Chemistry of Natural Compounds and Bioorganic Chemistry

Effect of urea on the structure and functional activity of proteins. Chymotrypsinogen A and α-chymotrypsin

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The effect of urea on the structural stability and functional activity of globular proteins, *viz.*, chymotrypsinogen A (ChtG) and α-chymotrypsin (Cht), was studied over a wide range of concentrations (0.5—6 mol L^{-1}), and the existence of two different mechanisms of the action of urea on these proteins was demonstrated. No changes in the spatial structure of ChtG were observed in the concentration range from 0.5 to 3 mol L^{-1} (region *I*). Differential UV spectroscopy shows the redistribution of aromatic groups between the inner volume and the outer surface of a protein molecule (protein denaturation) at concentrations >3 mol L^{-1} (region *II*). In region *I*, urea changes the kinetic parameters of enzymatic reactions involving Cht, which is explained on the basis of millimeter spectroscopy data by its action on the structure and nucleophilic reactivity of water.

Key words: urea, chymotrypsinogen A, α-chymotrypsin, differential UV spectroscopy, millimeter spectroscopy, breaking of the water structure.

The use of urea in organic and bioorganic chemistry is associated with its unique physicochemical properties. The most spectacular example of their manifestation is the ability of urea to denature proteins.[1,2] The enhancement of the solubility of nonpolar substances in water[1,2] in the presence of urea is also significant, as well as the improvement of the possibility, in this connection, to perform organic reactions in aqueous media and to control their regioselectivity.[2] Therefore, considerable attention was systematically given to the thermodynamics of the urea—water system.[3] The specific properties of urea are mainly associated with its ability to destabilize the structure of water. The molecular mechanism of this action of urea on the water structure has been established rather recently, using the data of absorption millimeter spectroscopy (MMS).[4]

In the present work, the indirect effect of urea on the structure and functional activity of protein due to the action upon the state of an aqueous medium was studied by differential UV spectroscopy and enzymatic kinetics.

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Serine proteinase (α-chymotrypsin, Cht) and its nonreactive precursor, chymotrypsinogen A (ChtG), were used as objects for the study.

Experimental

α-Chymotrypsin activity 9000 ATEE units, *N*-succinyl-L-phenylalanine *p*-nitroanilide (**1**) (Sigma), chymotrypsinogen A with content of α-chymotrypsin not more than 1 %, *N*-acetyl-L-tyrosine ethyl ester (**2**), *p*-nitrophenyl acetate (**3**), imidazole (Reanal), and urea (special purity grade, Reakhim) were used.

Study of denaturation of chymotrypsinogen by differential spectroscopy. Differential spectra were obtained with 0.05 % solutions of ChtG in a 0.07 *M* phosphate buffer (pH 6.0) containing 1—6 mol L^{-1} of urea. Protein was kept under the specified conditions for not less than 24 h to establish the conformational equilibrium. Measurements were carried out on a Specord M-40 spectrophotometer (Carl Zeiss) at 20 °C in the range of 40000—30000 cm^{-1} (250—330 nm) using a system of four 1.00-cm cells.

Study of the kinetics of enzymatic reactions involving Cht. The enzymatic hydrolysis of substrate **1** was performed in a 0.07 *M* phosphate buffer, pH 7.0. The concentration of Cht was $0.5 \cdot 10^{-4}$ mol L^{-1} and that of the substrate was $(0.5—1.5) \cdot 10^{-3}$ mol L^{-1}. The rate of hydrolysis of **1** was determined from an increase in the absorption of the reaction product (*p*-nitroaniline) at 417 nm and 20 °C in the presence of urea (0.5—3 mol L^{-1}). The perturbation of the spectrum of *p*-nitroaniline under the action of urea was taken into account. The dependence of the molar extinction of *p*-nitroaniline (ε) on the concentration of urea (C_2) was established preliminarily: $\varepsilon = \varepsilon_0 + aC_2$, where $a = 230$ mol^{-2} cm^{-1}, $\varepsilon_0 = 6500$ mol^{-1} cm^{-1}.

The enzymatic hydrolysis of substrate **2** was carried out under the same conditions at [Cht] $= 0.5 \cdot 10^{-7}$ mol L^{-1} and concentrations of the substrate of $(0.25—1.5) \cdot 10^{-3}$ mol L^{-1} in the presence of urea (0.5—4 mol L^{-1}). The hydrolysis rate was determined by the Schwert—Takenaka method[5] from the change in absorption at 237 nm (20 °C). It has been shown preliminarily that urea exerts no effect on the absorption of both substrate **2** and the reaction products at 237 nm.

Study of the kinetics of nonenzymatic hydrolysis of *p*-nitrophenyl acetate (3). Compound **3** ($2 \cdot 10^{-4}$ mol L^{-1}) was hydrolyzed in an alkaline medium (0.1—0.3 *M* $NaHCO_3$—Na_2CO_3 buffer, pH 10.1) and in an imidazole buffer (0.1—0.5 mol L^{-1}, pH 7.0) in the presence of urea (0.5—2 mol L^{-1}). The reaction rate was determined by the absorption of the reaction product (*p*-nitrophenol) at 405 nm.

Results and Discussion

Denaturation of ChtG under the action of urea. The effect of urea on the stability of the spatial structure of ChtG was determined from the amplitude of the differential spectrum that appeared upon unfolding of the structure of the protein globule. The change in the absorption was observed mainly in the range of wavelengths typical of the UV absorption of tryptophan residues (292—295 and 284—286 nm). The dependence of the degree of denaturation of ChtG on the concentration of urea is shown in Fig. 1. As can be seen in Fig. 1, only an

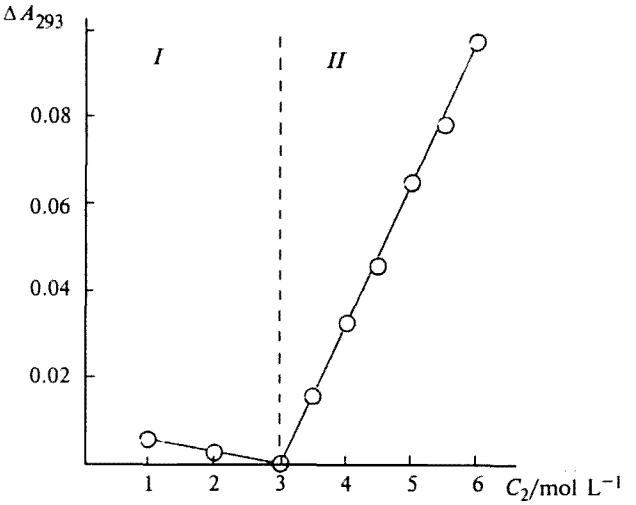

Fig. 1. Dependence of the amplitude of the differential spectrum ΔA_{293} of ChtG (0.05 mg mL^{-1}) on the concentration of urea C_2.

insignificant perturbation of the spectrum of the superficial chromophoric groups of ChtG is observed in region *I*, which does not indicate any substantial changes in the protein structure. The hypochromic shift in the absorption spectrum of ChtG appears in region *II*, which attests to the transfer of aromatic groups from the inner volume of the protein globule to the solvent due to its unfolding. In this region, the linear dependence of the extent of denaturation of ChtG on the concentration of urea is observed. The extrapolation of the $\Delta A(C_2)$ dependence in region *II* in Fig. 1 to its intersection with the abscissa gives the value of the critical concentration $C_2 = 3.0 \pm 0.1$ mol L^{-1}, which divides the region of stability of the protein globule and the region of its denaturation.

As is known from thermodynamic data, the preferential hydration of protein occurs at a concentration of urea lower than 3 mol L^{-1}.[6] The concentration of water in a two-component aqueous system is determined as

$$C_1 = (1000 - M_2C_2\bar{v}_2)/M_1,$$

where $\bar{v}$ is the specific partial volume of the dissolved substance; C_2 is its concentration; and M_2 and M_1 are the molecular masses of the dissolved substance and water, respectively. The average width of the water layer (L/nm) between the molecules of the dissolved substance, considering them spherical, can be calculated by the formula[7]

$$L = (C_2/N_A)^{1/3} - 2(3M_2\bar{v}_2/4\pi N_A)^{1/3} = \\ = 1.184(C_2)^{-1/3} - 0.145(M_2\bar{v}_2)^{1/3}.$$

At the critical urea concentration $C_2^* = 3$ mol L^{-1}, the width of the layer $L^* \approx 0.3$ nm, which is approximately equal to the diameter of one water molecule. This points to a considerable depletion of bulk (*i.e.*, unbound in hydrates) water in the solution. In this case,

the molar excess of water in the solution compared to urea is $C_1^*/C_2^* = 16$.

Previously, it has been shown directly by the MMS method that the equilibrium in the structure of liquid water in the presence of urea shifts toward the fraction of more mobile, chemically active molecules, *i.e.*, the thermodynamic temperature of water increases. This effect predominates at low concentrations (lower than 3 mol L^{-1}) of urea, and at higher concentrations the depletion of water in the system becomes more noticeable.[4] We suggested that it is the breaking of the water structure along with the depletion of the fraction of bulk water as the C_1/C_2 ratio decreases that characterizes the beginning of the phase transition in the protein denaturation.[7] Further increase in the urea concentration results in the binding of urea with the polypeptide chain, stabilizing its unfolded state. Therefore, the phase transition in a protein molecule in a 3 *M* solution of urea can occur due to both the direct interaction of urea with the protein and the indirect effect of urea on the protein *via* its action on the water structure.

Effect of urea on kinetic parameters of reactions involving Cht. The reactions of enzymatic hydrolysis of *N*-succinyl-L-phenylalanine *p*-nitroanilide (**1**) and *N*-acetyl-L-tyrosine ethyl ester (**2**) were studied to investigate the effect of urea on the functional properties of Cht in region *I*, where its preferential hydration occurs. The dependences of the hydrolysis rates of **1** and **2** on the substrate concentration are shown in Fig. 2 in the Lineweaver—Burk double reciprocal coordinates. It is noteworthy that the reactions involving Cht at all concentrations of urea give linear dependences in the coordinates $1/v(1/[S])$, *i.e.*, they are described by the simple Michaelis—Menten equation of enzyme kinetics: $v = k_{cat}[E][S]/(K_m+[S])$.

The effect of mixed retardation of the reaction by urea is observed upon the hydrolysis of **1**. This effect is characterized by an increase in K_m and a decrease in k_{cat} (Fig. 3, *a*,*b*). In the hydrolysis of **2**, the effect of urea is more complex: two points of intersection of lines $1/v(1/[S])$ are shown in Fig. 2, *b*. When the concentration is higher than 1.5 mol L^{-1}, urea acts as an inhibitor of the mixed type. However, when the concentration of urea is 0.5—1.5 mol L^{-1}, an anomalous increase in V_{max} of the enzymatic reaction is observed under the action of the effector. The complex character of the effect of urea on the rate of hydrolysis of **2** is manifested in the bell-shaped dependence of k_{cat} on the concentration of the effector (see Fig. 3, *b*).

It is known that the Cht-catalyzed hydrolysis of esters and amides occurs *via* the formation of an acyl-enzyme intermediate.

$$\text{EOH + RCOX} \underset{}{\overset{k_1}{\rightleftharpoons}} \text{[EOH·RCOX]} \xrightarrow[-\text{HX}]{k_2} \text{EOCOR} \xrightarrow[H_2O]{k_3} \text{EOH + RCOOH}$$

In the hydrolysis of the amide substrate **1**, $k_2 \ll k_3$, *i.e.*, acylation ($k_{cat} = k_2$) is the limiting stage of the reaction, while $k_2 \gg k_3$ and the decomposition of the acyl-enzyme compound ($k_{cat} = k_3$) is the limiting stage in the hydrolysis of ester **2**. In the latter case, a water molecule is one of the substrates of the catalyzed reaction.

Thus, we can follow the effect of urea on individual stages of the enzymatic reaction: acylation (hydrolysis of **1**) and deacylation involving a water molecule as the substrate (hydrolysis of **2**). Both the direct action of urea on the structure and function of protein and its indirect effect on the reaction course by binding or activation of substrates and changing the state of the aqueous medium can occur. The increase in K_m for reactions of both types also testifies to some weakening of binding of

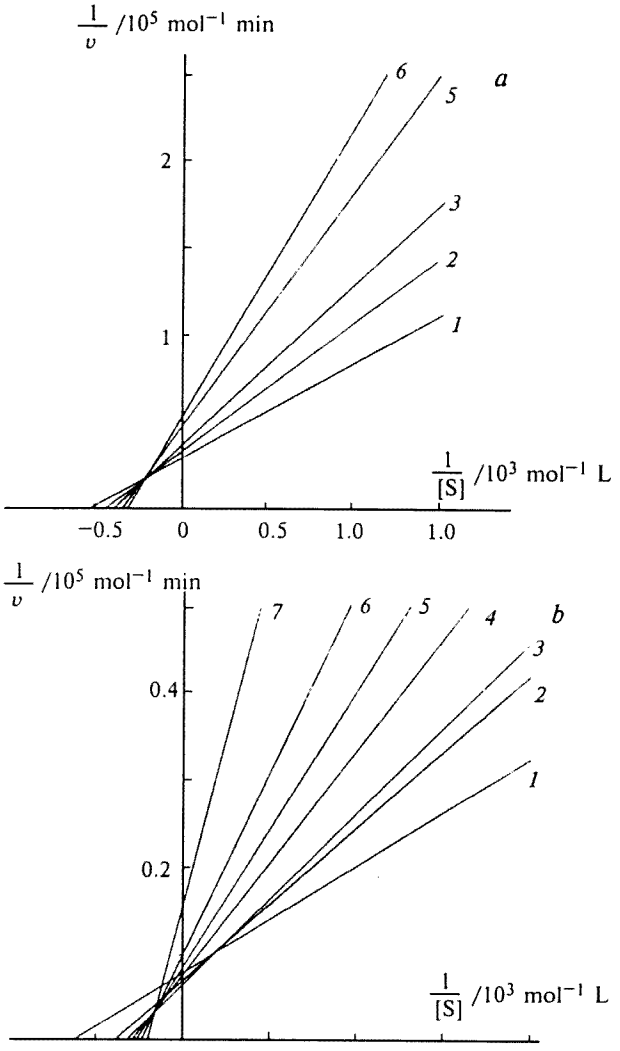

Fig. 2. Dependences of the rate of enzymatic hydrolysis of substrates **1** (*a*) and **2** (*b*) on their concentrations in double inverse coordinates $1/v(1/[S])$ at different concentrations of urea: control (*1*), 0.5 (*2*), 1 (*3*), 1.5 (*4*), 2 (*5*), 3 (*6*), 4 (*7*) mol L^{-1}.

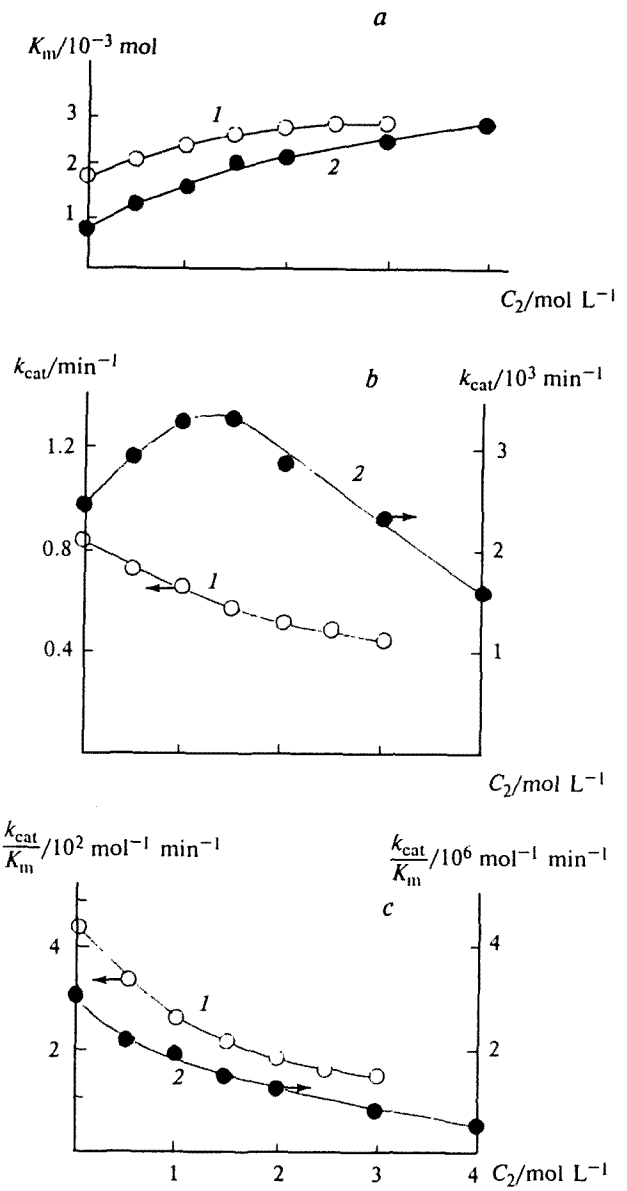

Fig. 3. Dependences of the Michaelis constant K_m (*a*), of the hydrolysis rate constant k_{cat} (*b*), and of the catalytic coefficient k_{cat}/K_m (*c*) on the concentration of urea C_2 for substrates **1** (*1*) and **2** (*2*).

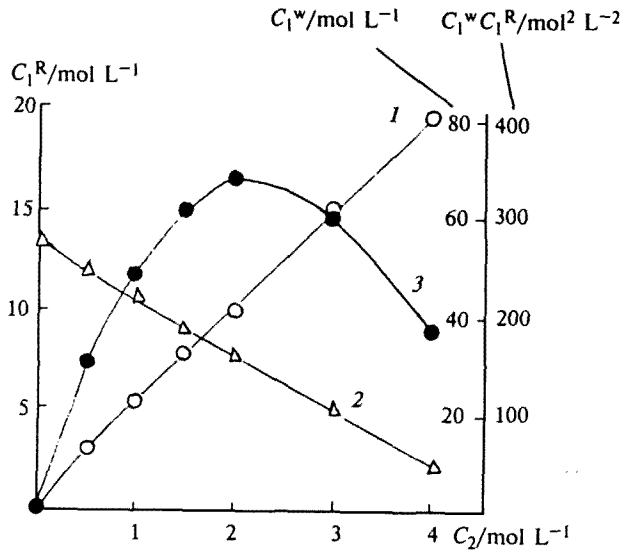

Fig. 4. Dependences of the concentration of rotators C_1^R (*1*), of the concentration of bulk water C_1^w (*2*), and of their product $C_1^R \cdot C_1^w$ (*3*) on the concentration of urea C_2 in the water—urea system.

the substrate with Cht in the formation of the enzyme-substrate complex. The decrease in k_{cat} in the case of hydrolysis of **1** characterizes the retardation in the formation of acyl-enzyme, because in this case $k_{cat} = k_2$. As can be seen from Fig. 3, *c*, the relative changes in k_{cat}/K_m for two different reactions have the same dependence on the concentration of urea. Since $k_{cat}/K_m = k_2/K_S$, it can be asserted that, at the stage of the formation of acyl-enzyme, urea acts only upon Cht to deteriorate its catalytic properties. In the case of the hydrolysis of **2** ($k_{cat} = k_3$) at low (0.5—1.5 mol L^{-1}) concentrations of urea, an anomalous acceleration of the hydrolysis of the acyl-enzyme intermediate, in which an H_2O molecule acts as the substrate, takes place. In this case, the nucleophilicity of the second substrate, *i.e.*, water molecules, increases in the presence of urea.

To check this assumption, the effect of urea on the course of a model reaction (hydrolysis of *p*-nitrophenyl acetate **3**) was studied. In the first case, the hydrolysis of **3** was carried out in an alkaline medium (pH 10.0). The presence of urea (0.5—2 mol L^{-1}) results in the acceleration of the reaction: $k_{eff} = 6.0 + 1.8 \cdot C_2$ (s^{-1}), where C_2 is the concentration of urea. In this case, the hydrolysis of **3** occurs *via* the mechanism of general base catalysis, *i.e.*, a water molecule is the second substrate of the reaction. The hydrolysis of **3** was also carried out in the presence of imidazole (pH 7.0), when the nucleophilic catalysis occurs predominantly to form *N*-acetylimidazole as the intermediate and to yield *p*-nitrophenol. In this case, the presence of urea exerts no effect on the rate of formation of *p*-nitrophenol.

Thus, the presence of urea in solution resulted in an increase in the nucleophilicity of water molecules in both the model and enzymatic reactions. The data obtained previously by the MMS method on the effect of urea on the structure of water make it possible to establish the mechanism of this action. The MMS method is based on the measurement of the selective absorption of the electromagnetic radiation in the 1—10 cm^{-1} range by water upon addition of various nonelectrolytes to the medium. Rotationally mobile water molecules make the main contribution to the absorption. The binding of H_2O molecules in hydrate shells of the majority of nonelectrolytes (N_θ^+ is the hydration number) is accompanied by a decrease in the fraction of rotators, and, hence, by a

decrease in the absorption of millimeter radiation (stabilization of the water structure).[3] However, it is shown by the MMS method that urea and some other compounds can retain water molecules with rotational mobility in the hydrate shell (breaking of the water structure that is characterized by the hydration number N_θ^-).[3] In the case of urea, this effect, known as negative hydration, results in the appearance of an additional ~5.5 mobile, chemically more reactive H_2O molecules per molecule of urea. To estimate the effect of urea on the structure and catalytic activity of the enzyme, two factors should simultaneously be taken into account: the increase in the content of the mobile fraction of water molecules (acceleration of the hydrolysis of esters) and the depletion of bulk water (destabilization of the protein structure). The dependences of the concentration of mobile water molecules $C_1^w = N_\theta^- C_2$ and of the content of free water (which is not included in the hydrate shell of urea) $C_1^w = C_1 - C_2 (N_\theta^+ + N_\theta^-)$ on the urea concentration are shown in Fig. 4. The dependence of the product of the magnitudes obtained on the urea concentration is also given; this dependence is bell-shaped, and the position of the maximum approximately coincides with that for the reaction rate constant k_{cat} in Fig. 3, *b*. This form of the dependence points to the predominant effect of the formation of rotators at a urea concentration lower than 2 mol L^{-1}, which then (at $C_2 > 2$ mol L^{-1}) is compensated by a decrease in the content of bulk water in the system.

Thus, it is shown that the complex effect of urea on the conformational state and the catalytic activity of the enzyme is determined by the peculiarities of the action of urea on the structure of water. At low concentrations of urea (0.5—1.5 mol L^{-1}), mobile, chemically more reactive water molecules play the main role. The increase in the concentration of rotators results in the acceleration of enzymatic and nonenzymatic reactions involving a water molecule as the substrate. At $C_2 >$ 1.5 mol L^{-1}, the increase in the concentration of rotators is compensated by the depletion of the reservoir of bulk water, which results in the retardation of all stages of the enzymatic reaction, and at $C_2 > 3$ mol L^{-1} this results in protein denaturation.

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