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Urea-generated free rotating water molecules are active in the protein unfolding process

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The critical urea concentration (C_3^*) which destabilizes the structure of bovine serum albumin and chymotrypsinogen was determined by UV difference spectroscopy. The increase of the relative content of mobile rotating water molecules in aqueous urea was formerly shown by millimeter spectroscopy [1]. The rise of rotator content at a urea concentration $C_3 \geq C_3^*$ when the bulk water is practically exhausted is suggested as a main driving force of protein unfolding.

Protein unfolding; Urea–water system; UV-difference and millimeter spectroscopy

1. INTRODUCTION

Changes in the water state may affect the stability of proteins in aqueous solutions. Liquid water is structurally inhomogeneous, since H_2O molecules are distributed between fractions characterized by the number of H-bonds from 1 to 4 [2]. H_2O molecules rotating around a single H-bond (confined rotators [3]) are of maximal chemical activity [4]. Denaturants as well as a temperature rise produce the breaking of the water structure which is accompanied by an increase of the rotator content in the water component of the solution [1]. The same factors affect protein structure unfolding.

To monitor the changes of the rotator content in aqueous systems we have recently suggested a new approach based on absorption measurements in the millimeter range (AMM), i.e. 1–3 cm^{-1} [1]. In this paper, AMM-data concerning the influence of some solutes on the water structure [1,5] were used to shed a new light on the urea- and guanidine-induced unfolding of chymotrypsinogen A (CTG) and bovine serum albumin (BSA), as studied by difference UV-spectroscopy.

2. EXPERIMENTAL

2.1. *Materials and chemicals*

Salt-free chymotrypsinogen A (Reanal), bovine serum albumin (puriss., Koch-Light) and urea (special purity grade, 3-3, Reachim) were used without further purification. Guanidine hydrochloride was twice recrystallized from water. DMSO and sulfolan were twice vacuum distilled. All protein solutions (0.5 mg/ml) were prepared in 0.05 M phosphate buffer pH 6.0.

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2.2. *UV difference measurements*

The difference spectra (SPECORD M40, Carl Zeiss) of proteins were recorded at 20°C using tandem cells (l = 1 cm) placed just before a photomultiplier window. Measurements were carried out 24 h after mixing. No autoactivation of CTG was detected with *N*-acetyl-L-tyrosine ethyl ester as a substrate. Negative UV difference spectra of CTG (tryptophane-rich) and BSA (tyrosine-rich) are shown in Fig. 1.

3. RESULTS AND DISCUSSION

The difference in absorbance (ΔA) between native and unfolded forms of proteins at 287 and 293 nm [6,7] was used to monitor BSA and CTG unfolding [8,9] in the presence of either urea or guanidine hydrochloride (Fig. 2). At low denaturant concentrations, the accessible tyrosines and tryptophanses were practically not revealed in BSA and CTG. In Fig. 2 the critical concentrations can be seen (C_3^*), which were evaluated as intersect points of linear parts of unfolding curves and had the following values. CTG: $C_3^* = 3.03 \pm 0.11$, $r = 0.96$ (urea), $C_3^* = 2.29 \pm 0.04$, $r = 0.96$ (GuHCl); BSA: $C_3^* = 2.94 \pm 0.12$, $r = 0.96$ (urea), $C_3^* = 1.01 \pm 0.04$, $r = 0.96$ (GuHCl).

In urea solutions the water content at C_3^* is

$$C_1^* = (1{,}000 - M_3 C_3^* \bar{v}_3)/M_1 = 48 \text{ M (Fig. 2)},$$

where $\bar{v}_3 = 0.765$ $cm^3 \cdot g^{-1}$ is a partial specific volume of the denaturant [1], M_3 and M_1 are molecular masses of the denaturant and water, respectively.

The state of the water–urea system at C_3^* may be conveniently characterized by the average separation length L^* (nm) between two cosolvent molecules considered as spheres:

$$L^* = (C_3^*/N_A)^{-1/3} - 2(3M_3\bar{v}_3/4\pi N_A)^{1/3} = 1.184(C_3^*)^{-1/3} - 0.145(M_3\bar{v}_3)^{1/3} \quad (1)$$

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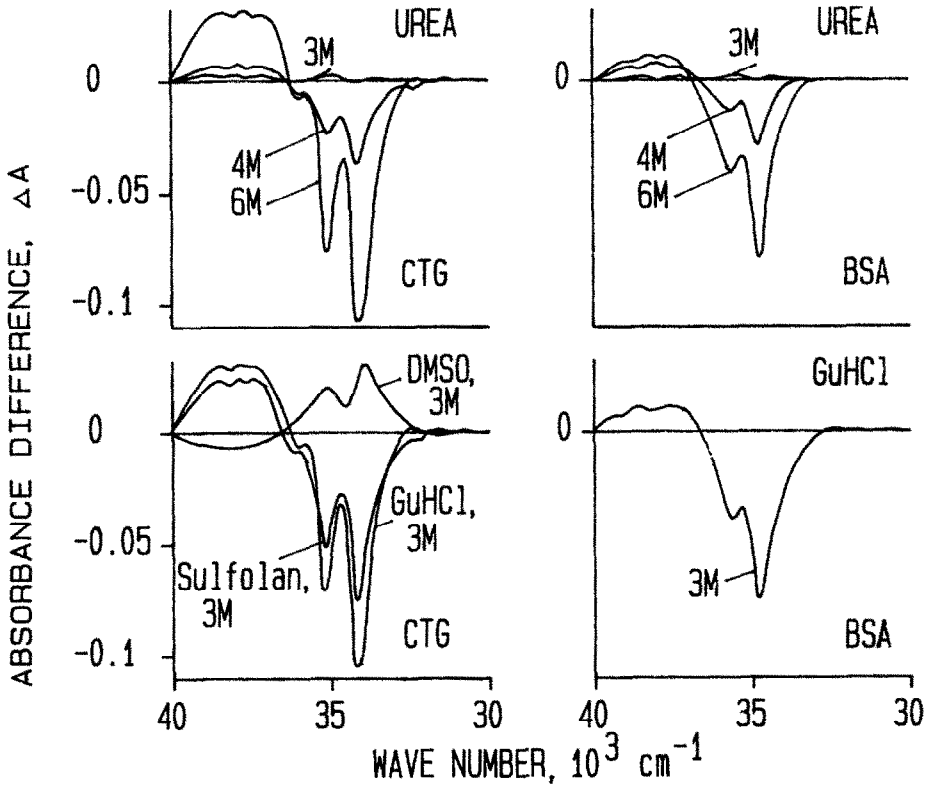

Fig. 1. Difference spectra of CTG and BSA denatured by urea, guanidine hydrochloride, sulfolan and dimethylsulfoxide. The protein concentration 0.5 mg/ml, 0.05 M phosphate buffer pH 6.0, 20°C.

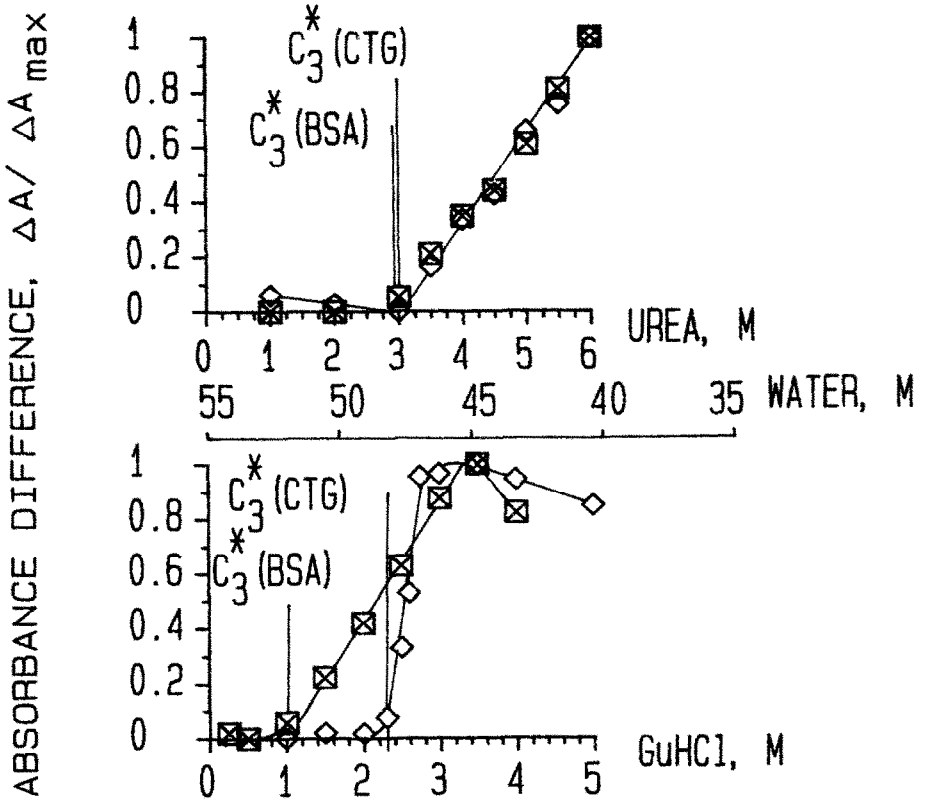

Fig. 2. Relative difference absorbance of CTG at 293 nm (◇) and BSA at 288 nm (⊠) in the presence of urea and guanidine hydrochloride at pH 6.0 and 20°C. The water concentration scale is the same for upper and lower curves.

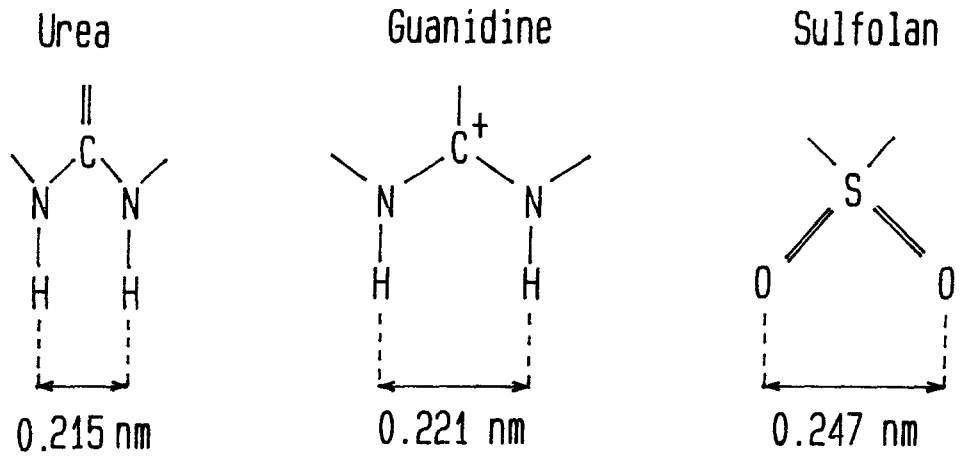

Fig. 3. The proposed fragments of denaturant molecules responsible for the water structure breaking. The distances between proximal centers in urea [1], guanidine hydrochloride [13] and sulfolan [14] are smaller than the Van der Waals diameter of the water oxygen atom $D = 0.36$ nm.

The value $C_3^* \cong 3.0$ M gives $L^* = 0.3$ nm which is about one diameter of a water molecule (one-layer separation). It is necessary to note that the molar excess of water at $C_3^* = 3.0$ M is $C_1^*/C_3^* = 16$.

The concentration dependence of urea hydration, studied by the AMM-method, also shows a critical point but at $\overline{C}_3^* = 1.0$ M, $\overline{C}_1^* = 51.3$ M and $\overline{C}_1^*/\overline{C}_3^* = 51.30$. The corresponding separation length $\overline{L}^* = 0.65$ nm is about two diameters of water molecule. At $C_3 > \overline{C}_3^*$, the urea–urea interactions are possible but the protein structure is unperturbed up to one-layer separatiaon at C_3^*.

It is known that at $C_3 < C_3^*$ proteins are preferentially hydrated [10] and cosolvents do not markedly destabilize their structure (see Fig. 2). We suggest that the observed denaturation effects ($C_3 \geqslant C_3^*$) are related to (1) the exhaustion of water and (2) the interactions of water molecules with urea and its breaking activity.

The exhaustion of bulk water by the cosolvent and its hydration are significant for protein precipitation and crystallization [11], but protein solutions used were dilute enough to neglect protein–protein interactions. The exhaustion of bulk water is typical for all classes of cosolvents and may be one of the denaturation factors. But in the presence of DMSO (Fig. 1) and other water-structure makers, the negative UV difference spectra typical for denaturation was not observed. The AMM-study has shown that DMSO lowers the rotator content [5].

In contrast, the raise of the urea concentration is accompanied by an increase in relative content of rotators in the water component of the solution [1]. The growth of temperature produces the same effect and also leads to protein denaturation [9]. We suggest that the breaking of the water structure, giving the high relative content of rotators, is one of the main driving forces of protein unfolding. An additional destabilizing factor may be the known urea binding by denatured proteins at $C_3 \geqslant C_3^*$ [10].

In the case of GuHCl (see Fig. 2) the parameters of unfolding curves depended on the type of protein. The distinction between the two denaturants may be explained by the ability of the guanidinium ion to interact strongly with protein anionic moieties [12]. It is interesting to note that sulfolan also produces the negative difference UV spectra (Fig. 1) typical for urea denaturation.

One should underline that urea, guanidine and sulfolan, having two close proximal donor or acceptor atoms, make it possible to form H-bonds with only one water molecule (Fig. 3) which retains its rotation mobility [1].

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