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Fluorescence studies of the effect of pH, guanidine hydrochloride and urea on equinatoxin II conformation

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Abstract

The solvent denaturation of equinatoxin II (EqTxII) in aqueous solutions of urea, guanidine hydrochloride (Gu-HCl) and at various pH values was examined by monitoring changes in the protein intrinsic emission fluorescence spectra and in the fluorescence spectra of the added external probe ANS. It has been observed that EqTxII denaturation is reflected in a strong red shift of intrinsic fluorescence emission maxima accompanied by a simultaneous decrease in fluorescence intensity and that guanidine hydrochloride is significantly more powerful denaturant than urea or changing of pH. Comparison of intrinsic fluorescence spectra of EqTxII denatured by one of the three denaturing agents has shown that the fully denatured states of the protein in Gu-HCl and urea are similar and substantially different from those induced by changing of pH. Furthermore, according to the measurements of the ANS-fluorescence in EqTxII solutions as a function of pH the protein exists at pH values below 2.0 in an acid-denatured compact state.

Keywords: Equinatoxin II; Toxin; A pore forming protein; Conformation; Fluorescence measurements; Protein denaturation

1. Introduction

Equinatoxin II (EqTxII), a protein isolated from the sea anemone *Actinia equina* L. is a lethal and cytolytic toxin with molecular mass of 19.8 kDa and p*I* 10.5 [1,2]. Determination of its primary structure, which consists of 179 amino acid residues, revealed relatively high contents of aromatics. It contains five tryptophan (Trp) and eleven tyrosine (Tyr) residues [3] that can serve as intrinsic fluorophores reporting changes in local environment. It was estimated by CD measurements [3] that native EqTxII contains between 29–33% of α helical, 53–58% of β strand + β turn and 10–16% of random structure. Tertiary structure of EqTxII has not been determined yet.

As shown by Belmonte et al. [4] EqTxII associates with native and model lipid membranes forming cation selective pores. The rate of toxin-induced permeabilization was found to be pH dependent with a maximum increase at around pH 9. Recently, several studies performed on some other membrane penetrating proteins have shown that a partial unfolding of protein molecules occurring only at certain conditions (low, high pH) and their ability to penetrate the membranes are closely related [5–15]. Thus, one may expect that also for EqTxII a loosening of its tertiary structure will lead to an enhanced membrane penetration. Consequently, when studying the EqTxII membrane penetrating abilities one should first answer the question whether EqTxII can exist in solution in partially denatured states which upon interacting with the membranes increase their permeability by opening discrete ionic channels.

The purpose of this study was to investigate by means of fluorimetry the changes in EqTxII structure caused by varying denaturation conditions (acidic and alkaline pH and increasing concentration of strong denaturants such as guanidine hydrochloride or urea). The fact that Trp fluorescence depends strongly on the local environment of the residues in the protein molecule [16–23] enables us to follow the changes in Trp environment during the unfolding process. In addition, a fluorescent dye 1-anilinonaphthalene-8-sulfonate (ANS) was used as an external probe to detect the protein compact intermediate states [24] that may appear during denaturation.

Abbreviations: EqTxII, equinatoxin II; Gu-HCl, guanidine hydrochloride; ANS, 1-anilinonaphthalene-8-sulfonate; Trp, tryptophan; Tyr, tyrosine; Gly, glycine; Asp, aspartic acid; Glu, glutamic acid.

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2. Material and methods

2.1. Material

EqTxII was purified from the sea anemone *Actinia equina* L. and assayed as described previously [2]. Ultrapure urea was purchased from Kemika (Zagreb, Croatia) and guanidine hydrochloride (Gu-HCl) was a product of Fluka (Buchs, Switzerland). They were recrystallized from hot ethanol before use. Glycine buffer solutions were prepared by the standard procedure.

A series of dilute protein solutions with equal EqTxII concentration ($c = 5 \cdot 10^{-5}$ $g/g_{solution}$) at various pH values or at different denaturant concentrations were daily prepared from defrozen aqueous protein stock solution by weight using a precise analytical balance. The time elapsed between the preparation of the solution and the measurement was always the same (3.5 h).

Solutions used in ANS fluorescence measurements were prepared by adding 5 μl of ANS-stock solution ($c = 3.8 \cdot 10^{-3}$ M) to 2 ml of protein solutions to obtain solutions in which the protein was always saturated with the bound ANS as shown by unchanged ANS spectra accompanying further additions of ANS stock solution to the protein solution. Ammonium salt of ANS, a product of Fluka (Buchs, Switzerland), was used without additional purification.

2.2. Fluorescence measurements

All fluorescence measurements were performed in a Perkin-Elmer Model LS-50 Luminescence spectrometer with a water thermostated cell holder using a 1 cm path length quartz cuvette. The instrument was controlled with a personal computer. Slit widths with a nominal band pass of 5 nm were used for both excitation and emission beams. Intrinsic fluorescence emission spectra were recorded from 290 to 500 nm after exciting at 275, 280 and 295 nm. Excitation wavelengths 275 and 280 nm are commonly used for measuring the total protein fluorescence spectrum (Trp and Tyr fluorescence) and the excitation wavelength 295 nm is used to obtain the Trp fluorescence spectrum [16,21,23]. The wavelength at maximum emission intensity, λ_{max}, and the emission intensity values at $\lambda = 332$ nm and at $\lambda = 360$ nm were evaluated in each case from the scans.

With ANS fluorescence measurements an excitation wavelength of 365 nm was used and the emission spectra were recorded in the range from 380 to 580 nm. Changes of these emission spectra induced by the EqTxII conformational changes were followed either through corresponding changes in λ_{max} or by measuring the emission intensities at $\lambda = 483$ nm.

Temperature at which all fluorescence measurements were taken was 20°C and the scan rate was 250 nm/min. Background intensity in the samples without protein was always subtracted and 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.

2.3. Polarization measurements

Measurements of the fluorescence polarization were performed with the same samples using the same instrument (equipped with automated polarizing accessory) as for fluorescence emission measurements. The excitation and emission slit widths were 5 nm. Excitation wavelength of 295 nm and emission wavelength of 338 nm were used. The grating correction factor G and the degree of polarization P were obtained as [16,22]

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

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

2.4. Thermodynamic analysis of equilibria

Assuming that protein unfolding is a two-state process the corresponding standard Gibbs free energy of unfolding, ΔG_U^o, can be given as

$$\Delta G_U^o = -RT \ln K_U \tag{2}$$

where K_U is the apparent equilibrium constant for the unfolding process. When unfolding can be followed by monitoring the change in some parameter X, K_U takes the form

$$K_U = \frac{X_N - X}{X - X_U} \tag{3}$$

in which X_N and X_U represent the values of X for the native and unfolded state, respectively.

A number of different studies on urea and Gu-HCl denaturation of proteins have shown that over the concentration range in which the denaturation process can be followed, ΔG_U^o varies linearly with the denaturant concentration as [25–28]

$$\Delta G_U^o = \Delta G_{U,H_2O}^o - mc_{den} \tag{4}$$

where m is the rate of change of ΔG_U^o with denaturant concentration (c_{den}) and $\Delta G_{U,H_2O}^o$ is the standard Gibbs free energy of unfolding in the absence of denaturant.

3. Results

3.1. Intrinsic fluorescence and polarization

3.1.1. Effect of pH

Fluorescence emission spectra obtained for EqTxII in aqueous solutions are typical of those generally observed

for Trp-containing proteins [16–23], despite of relatively high content of Tyr residues within the studied protein molecules. In the range of the excitation wavelengths, λ_{ex}, between 270 and 300 nm the emission wavelength at the maximum intensity, λ_{max}, remains practically constant. Its value $\lambda_{max} = 332 \pm 1$ nm obtained in pure aqueous solutions (no buffers added, pH 5.5–6) is close to the corresponding values of some other globular proteins containing Trp residues buried in their hydrophobic interior [29].

The effect of changing pH conditions on the intrinsic fluorescence of the EqTxII is shown in Fig. 1. It can be seen that at pH below 2.0 the protein emission spectrum is slightly red shifted ($\lambda_{max} = 335 \pm 1$ nm at pH 1.15) and that with increasing pH the position of λ_{max} remained practically unchanged up to pH 10.0 where another slight red shift is observed ($\lambda_{max} = 333.5 \pm 1$ nm, pH 10.1). Inspection of Fig. 1 further shows that emission intensity observed at 332 nm remains constant over pH range between 3 and 6 while it gradually drops by about 50% when pH decreases from 3 to 1 or increases from 6 to 10. The observed predomination of Trp fluorescence and its dependence on pH agrees well with the results of fluorescence studies performed with some other proteins and model substances [8,9,16–19,21,23,30–32]. According to these studies the predominant Trp fluorescence that occurs even in proteins containing more Tyr than Trp is caused primarily by relatively high absorbance of Trp residues and relatively low fluorescence efficiency of most Tyr

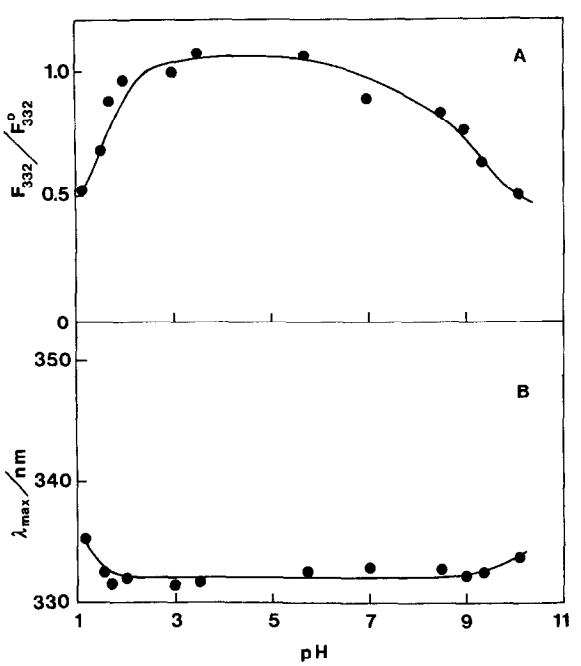

Fig. 1. Effect of changing pH conditions on the intrinsic fluorescence of the EqTxII ($c = 5 \cdot 10^{-5}$ g/$g_{solution}$). (A) Relative fluorescence intensity at 332 nm vs. pH. F^{o}_{332} is the fluorescence intensity at 332 nm of EqTxII pure water solution and F_{332} is the fluorescence intensity at 332 nm of EqTxII solution in appropriate glycine buffer. (B) λ_{max} of fluorescence emission spectra vs. pH. The measured curves are independent of the λ_{ex} value in the range between 275 and 295 nm.

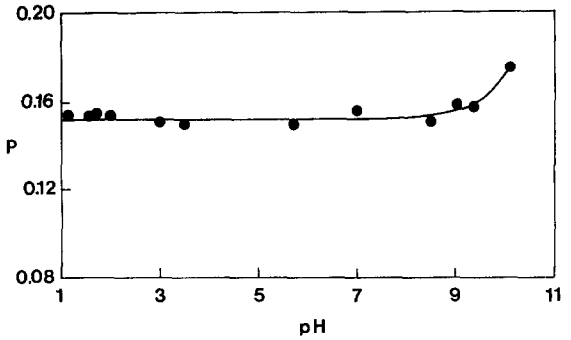

Fig. 2. Effect of changing pH conditions on the polarization of the EqTxII ($c = 5 \cdot 10^{-5}$ g/$g_{solution}$); $\lambda_{ex} = 295$ nm, $\lambda_{em} = 338$ nm.

residues and often also by an efficient transfer of excitation energy from Tyr to Trp [16–19,23]. Furthermore, they have also shown that the observed reduction of fluorescence intensity at low and high pH values can not be explained simply in terms of partial denaturation of the protein in acid or alkaline environment [8,30,31]. The reduction of fluorescence intensity at low pH values can as well be caused by quenching of fluorescence by hydrogen ions [19,21,32] and neutralisation of COO^- groups on aspartic (Asp) and glutamic (Glu) acid residues in vicinity of fluorophores [9,19,32]. There are 10 Asp and 2 Glu residues included in the EqTxII primary structure [3]. Similarly, the observed decrease of fluorescence at high pH values may be explained in terms of deprotonation of Tyr phenolic groups and the positively charged groups of amino acid side chains [9,19].

As shown in Fig. 2 the fluorescence polarization of EqTxII is much less sensitive to pH changes than fluorescence emission spectra. In the pH range between 1 and 8 the P value remains constant ($P = 0.147$), and only at pH > 8, close to the isoelectric point, a slow increase in P is observed ($P = 0.176$ at pH 10.1). This could indicate the presence of some intermediate states but it could as well be, for instance, a consequence of an association of protein molecules.

3.1.2. *Effect of Gu-HCl*

By measuring the intrinsic fluorescence of EqTxII as a function of added Gu-HCl we examined the effect of increasing Gu-HCl concentration on the protein fluorescence emission spectra. At Gu-HCl concentrations higher than 2 M an increasing red shift in λ_{max} accompanied by a decrease in fluorescence intensity was observed (Figs. 3 and 4) indicating significant changes in the EqTxII tertiary structure. At Gu-HCl concentrations higher than 4 M a constant red shift of λ_{max} to around 350 nm, a value characteristic for the fluorescence spectra of Trp residues in an aqueous environment, was observed. Thus, one may conclude that at Gu-HCl concentrations higher than 4 M EqTxII exists in a denatured state in which all Trp residues are fully exposed to the solvent. This conclusion is supported also with the appearance of the Tyr peak at 303 nm

at concentrations of Gu-HCl above 4 M (Fig. 4, spectra 9–11) which suggests that under these conditions the energy transfer from Tyr to Trp residues does not occur any more, a phenomenon characteristic for denatured proteins [16].

As can be seen from Fig. 3A,C and Fig. 4 the addition of Gu-HCl produces also a substantial reduction of fluorescence intensity. At Gu-HCl concentrations higher than 4 M the fluorescence intensity measured at 332 nm drops to about 27% of its value observed in solutions with no Gu-HCl present. This decreasing of fluorescence intensity obviously results from the denaturation of protein and may be explained in terms of increasing polarity of the Trp environment as it becomes fully exposed to the solvent. It has been suggested that the excited singlet states of Trp

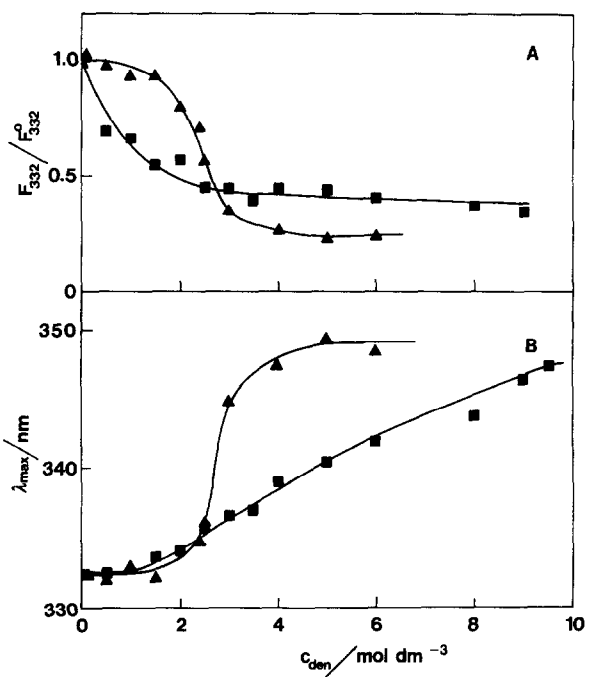

Fig. 3. Effect of increasing denaturant concentration on the intrinsic fluorescence of the EqTxII ($c = 5 \cdot 10^{-5}$ g/g$_{solution}$): Gu-HCl (▲), urea (■). (A) Relative fluorescence intensity at 332 nm as a function of denaturant concentration. F°_{332} is the fluorescence intensity at 332 nm of EqTxII pure water solution and F_{332} is the fluorescence intensity at 332 nm of EqTxII in the measured denaturant solution. (B) λ_{max} of fluorescence emission spectra as a function of denaturant concentration, (C) ratio of fluorescence intensities at 332 nm and 360 nm as a function of denaturant concentration. The measured curves are independent of the λ_{ex} value in the range between 275 and 295 nm.

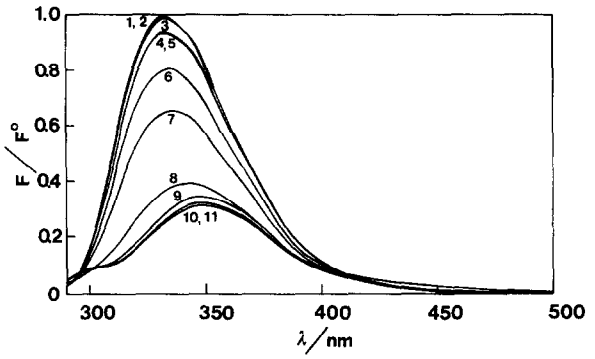

Fig. 4. Normalised corrected emission spectra of EqTxII ($c = 5 \cdot 10^{-5}$ g/g$_{solution}$) in the pure water (1) and in the presence of various concentrations of Gu-HCl: 0.1 M (2), 0.5 M (3), 1.0 M (4), 1.5 M (5), 2.0 M (6), 2.5 M (7), 3.0 M (8), 4.0 M (9), 5.0 M (10) and 6.0 M (11). F° is the fluorescence emission spectra of EqTxII in pure water; λ_{ex} = 280 nm.

interact with water molecules to form excited state complexes and that such a process competes with the radiative relaxation and leads to diminution of the fluorescence intensity [22,33–35].

From the measured dependence of the relative fluorescence intensity at 332 nm, F_{332}/F°_{332}, on the Gu-HCl concentration (Fig. 3A) we can see that the denaturant concentration at the protein half-transition, $c_{1/2}$, which indicates the stability of the protein, is 2.5 ± 0.3 M. Assuming the process of protein unfolding to be a two-state transition the corresponding equilibrium constants and ΔG°_{U} values were determined at different Gu-HCl concentrations from Eqs. 2 and 3 and Fig. 3A. Then, the apparent standard Gibbs free energy of denaturation in water, $\Delta G^{\circ}_{U,H_2O}$, was obtained by a linear extrapolation of ΔG°_{U} values to zero denaturant concentration (Eq. 4). Although such extrapolation from the region of high denaturant concentrations is rather unsafe, the extrapolated $\Delta G^{\circ}_{U,H_2O}$ value of 20 ± 10 kJ/mol is relatively close to the $\Delta G^{\circ}_{U,H_2O}$ value of 41 ± 10 kJ/mol determined directly from the corresponding DSC melting curve (unpublished data).

Measurement of fluorescence polarization as a function of Gu-HCl concentration show that the measured P values are similar to those observed with some other proteins [16,17,29,36,37] and that the addition of Gu-HCl causes only a minor decrease in polarization; P drops by only 20% when Gu-HCl concentration is increased from 0 to 4 M and above (Fig. 5). The effect is obviously much weaker than the corresponding decrease in the fluorescence emission intensity (Fig. 3A,C), but it suggests anyway that additions of Gu-HCl induce loosening of the EqTxII conformation and consequently the rotational freedom of the Trp residues is increased.

3.1.3. Effects of urea

Like in the case of Gu-HCl we have used the relative fluorescence intensity at 332 nm (Fig. 3A), the ratio of intensities at 332 nm and 360 nm (Fig. 3C) and λ_{max} of the Trp fluorescence (Fig. 3B) as probes for urea induced

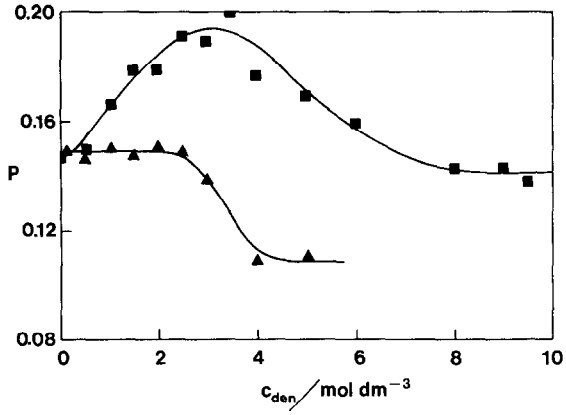

Fig. 5. Effect of increasing denaturant concentration on the polarization of the EqTxII ($c = 5 \cdot 10^{-5}$ g/g$_{solution}$): Gu-HCl (▲), urea (■); λ_{ex} = 295 nm, λ_{em} = 338 nm.

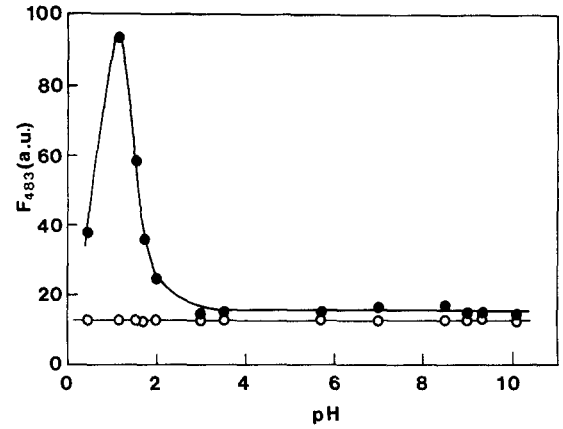

Fig. 6. ANS fluorescence intensity at 483 nm as a function of pH: (○) only in glycine buffer solutions (base line); (●) in glycine buffer solutions in the presence of EqTxII; $c_{EqTxII} = 5 \cdot 10^{-5}$ g/g$_{solution}$, $c_{ANS} = 9.5 \cdot 10^{-6}$ M, λ_{ex} = 365 nm.

EqTxII denaturation at 20°C. As can be seen from Fig. 3A and B at urea concentrations between 0 and 2.5 M a large drop of the fluorescence intensity with increased urea concentration is observed (by about 60% relative to the native protein), whereas the accompanying shift in λ_{max} is only small. At higher urea concentrations the emission maximum, λ_{max}, shifts significantly without much change in the intensity and the λ_{max} values of about 350 nm, corresponding to the final value obtained with Gu-HCl, were reached only in 9 M urea solution. The corresponding transition curve shown in Fig. 3B is very broad and suggests that the EqTxII transition in urea is not a two-state one. This notion is consistent with the dependence of F_{332}/F_{360} ratio on urea concentration, presented in Fig. 3C. From this figure it is clearly seen that EqTxII undergoes a three-state transition with midpoints of the first and second transition at urea concentrations around 1.9 M and 6.5 M, respectively. As can be seen from Fig. 5 the observed dependence of polarisation on the urea concentration supports the findings deduced from fluorescence emission measurements. Polarization first increases with increasing urea concentration until in about 3.5 M urea a maximum P value of 0.2 is observed. With further increasing of urea concentration P starts to decrease and in 9 M urea its value drops back to around 0.14.

3.2. ANS fluorescence

ANS fluorescence measurements were made with the purpose to detect possible compact intermediates occurring during the denaturation of EqTxII caused by changing pH or by increasing concentrations of urea or Gu-HCl.

3.2.1. Effect of pH

Over the pH range between 2.5 to 10.1 the ANS fluorescence in EqTxII solutions remains unchanged and practically equal to the ANS fluorescence in aqueous solutions with no protein present (Figs. 6 and 7). Lowering of pH below 2.5 induced a rapid increase in ANS emission intensity and a significant blue shift in λ_{max}. At pH around 1.1 maximum changes were observed (five times increased intensity, blue shift of 28 nm in λ_{max}) whereas further lowering of pH caused again emission intensities to decrease and λ_{max} values to increase. Such blue shifting of λ_{max} and accompanying increasing of emission intensity is typically observed when the environment of ANS molecules becomes less polar [16,22,23] as in the case of ANS binding within the hydrophobic interior of the acid-denatured protein molecules [24,38,39]. In other words, according to the ANS fluorescence changes observed below pH 2.5 one may speculate that at these low pH values EqTxII molecules exist in an acid-denatured form whose hydrophobic core permits binding of ANS molecules.

3.2.2. Effect of denaturants

The measured ANS fluorescence spectra in aqueous EqTxII solutions show almost no change after protein denaturation by urea or Gu-HCl. The difference between

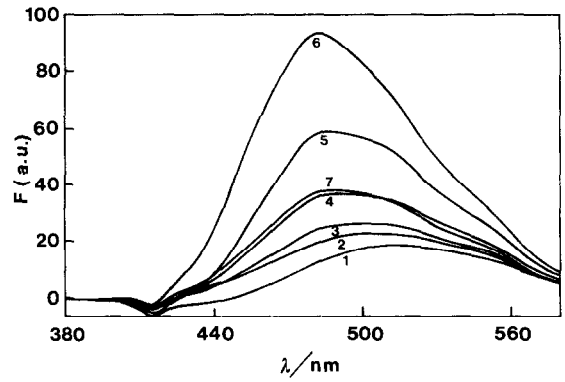

Fig. 7. ANS fluorescence spectra in pure water and in all glycine buffers (base line) (1) and ANS fluorescence spectra in presence of EqTxII as a function of pH: in pure water and in glycine buffers with pH 2.5–10.1 (2), in glycine buffer with pH 2.0 (3), pH 1.7 (4), pH 1.5 (5), pH 1.1 (6) and pH ~ 0.5 (7).

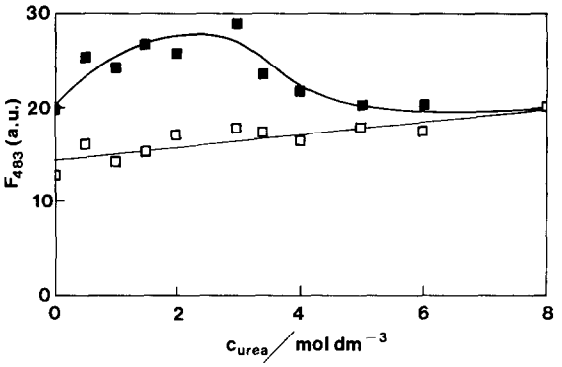

Fig. 8. Change in the ANS fluorescence intensity at 483 nm caused by increasing urea concentration: (□) only in aqueous urea solutions (base line); (■) in aqueous urea solutions in the presence of EqTxII; $c_{EqTxII} = 5 \cdot 10^{-5}$ g/g$_{solution}$, $c_{ANS} = 9.5 \cdot 10^{-6}$ M, $\lambda_{ex} = 365$ nm.

the ANS emission spectra in Gu-HCl solutions measured in the presence and absence of EqTxII is negligible over the entire Gu-HCl concentration range. In urea solutions ANS fluorescence shows slightly different behaviour (Fig. 8). At low urea concentrations a minor increase in ANS emission intensity and a minor blue shift of λ_{max}, compared with the corresponding values in urea solutions with no protein present is observed. This effect disappears when urea concentration is increased above 4 M.

4. Discussion

The λ_{max} value of 332 ± 1 nm in the fluorescence emission spectrum of EqTxII in aqueous, denaturant free solution (pH 5.5–6) indicates that in pure aqueous solutions the Trp residues of EqTxII molecules are located in a relatively hydrophobic environment [29]. When denaturants, Gu-HCl or urea, are added the position of λ_{max} of EqTxII fluorescence is red shifted from 332 nm in water to approximately 350 nm obtained in 4 M Gu-HCl or in 9 M urea (Fig. 3B). This red shifting of λ_{max} may be, as pointed out by numerous authors, explained in terms of increased exposure of Trp residues to aqueous environment, resulting from the process of protein denaturation [16,11,22,23,29]. The observed similarity of the final λ_{max} values in Gu-HCl and urea indicates that the protein final states in the two denaturant solutions are similar or, at least, that in both final states all fluorescing Trp residues are fully exposed to the solvent. Unfortunately, the question whether these final states are random coils or not can not be answered because the changes in the local environment of the Trp residues and the corresponding shifts in λ_{max} are not simply related to the protein structural changes accompanying the process of its unfolding [36]. It is interesting, however, that denaturation of EqTxII with Gu-HCl and urea does not follow the same mechanism. In the case of Gu-HCl, EqTxII seems to undergo a two-state transition, i.e., the concentration of states intermediate between native and denatured is negligibly small, as can be concluded on the basis of all performed measurements. In urea somewhat different behaviour is observed. The observation that both fluorescence polarization and ANS fluorescence show changes at low urea concentrations is a strong hint for the presence of a predenaturation conformational change, an effect that has been observed at low denaturant concentrations with some other proteins [24,40–46]. It should be noted that the change in P does not necessarily reflect a change in the average rotational motion of Trp residues caused by a conformational change of the protein. The small increase in P of EqTxII at low urea concentrations could as well be induced by small changes in the Trp local environment caused by urea binding to the peptide bonds. However, the fact that increased Trp fluorescence polarization (Fig. 5) and ANS fluorescence intensity (Fig. 8) are observed in the range of low urea concentrations in which the measured Trp emission intensity (Fig. 3A) significantly drops with the increased urea concentration (an effect that suggests the loss of tertiary structure [46]) and in which the first transition in the F_{332}/F_{360} vs. urea concentration curve is observed (Fig. 3C) points at the loss of the tertiary structure in this transition and at simultaneous formation of a partially folded protein structure. The observation that a λ_{max} value of about 350 nm, characteristic of Trp residues fully exposed to aqueous environment, is reached only in 9 M urea (Fig. 3B) indicates that the process of the protein unfolding is completed only at this extremely high urea concentration. Since the same λ_{max} value is observed already in 4 M Gu-HCl one can conclude that Gu-HCl has significantly stronger denaturation effect on EqTxII than urea.

The measured dependence of λ_{max} on pH (Fig. 1) clearly shows that even in very acidic or basic solutions the final changes in EqTxII conformation induced by changing of pH are much smaller than changes caused by adding Gu-HCl or urea. Small red shifts of λ_{max} observed below pH 2.0 and above pH 9 (Fig. 1) may be interpreted in terms of slight denaturation of EqTxII. This suggestion is in line with the fluorescence spectra of external probe ANS observed when pH of EqTxII solutions to which ANS is added drops below 2.5 (Figs. 6 and 7). It is also consistent with the results of similar investigation of pH and denaturant concentration influence on the conformation of Diphtheria toxin, another membrane-penetrating protein, [8,9]. The findings for this protein, a small red shift in λ_{max} (3 nm) at low pH and a much larger red shift (21 nm) in 3 M Gu-HCl, are comparable with our results. The small shift in λ_{max} of Diphtheria toxin fluorescence spectra observed at low pH has been attributed to the acid induced partial unfolding of the protein. Similar existence of partially denatured states caused by acid denaturation and accompanied by only minor shifts in λ_{max} has been observed also with some other proteins [5,14,47]. For

colicin A, for example, fluorescence and CD studies of its pH dependent conformational changes produced results that are very close to ours. Between pH 2 and pH 7 a constant emission λ_{max} of 332 nm was observed while according to the interpretation of CD spectra the protein adopts below pH 4 a structure with the features of the molten globule state [5]. We believe that the observed similarity between these results and those obtained with EqTxII additionally supports our suggestion that at low pH values a partial denaturation of EqTxII takes place.

As mentioned previously, changes in EqTxII conformation caused by lowering the pH of the protein solution were also followed by measuring the fluorescence emission spectra of the added ANS. It has been reported [24,38,39] that the hydrophobic external fluorescent probe ANS has a different binding affinity to various conformations of polypeptide chains. Generally, it does not bind to the proteins in their native or fully unfolded states, but has high binding affinity to the protein intermediates with sufficiently large and accessible hydrophobic core to bind the probe. Since ANS-fluorescence spectra of EqTxII at low pH values show drastically increased intensity and large blue shifts of λ_{max} (Figs. 6 and 7), one may assume that our protein exists at low pH values in a compact acid-denatured state. According to the definition of the molten globule state [44,48,49] additional CD measurements have to be made to determine whether this state is a molten globule or not.

It has been reported for many membrane-pore-forming proteins, that they can insert into lipid bilayers only in a partially denatured state, which in most cases is an acid denatured molten globule state [5–15]. According to our results EqTxII could have such properties at low pH (ANS, λ_{max} shift) and possibly at higher pH (slight λ_{max} shift, slight increase in P). In their study on EqTxII-membrane interactions in the pH range between 5 and 10 Belmonte et al. [4] have shown that permeabilization of lipid vesicles for EqTxII increases with increased pH. They report an optimum permeability at around pH 9 where our results show that EqTxII may exist in a slightly altered conformation. However, to obtain stronger evidence that a partial denaturation of EqTxII is necessary for its successful penetration into membranes an investigation of EqTxII-induced pore formation and the related membrane permeabilities should be made at low pH values where according to our results EqTxII quite clearly exists in a partially denatured conformation.

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