethylurea and butylurea. Apparently, the denaturation effectiveness of alkylureas with bulkier hydrophobic alkyl groups cannot be described only in terms of denaturant-protein hydrogen bonding but also van der Waals and hydrophobic interaction should

be taken into account. Makhatadze and Privalov (1992) found that increased binding of urea and GuHCl by proteins is accompanied by a significant decrease of binding enthalpy and entropy. Therefore, they have suggested that the protein binding sites for urea and GuHCl are likely to be formed by several hydrogen bonding groups. Recently published X-ray crystal structures of RNase A with GuHCl and dihydrofolate reductase with urea have shown that GuHCl and urea molecules are bound to the protein by hydrogen bonds and van der Waals interactions (Dunbar et al., 1997). The fact that, in spite of their lower ability to form hydrogen bonds, some alkylureas and aliphatic alcohols can be even stronger denaturants than GuHCl or urea means that the ability of these denaturants to change the properties of the solvent (water) and through that the strength of hydrophobic interactions may be at least as important as the ability of GuHCl or urea to form hydrogen bonds. In other words, hydrogen bonding and hydrophobic interactions may be considered as the most important forces contributing to the conformational stability of globular proteins (Pace, 1992; Shirley et al., 1992). As we observe with RNase A, it is the destabilization of these forces caused by the addition of denaturants that leads to the global changes of the protein conformation.

Near- and far-UV CD spectra of RNase A

The changes in tertiary and secondary structure of RNase A as a function of pH, concentration of GuHCl, urea, and alkylureas and temperature can be followed by measuring the protein near- and far-UV CD spectra, respectively.

Figure 3 presents near-UV (panel A) and far-UV (panel B) CD spectra of RNase A as a function of pH in the range from 3.5 to 1.1 in comparison with the corresponding CD spectra of RNase A in triple distilled water (pH = 7.0–7.4). The observed spectra clearly show that the amount of the RNase A tertiary and secondary structure decreases with decreasing pH, which is fully consistent with the data obtained from DSC and UV measurements (Table 1). As we can see from Figure 3A, RNase A possesses at pH 1.1 some tertiary structure indicating that even at very acidic pH this protein unfolds only partially (Fink et al., 1994). Similarly, measurements of $[\Theta]_{275}$ as a function of denaturant concentration clearly show that at 25 °C RNase A retains some of its tertiary structure even at the highest possible concentrations of methyl-, *N,N'*-dimethyl-, and ethylurea (Fig. 4). Thus, to achieve a complete disruption of the RNase A native state in solutions of less effective denaturants, a combination with thermal denaturation is needed. From the CD melting curves resulting from the thermally induced disruption of the RNase A secondary ($[\Theta]_{222}$ vs. T) and tertiary ($[\Theta]_{275}$ vs. T) structure, the corresponding melting temperatures T_d^{222} and T_d^{275} are obtained (Table 2). The T_d^{222} values are in all denaturants for about 3 °C higher than the corresponding T_d^{275} values indicating that the thermally induced disruption of the RNase A secondary and tertiary structure are not overlapping processes. This further means that the thermal denaturation of RNase A cannot be considered as a 100% two-state transition. Similar conclusion was reached by Reinstädler et al. (1996) who observed that during the refolding of RNase A the formation of the secondary structure precedes the formation of stable tertiary contacts and by Houry et al. (1996) who showed, using CD, the existence of burst-phase intermediates in the conformational folding pathway of RNase A.

The measured near UV-CD spectra show (Fig. 5A) that the thermally denatured states of RNase A observed at 80 °C do not possess any tertiary structure in the triple distilled water and in

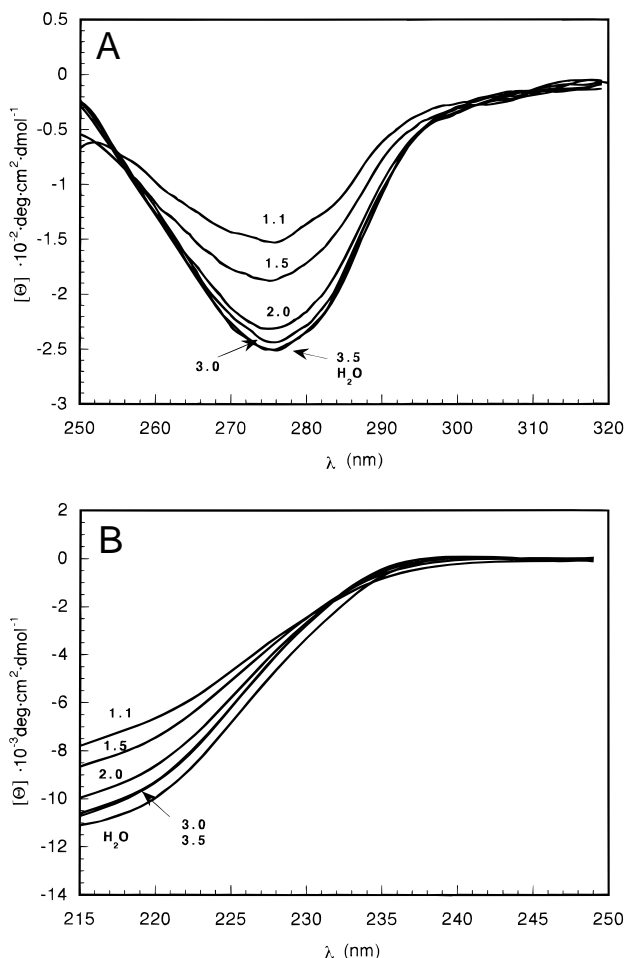


Fig. 3. (A) Near-UV CD spectra and (B) far-UV CD spectra of RNase A as a function of pH from 1.1 to 3.5 and in triple distilled water (H_2O) with pH 7.0–7.4 at 25 °C. Protein concentration was 0.45 mg/mL.

mild denaturant solutions (1 M GuHCl, 3 M and 5 M urea, 3 M methylurea, 3 M N,N' -dimethylurea, 3 M ethylurea, and 0.8 M butylurea). Furthermore, according to the near UV-CD spectra, these states are the same as those observed at 25 °C at high concentrations of urea or GuHCl at which RNase A is considered to be fully denatured. The far-UV CD spectra (Fig. 5B) of RNase A show that the amount of secondary structure observed at 80 °C in triple distilled water is the same as in the above-mentioned mild denaturant solutions. However, regarding the amount of the secondary structure at 80 °C, these final states differ significantly from those observed at 25 °C in 4.5 M GuHCl. As shown in Figure 5B, the far-UV molar ellipticity of RNase A denatured by 4.5 M GuHCl decreases with increasing temperature and at around 80 °C becomes close to the values measured at 80 °C in mild denaturant solutions (Fig. 5B). The effect of temperature on $[\Theta]$ value of RNase A in concentrated GuHCl solution could be explained in terms of reduced GuHCl binding at high temperatures that results from its negative enthalpy of binding (Privalov & Makhatadze, 1992). Since at high denaturant concentrations the bound GuHCl appears to favor a formation of local helices within the unfolded RNase A molecule (Bierzyński & Baldwin, 1982), increased tem-

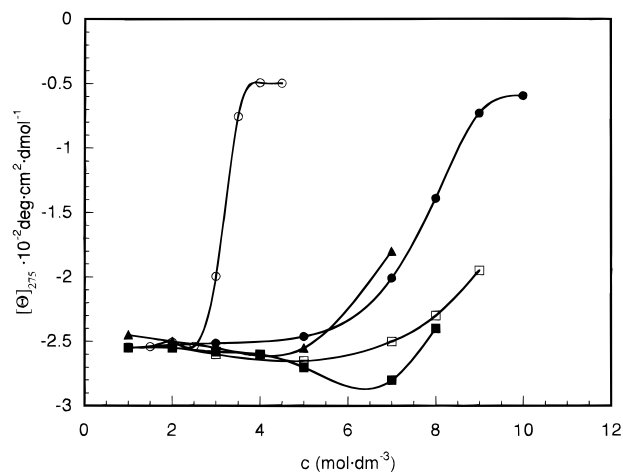


Fig. 4. Changes in molar ellipticity at 275 nm $[\Theta]_{275}$ of RNase A as a function of concentration of GuHCl (○), urea (●), methylurea (□), N,N' -dimethylurea (■), and ethylurea (▲) at 25 °C. Protein concentration was 0.45 mg/mL.

perature accompanied by a reduction in GuHCl binding will lead to a decrease of the content of local helices. Such decrease will be further manifested in changes of ellipticity and only at higher temperatures, the measured far UV-CD spectra will become similar to those observed at high temperatures in other denaturants (Fig. 5B). It is still a matter of discussion whether the use of strong denaturants like GuHCl or urea is more effective in RNase A unfolding than temperature. Using the far-UV CD spectroscopy

Table 2. The denaturation transition temperatures of RNase A in the presence of different denaturants obtained by following the changes in ellipticity as a function of temperature at 275 nm (T_d^{near}) and at 222 or 227 nm (for N,N' -dimethylurea and ethylurea) (T_d^{far})^a

	T_d^{near} (°C)	T_d^{far} (°C)
pH 7.0–7.4 (triple distilled water)	62.6	65.6
GuHCl		
1 M	52.8	55.0
Urea		
3 M	51.4	53.8
5 M	42.5	43.8
Methylurea		
3 M	49.2	51.5
N,N' -dimethylurea		
3 M	48.0	51.1
Ethylurea		
3 M	44.1	48.3
Butylurea		
0.8 M	47.6	51.4

^aProtein concentration was 0.45 mg/mL for following the changes in near-UV (275 nm) and far-UV (222 or 227 nm) CD range. Estimated error in T_d is ± 0.5 °C.

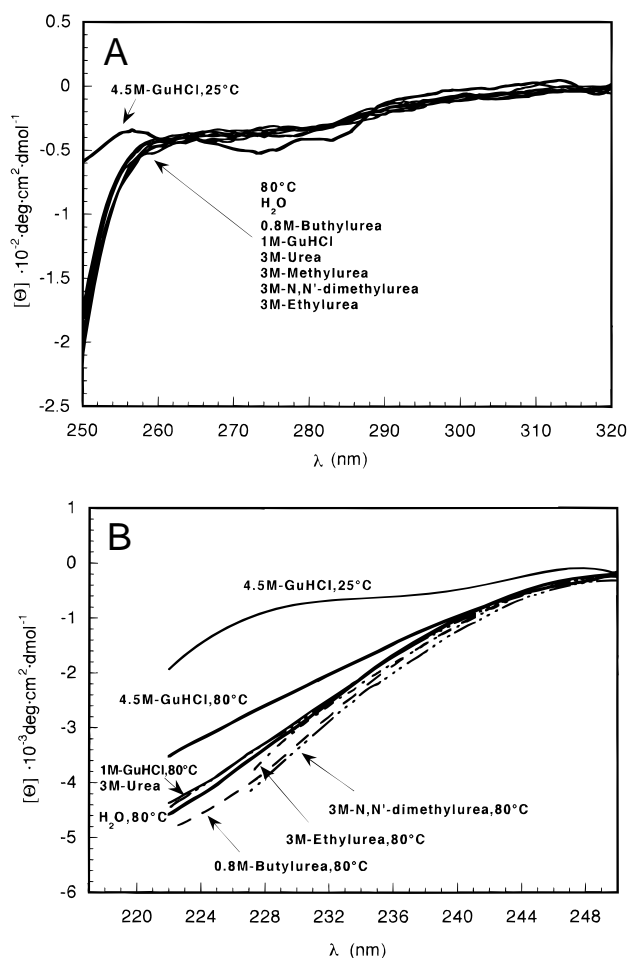


Fig. 5. (A) Near-UV CD spectra and (B) far-UV CD spectra of RNase A at 80°C in 1 M GuHCl, 3 M urea, 3 M methylurea, 3 M *N,N'*-dimethylurea, 3 M ethylurea, and 0.8 M butylurea in comparison with those in 4.5 M GuHCl at 25 and 80°C. Protein concentration was 0.45 mg/mL.

(Labhardt, 1982) has shown that heat denatured RNase A possesses a residual secondary structure that disappears on addition of GuHCl. Further support to this conclusion has been given by Sosnick and Trewella (1992), who have shown on the basis of their small-angle X-ray scattering and Fourier transform infrared spectroscopy data that RNase A in the presence of GuHCl thermally unfolds into much looser structure than in the absence of GuHCl. These results are in excellent agreement with our far-UV CD data, which indicate that only at high temperatures and high denaturant concentrations the final states of RNase A appear to be fully denatured. In contrast, using infrared spectroscopy data on the re-folding kinetics of RNase A, Reinstädler et al. (1996) provide evidence for high structural similarity of urea-denatured and heat-denatured states of the protein. As discussed by Fabian and Mantsch (1995) and Reinstädler et al. (1996), a possible source of this discrepancy could be the existence of native-like regions of secondary structure (e.g., hydrophobic clusters) in the heat-denatured RNase A state that can be detected only by the far-UV CD spectroscopy.

Preferential solvation changes of RNase A upon heat denaturation

Preferential solvation is a purely thermodynamic measure of the relative interaction of solvent i ($i = 1$ or 3) with macromolecules. According to conventional notation component 1 is water, component 2 is the protein, and component 3 is the cosolvent (Scatchard, 1946). The preferential solvation parameter Γ_{23} that originates from the studies of equilibrium dialysis is defined as the number of moles of component 3, m_3 , which should be added to the solution per added mole of component 2, the macromolecule, to restore the chemical potential of component 3, μ_3 (Schellman, 1990).

$$\Gamma_{23} = \left(\frac{\partial m_3}{\partial m_2} \right)_{T, \mu_3} \quad (4)$$

This value has been shown to be the excess number of molecules (moles) of component 3 in the solvation shell of the macromolecule relative to its values in the bulk solvent (Schellman, 1978). Since denaturation of a protein molecule is always accompanied by a change of its surface, one can expect that it will be also accompanied by a corresponding change in the preferential solvation $\Delta\Gamma_{23}$ defined as

$$\Delta\Gamma_{23} = \Gamma_{23}^D - \Gamma_{23}^N \quad (5)$$

where D and N stand for denatured and native state, respectively. Recently, an expansion of Schellman's binding theory (Schellman, 1993, 1994) made possible the calculation of $\Delta\Gamma_{23}$ directly from the measured DSC data (Plaza del Pino & Sanchez-Ruiz, 1995; Kovrigin & Potekhin, 1997; Xie & Timasheff, 1997a, 1997b):

$$\Delta\Gamma_{23} = - \frac{(\Delta H_{T_d})_{\text{DSC}} \cdot \left(\frac{\partial T_d^{\text{DSC}}}{\partial m_2} \right)_{\text{pH}, m_3}}{R \cdot (T_d^{\text{DSC}})^2 \cdot \left(\frac{\partial \ln(a_3)}{\partial m_3} \right)_{T_d^{\text{DSC}}, \text{pH}, m_2}} \quad (6)$$

This formula applies for a reversible transition between two conformational states and gives a denaturational change of preferential solvation of protein (component 2) by denaturant (component 3) at the transition temperature T_d^{DSC} . $(\Delta H_{T_d})_{\text{DSC}}$ is the measured enthalpy of transition at T_d^{DSC} and R is the gas constant. $[\partial T_d^{\text{DSC}} / \partial m_3]_{\text{pH}, m_2}$ term is the rate of variation in T_d^{DSC} on increasing m_3 at constant pH. It can be determined experimentally from T_d^{DSC} vs. m_3 plots only for those denaturant solutions that show practically no pH dependence on the denaturant concentration. The term $[\partial \ln(a_3) / \partial m_3]_{T_d^{\text{DSC}}, \text{pH}, m_2}$ in the denominator of Equation 6 contains activity of denaturants a_3 . Because the protein concentration m_2 was very low, we disregarded its possible influence on the activity coefficients of denaturants and we determined their values from literature data reported for the corresponding binary water-denaturant solutions (Stokes, 1967; Barone et al., 1972).

The $\Delta\Gamma_{23}$ values determined from Equation 6 refer to different T_d^{DSC} values that depend on the denaturant species and denaturant concentration. To enable the comparison between different denaturants regarding the changes of their preferential solvation induced by the unfolding of RNase A, we calculated the $\Delta\Gamma_{23}$ values at 25°C. In these calculations we used the $(\Delta H_{298})_{\text{DSC}}$

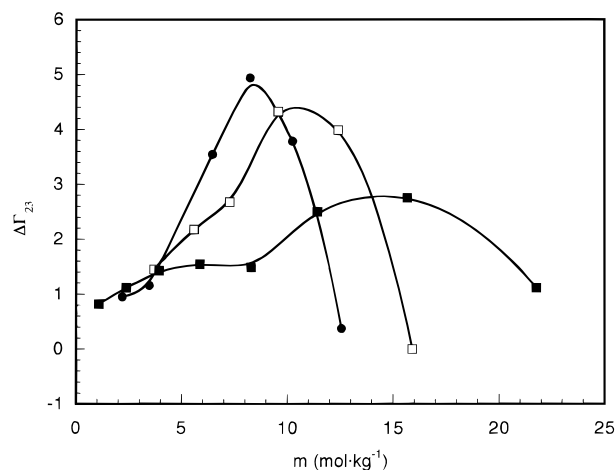


Fig. 6. Preferential solvation changes $\Delta\Gamma_{23}$ of RNase A at 25 °C as a function of molality of aqueous urea (●), methylurea (□), and *N,N'*-dimethylurea (■) solution.

values calculated from the corresponding $(\Delta H_{T_d})_{DSC}$ data,² the experimental $[\partial T_d^{DSC}/\partial m_3]_{pH,m_2}$ terms that appear to be independent of the denaturant concentration, the literature data on molal activity coefficients at 25 °C (Stokes, 1967; Barone et al., 1972), and assumptions that $[\partial \ln(a_3)/\partial m_3]_{T_d^{DSC},pH,m_2}$ and $[\partial T_d^{DSC}/\partial m_3]_{pH,m_2}$ terms are independent of temperature. In urea, methylurea, and *N,N'*-dimethylurea solutions, each of these $\Delta\Gamma_{23}$ values increases with the denaturant concentration, passes a maximum, and starts to decrease (Fig. 6). The observed positive sign of $\Delta\Gamma_{23}$ values indicates that urea and alkylureas have greater affinity for the protein surface exposed upon thermal denaturation. $\Delta\Gamma_{23}$ in urea solutions has a maximum at the molality of about 8 mol/kg_{H₂O} (~6 M) and reaches the zero value at the molality of about 13 mol/kg_{H₂O} (~8.2 M). In methylurea solutions $\Delta\Gamma_{23}$ shows maximum at a little higher concentration (~11 mol/kg_{H₂O}) and reaches zero value at around 18 mol/kg_{H₂O} (~10 M). In *N,N'*-dimethylurea $\Delta\Gamma_{23}$ shows only slightly expressed maximum at very high concentrations (~15 mol/kg_{H₂O}) and does not reach the zero value even at the highest measurable denaturant concentrations, which means that RNase A in solution of *N,N'*-dimethylurea cannot be fully denatured at 25 °C. At this point, it should be noted that the $\Delta\Gamma_{23}$ values extrapolated to 25 °C may be rather unreliable since among the already mentioned approximations their calculation involves the ΔH_{298}° data obtained from experimental $(\Delta H_{T_d})_{DSC}$ values and highly uncertain ΔC_p values. Nevertheless, comparison of the $\Delta\Gamma_{23}$ curves determined at 25 °C for the three denaturants shows good correlation with the thermodynamic characterization of the corresponding denaturant effectiveness presented in Table 1 and Figure 4.

² ΔH_{298}° is the enthalpy of denaturation at 25 °C calculated by applying the following equation: $\Delta H_T^{\circ} = (\Delta H_{T_d})_{DSC} - \Delta C_p(T - T_d^{DSC})$ by using the experimental $(\Delta H_{T_d})_{DSC}$ and T_d^{DSC} data (Table 1), ΔC_p used in this calculation was 10.6 kJ/mol·K for GuHCl, 9.6 kJ/mol·K for urea, 7.6 kJ/mol·K for methylurea, 4.8 kJ/mol·K for *N,N'*-dimethylurea, 5.0 kJ/mol·K for ethylurea, and 10.2 kJ/mol·K for butylurea. These ΔC_p values are average values over the measured concentration range obtained for each denaturant from corresponding $(\Delta H_{T_d})_{DSC}$ vs. T_d^{DSC} plot. The relative error in ΔH_{298}° values at 25 °C is estimated to be $\pm 20\%$.

Materials and methods

Materials

Highly purified lyophilized ribonuclease A type XII-A from bovine pancreas (RNase A) was purchased from Sigma Chemical Co. (St. Louis, Missouri) and used without further purification. Ultrapure urea was a product of Kemika (Zagreb, Croatia). Guanidinium hydrochloride (GuHCl), methylurea, *N,N'*-dimethylurea, ethylurea, and butylurea were supplied by Fluka (Buch, Switzerland). Before use, all ureas and GuHCl were recrystallized from hot ethanol and dried for 48 h in vacuum at 40 °C in the presence of phosphorus pentoxide.

Protein stock solutions in triple distilled water were prepared daily. Protein concentration in aqueous solution at 20 °C was determined by using $E_{1\text{cm}}^{1\%} = 7.38$ at 278 nm (Scott & Scheraga, 1963). Glycine buffers (0.1 M glycine, 0.1 M NaCl/0.1 M HCl) with appropriate pH (1.1, 1.5, 2.0, 3.0, and 3.5) were used.

Differential scanning calorimetry

DSC measurements were performed in Seteram micro DSC calorimeter (Celuire, France) described elsewhere (Lapanje & Poklar, 1989). The sample and the reference cells of optimal volume of 0.8 mL were used. Protein concentration in all DSC experiments was 4 mg/g_{solution}. Calibration of the instrument was performed with special cells using the Joule effects. Thermograms of RNase A in the presence of different concentration of denaturants were recorded over the temperature range between 20 and 80 °C at a heating rate of 0.5 °C/min. The base lines obtained by vessels filled with equal quantities of solvent were subtracted from the thermograms of protein solutions. The area under such corrected thermogram is proportional to the enthalpy of denaturation $(\Delta H_{T_d})_{DSC}$ at T_d^{DSC} at which the ratio between the native and denatured form of the protein is supposed to be one.

Reversibility of the transition was checked by reheating the sample and reference cell (second scan) after cooling them to the room temperature. The degree of reversibility was obtained by comparing the enthalpy of the denaturation of the second scan with the enthalpy of denaturation obtained from the first one.

UV spectroscopy

The UV spectra of RNase A at different pH, in triple distilled water and at different concentrations of GuHCl, urea, and alkylurea solutions were recorded on a Cary 1 UV-visible spectrophotometer equipped with thermoelectrically controlled cell holder (Varian, Australia) using matched 1 cm pathlength quartz cuvettes. Equilibrium thermal unfolding of RNase A was monitored at 286 nm as a function of temperature over an appropriate temperature range (in most cases from 10 to 90 °C) with heating rate of 0.5 °C/min. The wavelength 286 nm was chosen because RNase A contains six tyrosines, two phenylalanines, and no tryptophanes (Scheraga, 1967). The sample cuvettes were filled with protein solution, whereas the reference cuvettes were filled with a pure solvent. The protein concentration in all UV experiments was 0.5–0.6 mg/g_{solution}.

Circular dichroism (CD) spectropolarimetry

CD measurements were performed on an AVIV model 62A DS Spectropolarimeter (AVIV Associates, Lakewood, New Jersey)

equipped with a thermoelectrically controlled cell holder. CD spectra of RNase A in triple distilled water and in the presence of different concentration of GuHCl, urea, and alkylureas were measured in the far-UV range (200–250 nm) in 1 mm and in the near-UV range (250–300 nm) in 10 mm pathlength quartz cuvettes. Spectra were measured at 5 °C intervals in the temperature range from 0 to 95 °C with an averaging time of 3 s, an equilibrium time of 3 min, and a bandwidth of 1 nm. The concentration of protein solutions used for the CD measurements in the near- and far-UV CD range was 0.45 mg/g_{solution}. From each spectrum the spectrum of the solvent was subtracted. The mean residue ellipticity $[\Theta]_{\lambda}$ was calculated by using the following relation:

$$[\Theta]_{\lambda} = \frac{M_o \cdot \Theta_{\lambda}}{100 \cdot c \cdot l} \quad (7)$$

in which Θ_{λ} is the measured ellipticity, M_o is the protein mean residue molar weight, c is the concentration of RNase A in g/mL, and l is the path length in dm. The value of $M_o = 110.5$ g/mol is obtained from MW of RNase A (13.7 kDa) by dividing it with the number of amino acid residues (124) constituting the RNase A molecule (Scheraga, 1967). $[\Theta]_{\lambda}$ was expressed in [deg·cm²·dmol⁻¹].

Conclusion

With the combination of calorimetric (DSC) and spectroscopic techniques (UV spectroscopy and CD spectropolarimetry), the effect of pH, guanidinium hydrochloride (GuHCl), urea, methylurea, *N,N'*-dimethylurea, ethylurea, and butylurea on the thermodynamic stability, structural properties, and preferential solvation changes during the thermal unfolding of ribonuclease A (RNase A) has been investigated. Our results exhibit the following features: (1) the thermal stability of ribonuclease A decreases with increasing concentration of denaturants and the size of hydrophobic group substituted on the urea molecule; (2) the effect of butylurea on thermal and thermodynamic stability of RNase A is more pronounced than that of guanidine hydrochloride, meaning 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; (3) the tertiary structure of RNase A melts at lower temperature 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 is valid; (4) far-UV CD spectra show that the denatured states of RNase A in the presence of different denaturants at higher temperatures are the same but differ from the denatured states that exist at higher concentrations of urea or guanidine hydrochloride at 25 °C; (5) alkylureas do not induce α -helix formation in RNase A ($\alpha + \beta$ type of protein) as they do in β -lactoglobulin (β -sheet protein); (6) the values of $\Delta\Gamma_{23}$ accompanying the process of thermal denaturation of RNase A are positive indicating that all denaturants favor denatured state and they approach $\Delta\Gamma_{23} = 0$ value at concentrations at which RNase A is fully denatured.

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