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INFLUENCE OF ALKYLUREAS ON THE FLUORESCENCE PROPERTIES OF MODEL DIPEPTIDES AND RIBONUCLEASE A

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Abstract: The influence of guanidinium hydrochloride (GuHCl), urea and some alkylureas on the stability of bovine ribonuclease A (RNase A) in aqueous solutions at 25 °C was investigated by measuring the protein intrinsic fluorescence emission as a function of the added denaturant concentration. It was shown that GuHCl is significantly stronger denaturing agent than urea and that in solutions of alkylureas a full RNase A denaturation cannot be achieved even at the highest possible denaturant concentrations. Such behavior was ascribed to lower denaturing efficiency and/or lower solubility of alkylureas. These findings were fully supported by the results of RNase A fluorescence polarization measurements performed in the same denaturant solutions. The fluorescence emission spectra of RNase A were also compared with the corresponding spectra of the model dipeptide containing one tyrosine residue. It was shown that the changes in the RNase A intrinsic fluorescence emission observed at high denaturant concentrations are due primarily to the unfolding of the protein.

INTRODUCTION

It is well known that almost all proteins contain natural fluorophores tyrosine (Tyr), tryptophan (Trp) and phenylalanine (Phe) and that upon excitation at 280 nm where only Tyr and Trp absorb the fluorescence emission is due primarily to Trp [1-3]. The emission spectra of Trp residues are highly sensitive to the polarity of their immediate surrounding and to the presence of all kinds of quenchers. As a result, the

position and the magnitude of Trp emission maximum depends on those factors which affect the exposure of Trp residues to the water phase. In other words, measuring of the Trp fluorescence excited at 280 nm is suitable for following the unfolding equilibria of those proteins that contain Trp alone or Trp together with Tyr. The emission of the other natural fluorophore, Tyr, occurs at around 303 nm and is almost insensitive to solvent polarity. In spite of its high absorption at 280 nm the Tyr emission from most proteins is small and frequently hard to detect. The main reason for that is quenching of Tyr emission by various groups and ions in the Tyr immediate vicinity and by energy transfer from Tyr to Trp residues. The observed weak Tyr emission from proteins thus depends on their three dimensional structure. Consequently, the unfolding of proteins that contain only Tyr residues can be followed by measuring their intrinsic fluorescence emission intensity at 303 nm [1-3].

Bovine ribonuclease A (RNase A) is a protein which has been frequently used, due to its structural simplicity and commercial availability, for studying the influence of various physicochemical agents on the conformational stability and the unfolding - refolding equilibria of proteins. RNase A is a small monomeric enzyme with molecular weight of 13.7 kDa that consists of 124 amino acids. It contains four disulfide bonds and six Tyr residues, three of them being buried within the protein molecule when it is in its native state [4-5]. According to the far-UV CD spectra it shows structural characteristics of $\alpha+\beta$ type proteins [6]. The three-dimensional X-ray structure of RNase A shows that it is a kidney-shaped molecule containing a N-terminal α-helix and two other short helices packed against a central twisted antiparallel β-sheet [7]. In this work we investigated the influence of denaturants GuHCl, urea and some alkylureas on the RNase A stability in aqueous solutions at 25^{o}C by measuring its intrinsic fluorescence as a function of added denaturant concentration. We also performed the same type of measurements on aqueous solutions of model dipeptides containing Tyr or Trp residues. From the comparison of the RNase A and the corresponding model dipeptide emission spectra we tried to estimate the contributions to the observed changes in the protein emission intensity that result from the RNase A unfolding induced by the addition of denaturants.

EXPERIMENTAL PROCEDURES

Materials

Two dipeptides containing tryptophan, Glycyl-L-Tryptophan (Gly-L-Trp) and L-Leucyl-L-Tryptophan (L-Leu-L-Trp) and two dipeptides containing tyrosine, Glycyl-L-Tyrosine (Gly-L-Tyr) and L-Leucyl-L-Tyrosine (L-Leu-L-Tyr) (Fig. 1) and ribonuclease A type XII-A from bovine pancreas (RNase A) were purchased from Sigma Chemical (St. Louis, MO) and used without further purification.

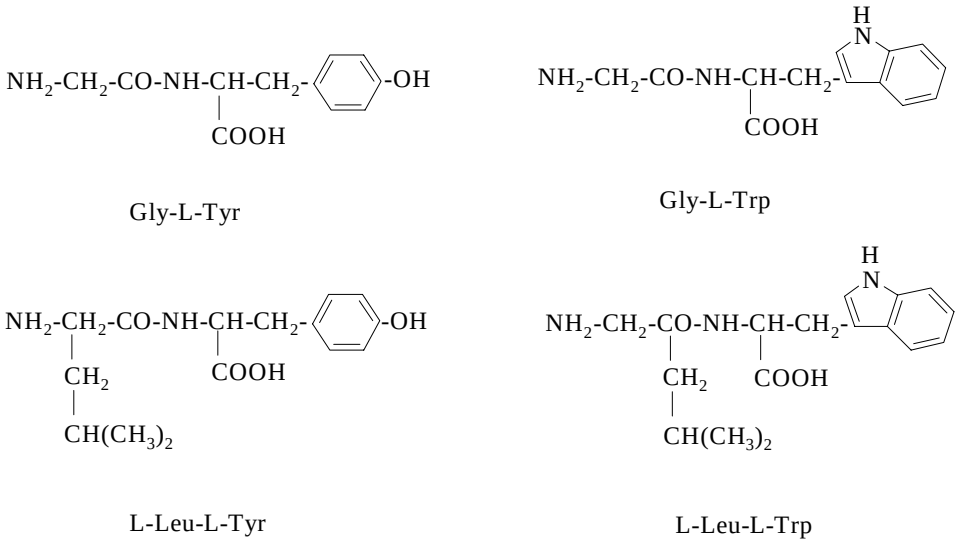

Figure 1**:** *Model dipeptides containing tyrosine or tryptophan residue.*

Ultrapure urea was a product of Kemika (Zagreb, Croatia). Guanidine hydrochloride, methylurea, N,N'-dimethylurea, ethylurea, and buthylurea were supplied by Fluka (Buch, Switzerland). Before use, all ureas and GuHCl were recrystalized from hot ethanol and dried for 48 hours under a vacuum at 40 ^{o}C in the presence of phosphorus pentoxide. Glycine buffers (0.1 M glycine, 0.1 M NaCl / 0.1 M HCl) with appropriate pH (1.1, 3.0, 3.5) and solution of RNase A in triple distilled water (pH = 7.0-7.4) were used.

Aqueous dipeptide stock solutions were prepared by weighing dried dipeptides on a precision analytical balance (Sartorius Research RC 210S, Goettingen, Germany) and dissolving them in a known amount of water. Solutions of dipeptides in aqueous solutions of the desirable denaturant concentration were then prepared by weighing into a given quantity of stock solution appropriate amounts of solid ureas and triple distilled water. The final concentration of dipeptides in all urea and alkylurea solutions was $1.5 \cdot 10^{-5}$ M.

Protein aqueous stock solutions were prepared daily by weighing a proper amount of the dry protein into the triple distilled water. Protein concentration in aqueous solution at 20 ^{o}C was determined spectrophotometrically by using $E_{1cm}^{1\%}$ = 7.38 at 278 nm [8]. From the stock solution of RNase A, solutions of the desirable denaturant concentration were prepared in the same way as the dipeptide solutions. The concentration of RNase A in all solutions used for fluorescence measurements was $1.0 \cdot 10^{-5}$ M.

Intrinsic Fluorescence measurements

Intrinsic fluorescence emission spectra of dipeptides and RNase A in the presence of different concentrations of denaturants were measured on a Perkin-Elmer Model LS-50 Luminescence Spectrometer equipped with a water thermostated cell holder using a 1 cm pathlength quartz cuvette. Slit widths with a nominal band pass of 5 nm were used for both excitation and emission beams. Intrinsic fluorescence emission spectra were recorded in the range from 280 to 460 nm after exciting at 275, 280 or 295 nm. All fluorescence measurements were taken at 20 ^{o}C with the scan rate of 250 nm / min. The fluorescence emission spectrum of pure solvent (background intensity) was always subtracted from the corresponding emission spectrum of the model dipeptide or RNase A. The intrinsic fluorescence spectra were corrected for PM-tube response using fluorescence spectrum of Quinine sulfate ($c = 2.5 \cdot 10^{-7}$M) in 0.1 M solution of perchloric acid as a standard.

Polarization measurements

Fluorescence polarization measurements of protein were made on the same spectrophotometer equipped with an automated polarizing accessory. The excitation and emission slit widths were 5 nm. All measurements were carried out by exciting at 275 nm, while corresponding emission wavelength was 303 nm. The grating correction factor G and degree of polarization P were obtained as [9]

$$G = \frac{F_{HV}}{F_{HH}}; \qquad P = \frac{F_{VV} - GF_{VH}}{F_{VV} + GF_{VH}} \tag{1}$$

where F_{VV}, F_{VH}, F_{HV}, and F_{HH} are the fluorescence intensity components in which the subscripts refer to the horizontal (H) and vertical (V) position of the excitation and emission polarizers, respectively.

Thermodynamic Analysis of Equilibria

Assuming that the protein unfolding is a reversible "all or none" transition, the standard Gibbs free energy of denaturation, ΔG_D^o, can be expressed as

$$\Delta G_D^o = -RT \cdot \ln K_D \tag{2}$$

where the apparent equilibrium constant for the denaturation process, K_D, is given by the equation

$$K_D = \frac{f_D}{f_N} = \frac{y_N - y}{y - y_D} \tag{3}$$

in which y is the measured property (in this study the protein intrinsic emission intensity) and N and D refer to the native and denatured state, respectively. Numerous studies of protein denaturation by denaturing agents such as GuHCl, urea or alkylureas have shown that over the denaturant concentration range in which the denaturation process can be followed, ΔG^o_D varies at a constant temperature linearly with the denaturant concentration as [10-11]

$$\Delta G_D^o = \Delta G_{D,H_2O}^o - m \cdot c_D \tag{4}$$

In this empirical relation $\Delta G^o_{D,H_2O}$ is the standard Gibbs free energy of denaturation in the absence of denaturant, obtained with a linear extrapolation of ΔG^o_D to zero denaturant concentration and factor m is the rate of change of ΔG_D^o with denaturant

concentration, c_D. The validity of the described linear extrapolation has been discussed by a number of authors from both the theoretical [12-14] and experimental point of view [15-16].

RESULTS AND DISCUSSION

Fluorescence emission of model dipeptides containing Tyr or Trp residues.

Measurements of the fluorescence emission spectra of the model dipeptides Gly-L-Tyr, L-Leu-L-Tyr, Gly-L-Trp and L-Leu-L-Trp dissolved in the triple distilled water showed no dependence on the excitation wavelength, λ_{exc} (Table 1). They did show, however, that the fluorescence intensities of Tyr and Trp combined with Leu are higher than those obtained from Tyr and Trp combined with Gly. It seems, that leucine residue which is bulkier than the glycine one creates less polar environment around Tyr and Trp causing their fluorescence to increase. Since both Tyr and Trp are known to increase their fluorescence emission significantly already in slightly less polar solvents [1-2] such explanation seems reasonable.

Table 1: *The wavelength at the emission maximum, λ_{max}, and the corresponding fluorescence intensity, $F_{\lambda_{max}}$, of the model dipeptides in aqueous solutions at 25°C ($c = 1.5 \cdot 10^{-5}$ M) as a function of the excitation wavelength, λ_{exc}.*

	λ_{exc} nm	λ_{max} nm	*$F_{\lambda_{max}}$ arbitrary units
Gly-L-Tyr	275	303.0 ± 1	485
	280	302.0 ± 1	485
	295	305.0 ± 1	6
L-Leu-L-Tyr	275	302.5 ± 1	597
	280	302.0 ± 1	549
	295	-	-
Gly-L-Trp	275	359.5 ± 1	562
	280	358.5 ± 1	570
	295	358.5 ± 1	218
L-Leu-L-Trp	275	359.5 ± 1	892
	280	359.0 ± 1	886
	295	358.5 ± 1	368

*The relative error involved in measurement the fluorescence emission intensity is estimated to not exceed 3 %.

The concentration dependence of the emission intensity at λ_{em} = 358 nm, F_{358}, of 1.5×10^{-5} M Gly-L-Trp in urea and alkylurea solutions relative to the corresponding fluorescence intensity in water, F^{o}_{358}, is shown in Fig. 2A.

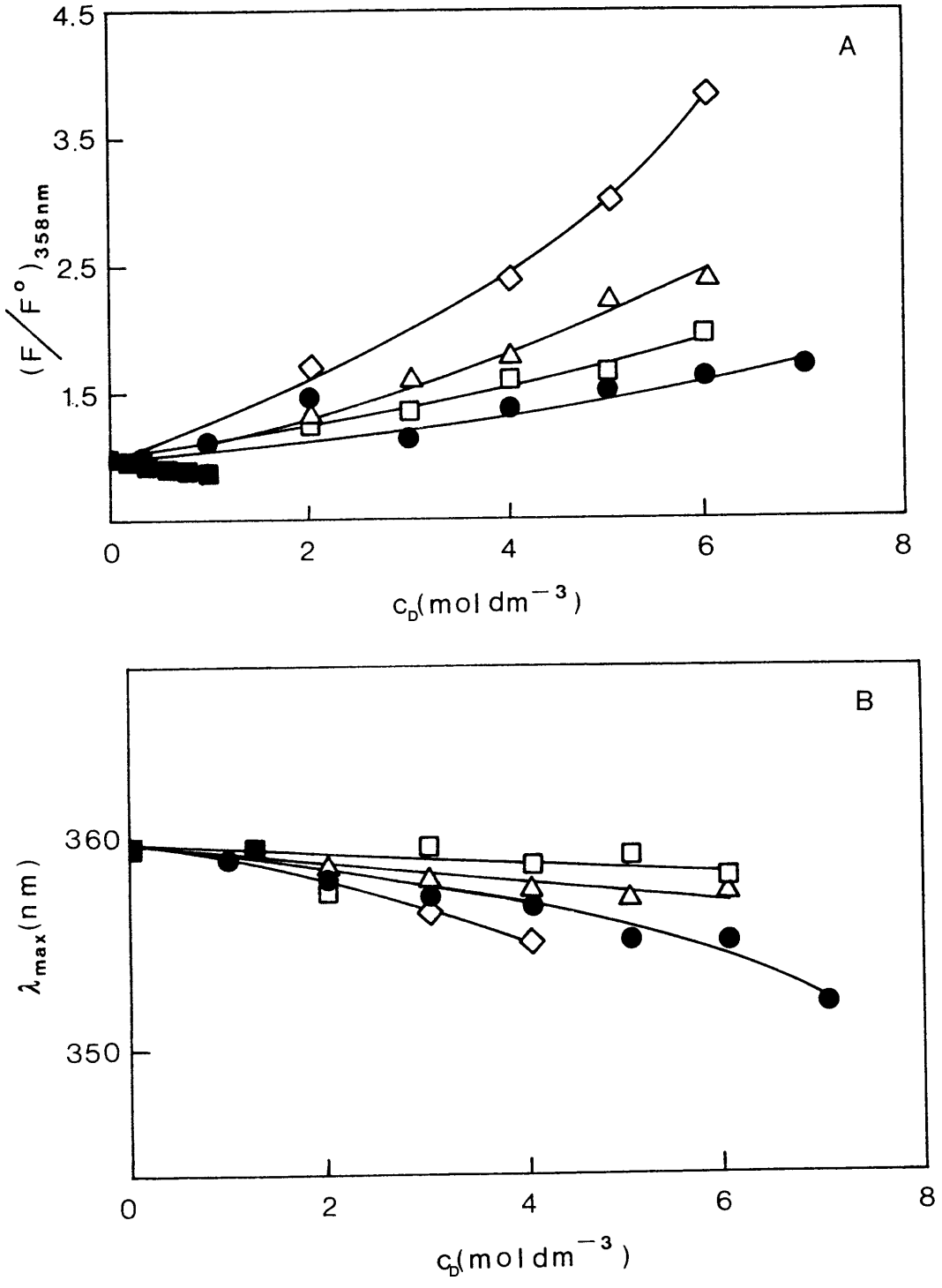

***Figure 2**: The relative fluorescence emission intensity measured at 358 nm $(F/F^o)_{358}$ (Panel A) and the emission maximum wavelength shift λ_{max} (Panel B) of Gly-L-Trp in aqueous solutions($c = 1.5 \cdot 10^{-5}$ M) at 25 °C in the presence of urea ( ), methylurea (Δ), N,N'-dimethylurea (◊), ethylurea (●) and butylurea (■) as a function of denaturant concentration, c_D. λ_{exc} was 295 nm. F^o refers to the fluorescence intensity of Gly-L-Trp in triple distilled water.*

It can be seen that the observed relative fluorescence intensity, $(F/F^o)_{358}$, increases with increasing urea and alkylurea concentration in the following order: butylurea < ethylurea < urea < methylurea. < N,N'-dimethylurea. Similar dependence upon the solvent composition is observed also with the wavelength of the Gly-L-Trp emission maximum, λ_{max}. With increasing denaturant concentration and increasing size of the alkylgroup substituted on the urea molecule λ_{max} shifts toward lower values (Fig. 2B).

For Gly-L-Tyr the relative fluorescence intensities measured at λ_{em} = 303 nm, $(F/F^o)_{303}$, are not so high as for Gly-L-Trp and increase with denaturant concentration only in urea and N,N'-dimethylurea solutions (Fig. 3A). Furthermore, λ_{max} does not shift with increasing concentration or hydrophobicity of alkylureas and stays at 303 nm (Fig. 3B).

Comparison of our results with the literature data on the fluorescence spectra of Tyr and Trp derivatives shows good consistency. The fluorescence emission spectrum of Trp is known to be highly sensitive to solvent polarity and to quenching by variety of reasons [2]. Numerous works have shown that the emission maximum of Trp-derivatives which occur in water at 350-360 nm shifts in less polar solvents to significantly lower values (in hexane ~ 300 nm) [2, 17-18]. In contrast, the reported emission of Tyr and its derivatives occurs in water at around 303 nm and shows no, or very little sensitivity to solvent polarity [2, 18]. Similar behavior of both fluorophores was observed also in this work.

The observed shifts of Trp emission spectra were attributed to the changes of local solvent polarity caused by the addition of urea and its derivatives. With the increasing urea and alkylurea concentration and also with the increasing size of the alkyl groups on the urea molecules the Gly-L-Trp, Gly-L-Tyr, L-Leu-L-Trp or L-Leu-L-Tyr surrounding environment becomes less polar. As a result, the observed emission spectra of Trp-residues which reflect the polarity of their immediate surroundings are shifted toward blue. In contrast, the corresponding spectra of Tyr-residues remain unchanged due to the poor sensitivity of this fluorophore to changes in solvent polarity. The fluorescence intensity studies performed on solutions of Trp, Tyr and some of their derivatives have shown that the fluorescence emission intensity of these

fluorophores in general increases (with Tyr to smaller extent) as the solvent becomes less polar. It has been suggested that the excited singlet states of Trp and Tyr interact with neighboring water molecules to form excited state-water complexes and that such complexation competes with the radiative decay of the fluorophore excited state [2, 18]. Thus, the observed enhancement of the Trp and Tyr fluorescence intensity that accompanies the addition of urea and alkylureas to aqueous solutions of Trp and Tyr dipeptides may be due to a decreased water concentration in the immediate vicinity of the fluorophores. The consequent reduction of the amount of the excited state-water complexes or formation of weaker complexes than those derived from water will lead to a less pronounced nonradiative deactivation of the excited states and thus to an increase in the fluorescence quantum yield.

As already mentioned, a process that competes with the enhancement of the fluorescence emission intensity is fluorescence quenching. This complex process is due to the fluorophore - neighboring molecules interactions such as hydrogen bonding, acid-base chemistry or charge transfer, to name a few. The Tyr fluorescence is known to be quenched by the presence of nearby uncharged amino groups [2]. Apparently, a transfer of protons from the Tyr aromatic hydroxyl groups to these proton acceptors takes place during the lifetime of the excited state leading to a quenching of the tyrosine fluorescence. Therefore, the addition of urea and alkylureas to Tyr aqueous solutions will result in quenching of the emission fluorescence of Tyr-fluorophores. This quenching will compete with the previously described enhancement of fluorescence intensity due to the less aqueous solvent in the immediate vicinity of Tyr fluorophores and the resulting relative fluorescence $(F/F^o)_{303}$ will be lower than the corresponding value observed for Trp. In some solvents the $(F/F^o)_{303}$ value may even drop below 1. Inspection of Figs. 2 and 3 clearly shows that the suggested qualitative explanation for the dependence of fluorescence intensity of Trp and Tyr residues in model dipeptides upon the addition of urea and alkylureas is in good agreement with experimental data.

A variety of reasons have been suggested for the well known absence of tyrosine fluorescence in proteins and one of them is the energy transfer from Tyr to Trp. The efficiency of this transition depends strongly upon the distance between the

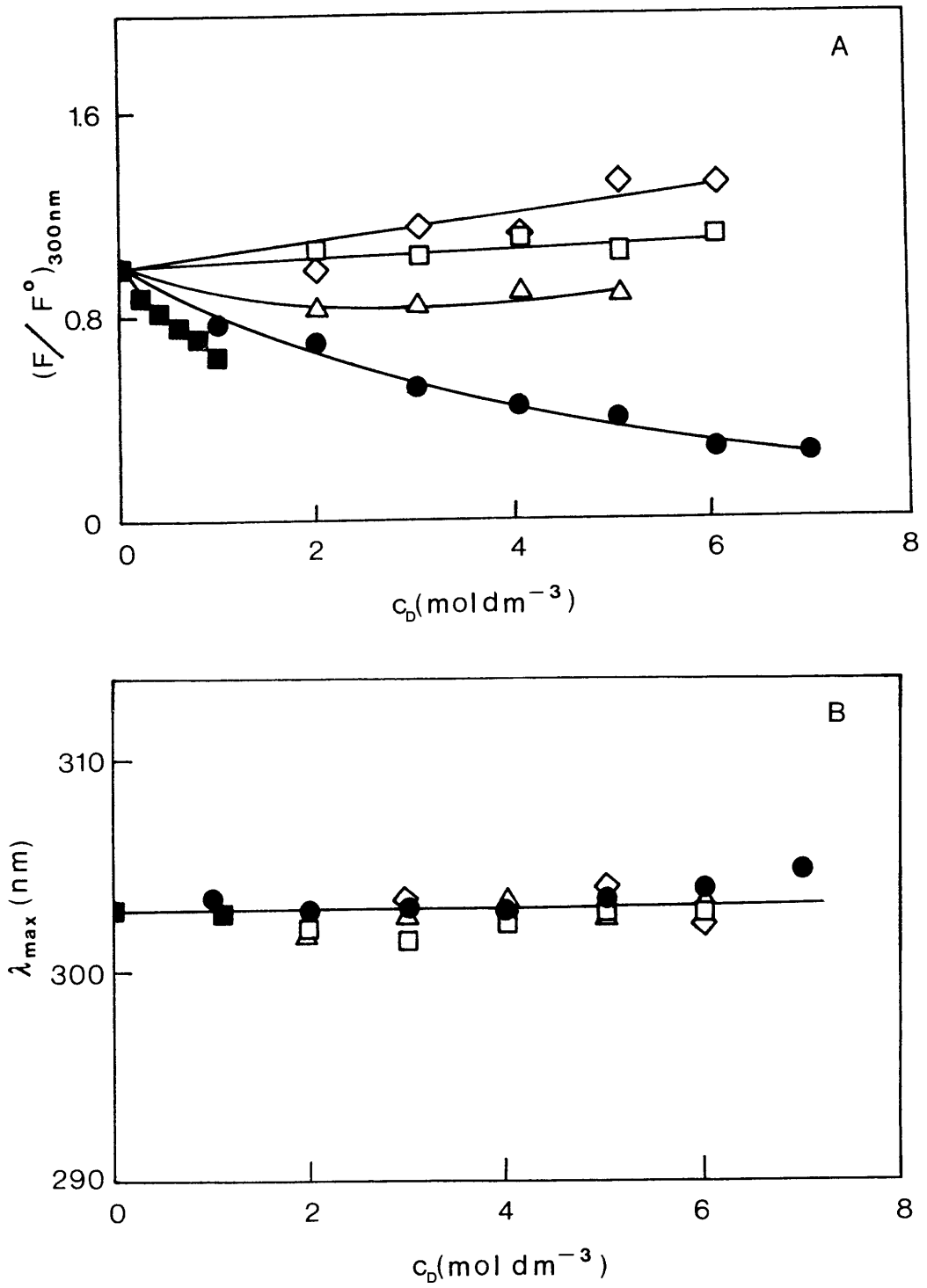

***Figure 3**: The relative fluorescence emission intensity measured at 303 nm $(F/F^o)_{358}$ (Panel A) and the emission maximum wavelength shift λ_{max} (Panel B) of Gly-L-Tyr in aqueous solutions($c = 1.5 \cdot 10^{-5}$ M) at 25 °C in the presence of urea ( ), methylurea (Δ), N,N'-dimethylurea (◊), ethylurea (●) and butylurea (■) as a function of denaturant concentration, c_D. λ_{exc} was 275 nm. F^o refers to the fluorescence intensity of Gly-L-Tyr in triple distilled water.*

Tyr-donors and Trp-acceptors and therefore, as numerous studies have shown, it depends on the three dimensional structure of the protein [1-2]. Consequently, the addition of denaturants, such as urea or alkylureas, to protein solutions is accompanied by changes in the energy transfer efficiencies which are reflected in changes of the measured fluorescence emission intensities. The question is, whether these changes are due only to changes of protein conformation or also to specific interactions of denaturant molecules with Tyr and Trp residues. In an attempt to clarify this question we performed a series of fluorescence measurements in solutions of the model dipeptides, Gly-L-Tyr and Gly-L-Trp, at constant total dipeptide concentration of 10^{-5} M and at Tyr:Trp ratios of 1:1, 1:2 and 11:5 to which different amounts of urea and alkylureas were added. At 1:1 and 1:2 ratios we did not observe any difference between the measured spectra and those calculated using the principle of additivity of pure Gly-L-Tyr and Gly-L-Trp spectra at the corresponding concentrations. At ratio of 11:5, however, the difference between the measured and calculated spectra is noticeable (Fig. 4).

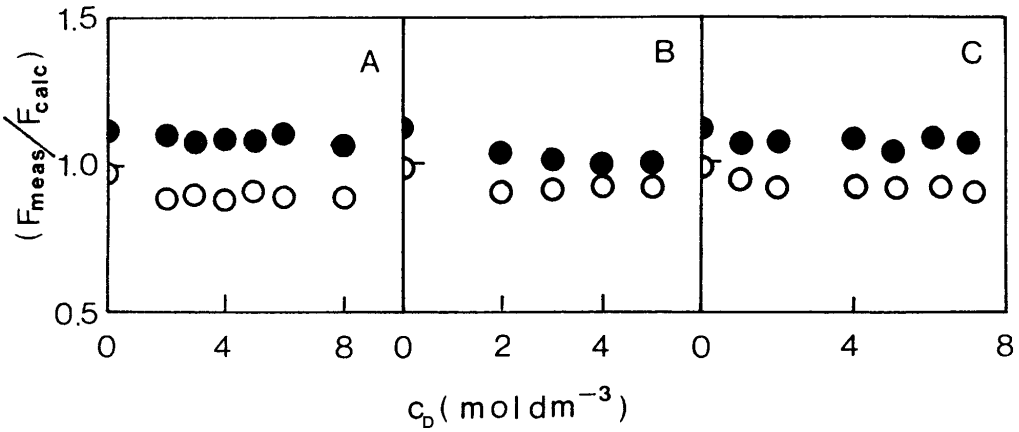

Figure 4: *The energy transfer from Tyr (O) to Trp (●) at 25 °C in aqueous solutions of the total dipeptide concentration of $1.5 \cdot 10^{-5}$ M and Gly-L-Tyr to Gly-L-Trp ratio of 11 to 5 in the presence of urea (Panel A), N,N'-dimethylurea (Panel B) and ethylurea (Panel C). λ_{exc} was 275 nm. The fluorescence emission intensity, F_{meas} for Tyr in the mixture solution was measured at 303 nm and for Trp at 358 nm, respectively. These intensities were compared to the corresponding values, F_{calc}, calculated for the Gly-L-Trp : Gly-L-Tyr ratio of 11:5 from the pure spectra of Gly-L-Trp and Gly-L-Tyr.*

The comparison of the measured and calculated fluorescence intensities shows that the measured Tyr emission at 303 nm is for about 10% lower and the measured Trp emission at 358 nm for about 10% higher than the corresponding emission intensities calculated using the principle of additivity. Inspection of Fig. 4 further shows that at the given experimental conditions the observed Try-to-Trp energy transfer of about 10% does not depend on the urea or alkylurea concentration and is thus not sensitive to any specific interactions between Tyr or Trp residues and urea or alkylurea molecules. This result is irrelevant for RNase A since it does not contain any Tyr residues, however, it should be taken into account in denaturation studies followed by fluorescence of those proteins that contain both Tyr and Trp.

Fluorescence emission and fluorescence polarization of RNase A.

Upon excitation at 275 nm, the fluorescence emission of RNase A in urea solutions increases with increasing urea concentration while the position of emission maximum (λ_{max} = 303 nm) remains unchanged (Fig. 5).

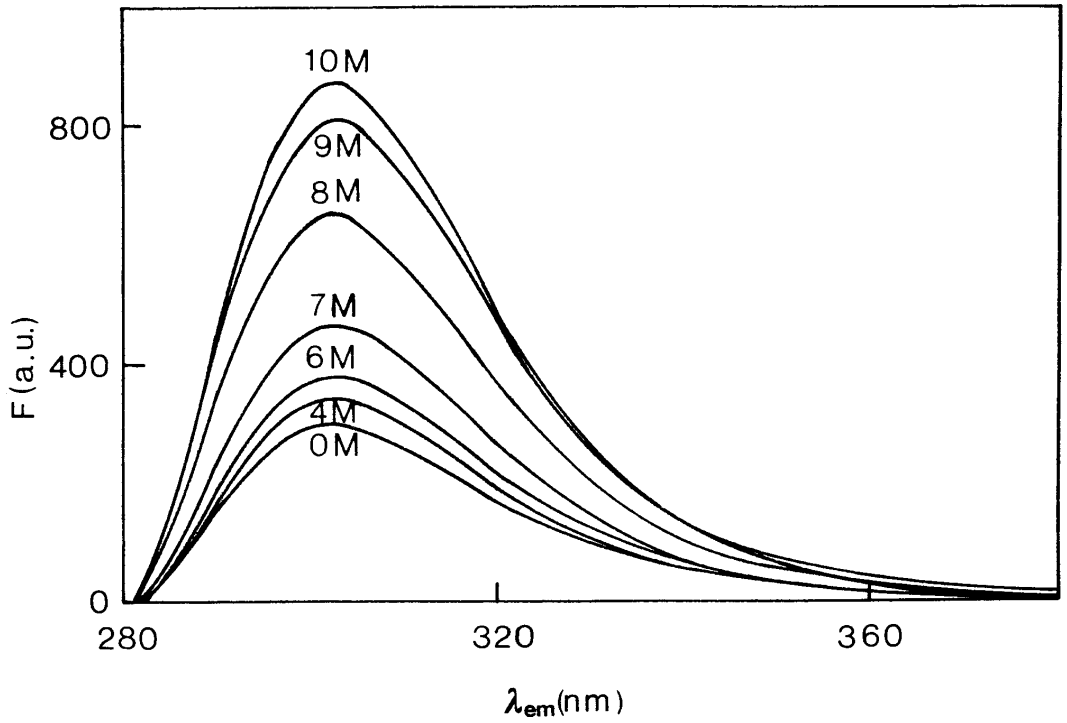

Figure 5: *The fluorescence emission spectra of RNase A (c = $1.0 \cdot 10^{-5}$ M) in the aqueous solutions of urea at urea concentrations between 0 to 10 M; λ_{exc} = 275 nm, F the fluorescence intensity in arbitrary units.*

Clearly, the unfolding of RNase A that occurs at high urea concentrations results in increased average exposure of the protein Tyr residues to the water phase. Because of that, quenching of Tyr by the nearby peptide bonds and charged and uncharged carboxyl and amino groups is reduced and as a result an increase in the fluorescence emission is observed. Similar dependence of RNase A fluorescence emission on denaturant concentration was observed also in solutions of GuHCl and some alkylureas. As can be seen from Fig. 6 where the relative fluorescence emission, $(F/F^o)_{303}$, is presented as a function of denaturant concentration, the unfolding of RNase A seems to be completed only in urea and GuHCl solutions. In solutions of methyl- and N,N'-dimethylurea which are obviously less efficient denaturants than GuHCl or urea only the beginning of RNase A unfolding can be observed at the highest possible denaturant concentrations.

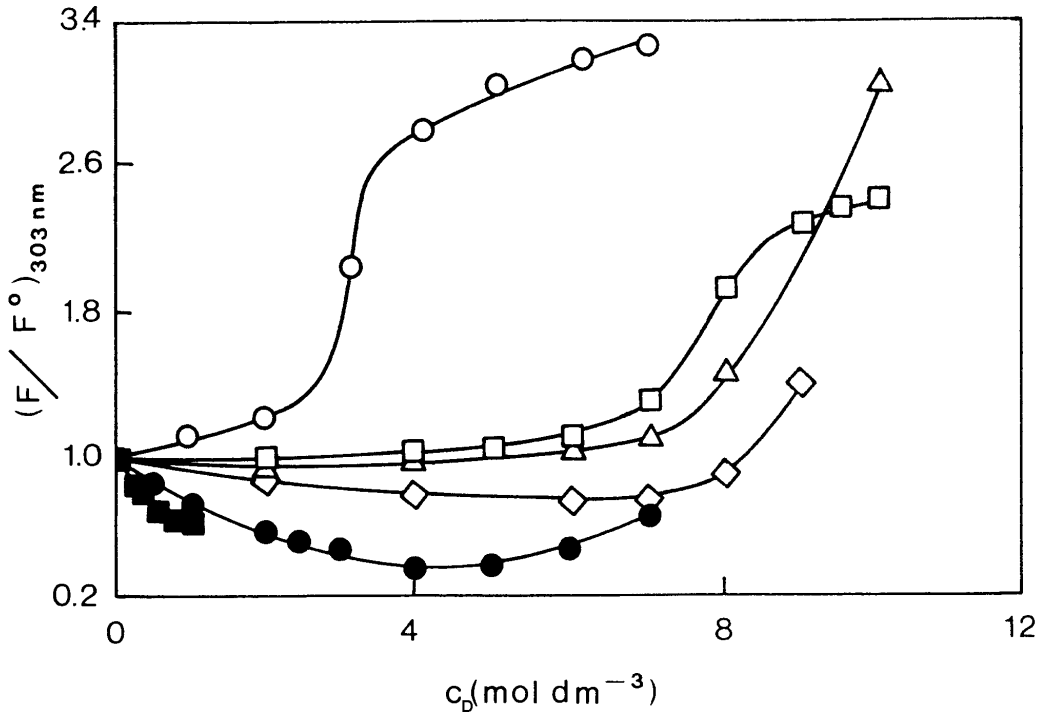

***Figure 6**: The relative fluorescence emission intensity of RNase A (c = 1.0·10^{-5} M) at 303 nm and 25 °C, $(F/F^o)_{303}$, in the presence of GuHCl (O), urea ( ), methylurea (Δ), N,N'-dimethylurea (◊), ethylurea (●) and butylurea (■) as a function of denaturant concentration. λ_{exc} was 275 nm. F^o refers to the fluorescence intensity of RNase A in triple distilled water.*

Furthermore, in buthylurea solutions in which very high denaturant concentration cannot be reached due to the solubility problem nothing but decreasing of $(F/F^o)_{303}$ with increasing denaturant concentration is observed (Fig. 6). This decreasing is also observed at low denaturant concentration in N,N'-dimethylurea and ethylurea solutions and is more pronounced with alkylureas possessing larger hydrophobic groups. It seems, that it is caused by some additional quenching of Tyr fluorescence by denaturant molecules. Evidently, the measured fluorescence emission dependence on the denaturant concentration results from the competition of two opposing effects; the increasing of the fluorescence emission due to increasing exposure of Tyr residues to the aqueous phase and the decreasing of the fluorescence emission due to the increased quenching by the denaturant molecules. The existence of this second contribution is confirmed by the results presented in Figs. 3A and 6 which show that at low denaturant concentrations the fluorescence of the RNase A Tyr residues and the corresponding fluorescence of Tyr residues in the model dipeptide Gly-L-Tyr are similarly affected by urea and alkylureas.

The effect of GuHCl, urea and methylurea on the fluorescence polarization of RNase A is presented in Fig. 7. Obviously, the measured fluorescence is sensitive to RNase A conformational changes, although this sensitivity is less pronounced than with fluorescence emission intensity. In fact, only the RNase A conformational changes induced by addition of GuHCl, urea and methylurea can be followed by measuring the accompanying changes in the protein fluorescence polarization. As shown in Fig. 7 the polarization of RNase A fluorescence in solutions of GuHCl, urea and methylurea decreases with increasing denaturant concentration indicating an increase in the flexibility of Tyr side chains and an increase in the randomization of the RNase A tertiary structure.

Comparison of the results presented in Figs. 6 and 7 further shows that the denaturant concentration region in which according to fluorescence emission measurements the protein undergoes conformational transition overlaps for GuHCl, urea and methylurea with the denaturant concentration region in which changes in fluorescence polarization are observed.

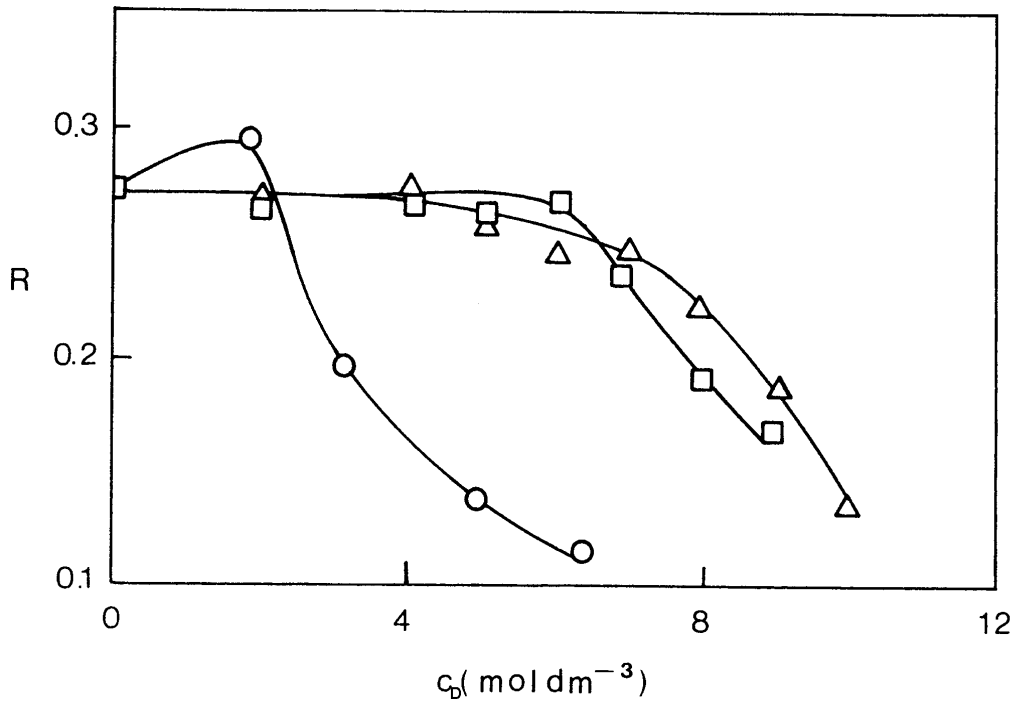

***Figure 7**: The fluorescence polarization of RNase A in aqueous solutions (c = 1.0·10^{-5} M) at 303 nm and 25 °C in the presence of GuHCl (O), urea ( ) and methylurea (Δ) as a function of denaturant concentration, c_D. λ_{exc} = 275 nm, λ_{em} = 303 nm.*

Thermodynamic analysis of RNase A fluorescence emission data.

Assuming that the unfolding of RNase A is a two state process the corresponding equilibrium constants and ΔG^o_D values can be calculated from eqs. 2 and 3. Using these values we were able to show that over the GuHCl or urea concentration range in which the RNase A denaturation could be followed the calculated ΔG^o_D varies linearly with the denaturant concentration as predicted by eq. 4. The characteristic values of $\Delta G^o_{D,H2O}$, the denaturant concentration $c_{1/2}$ at which half of RNase A molecules are unfolded and at which $\Delta G^o_D = 0$ and factor *m* are presented in Table 2. The $c_{1/2}$ value in GuHCl ($c_{1/2}$ = 3.0 M) is lower than in urea ($c_{1/2}$ = 7.6 M) indicating that GuHCl is a more efficient denaturant than urea. With other denaturants the fully denatured state of RNase A could not be reached because of their less pronounced denaturation abilities and/or to low solubilities. Inspection of Table 2 shows that values of the standard Gibbs free energy of denaturation in water at 25 °C, $\Delta G^o_{D,H2O}$, obtained in GuHCl and urea solutions by linear extrapolation of ΔG^o_D values

to zero denaturant concentration are 27.5 and 28.0 kJ/mol, respectively. Although such extrapolations from regions of high concentrations are rather unsafe, the agreement of $\Delta G^{o}_{D,H2O}$ values determined from solutions of both denaturants is excellent. Furthermore, these $\Delta G^{o}_{D,H2O}$ values are very close to the corresponding $\Delta G^{o}_{D,H2O}$ values obtained in GuHCl (26.5 kJ/mol) and urea (34 kJ/mol) solutions from DSC measurements [19]. Another quantity that may be used for the characterization of the denaturation efficiency of a given denaturant is the factor *m* appearing in eq. 4. Its physical significance is not completely clear although according to several studies [12-16] it may reflect the difference between the accessibility of the surface areas of the denatured and native state for a given denaturant. In other words, its value may be considered as a measure of the compactness of the protein denatured state. According to such characterization GuHCl with its high *m* value of 9.2 can be considered as much stronger denaturing agent of RNase A than urea whose *m* value is only 3.7. It is to be noted that similar results were obtained also from DSC studies of RNase A denaturation from which *m*-values of 8.0 for GuHCl and 4.1 for urea were derived [19].

Table 2: *Thermodynamic characteristic of solvent denaturation of RNase A at 25°C in aqueous GuHCl and urea solution obtained from fluorescence intensity measurements at 303 nm by applying the eqs. 2 - 4;* λ_{exc} *was 275 nm.*

	$\Delta G^{o}_{D,H2O}$ (kJ/mol)	m (kJ·L/mol^2)	$c_{1/2}$ (mol/L)
GuHCl	27.4 ± 1.5 (26.5 ± 5)	9.2 ± 0.6 (8.0 ± 1.6)	3.0 ± 0.4 (3.3 ± 0.6)
Urea	28.0 ± 0.2 (34.2 ± 7)	3.7 ± 0.2 (4.1 ± 0.8)	7.5 ± 0.5 (8.3 ± 1.6)

The data in parenthesizes are from DSC measurements [19].

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POVZETEK

Vpliv guanidinijevega hidroklorida (GuHCl), sečnine in nekaterih alkil sečnin na stabilnost ribonukleaze A (RNase A) v vodnih raztopinah pri 25 °C smo raziskovali z merjenjem lastne emisijske fluorescence proteina v odvisnosti od koncentracije dodanega denaturanta. Pokazali smo, da je GuHCl znatno močnejši denaturant od sečnine in da v raztopinah alkil sečnin ne moremo doseči popolne denaturacije RNase A celo pri najvišjih možnih koncentracijah denaturanta. Vzrok za takšno obnašanje smo pripisali nižjim denaturacijskim sposobnostim in / ali nižjim topnostim alkil sečnin. Rezultati merjenja emisijske fluorescence se za RNase A v raztopinah omenjenih denaturantov dobro ujemajo z rezultati merjenj odgovarjajoče fluorescenčne polarizacije, ki smo jih izvedli pri enakih pogojih merjenja. Fluorescenčne emisijske spektre RNase A smo primerjali tudi z ustreznimi izmerjenimi spektri modelnega dipeptida, ki vsebuje eno tirozinsko skupino. Pokazali smo, da so spremembe lastne emisijske fluorescence RNase A, ki jih opazimo pri visokih koncentracijah denaturantov v prvi vrsti posledica razvitja proteina.