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Ethylene Glycol and the Thermostability of Trypsin in a Reverse Micelle System

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Abstract—The influence of ethylene glycol (EG) on the kinetics of hydrolysis of N-α-benzoyl-L-arginine ethyl ether catalyzed by trypsin encapsulated in sodium bis-(2-ethylhexyl)sulfosuccinate (AOT)-based reverse micelles was studied at different temperatures. Ethylene glycol was shown to shift the range of the trypsin activity in the reverse micelles towards higher temperatures. Infrared spectroscopy showed a stabilizing effect of EG on the secondary structure of the protein in the system of reverse micelles. Electron spin resonance spectroscopy showed that the solubilized protein affected the interactions of EG with the polar head groups of AOT and altered the rigidity of the micellar matrix. The results indicate that EG increases the thermostability of the solubilized enzyme in microemulsion media by two mechanisms.

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Key words: reverse micelles, ethylene glycol, trypsin, thermostability

It is known that sugars, amino acids, some salts [1, 2], and polyols [3, 4] increase the thermostability of proteins, shifting the maximum of their enzymatic activity to higher temperatures. Polyethylene glycols of different molecular weights (including ethylene glycol (EG) [4]) dehydrate proteins and bind to their surface. Increase in the temperature results in partial unfolding of the protein. The abovementioned compounds interact with hydrophobic residues of the protein exposed to the protein surface and decelerate its subsequent unfolding.

Similar changes in the properties of proteins were found *in vitro* under conditions that differed significantly from natural conditions. Dispersion of proteins in reverse micelles allows modeling the natural immobilization of enzymes and their microenvironment [5]. Many enzymes demonstrate significant stability and high activity in reverse micelles, although they can exhibit specific properties compared to those in aqueous solutions. Micellar enzymology [6] considers different aspects of the investigation of enzymes in microemulsions (ternary water–surfactant–organic solvent systems), including the search for factors increasing the thermostability of the enzymes. An enzyme solubilized in microemulsion is a single catalytic ensemble, in which the enzymatic activity depends on both the enzyme structure and the dynamic structure of the reaction medium.

There are some data concerning the effect of different additions including water miscible organic solvents on the structure of reverse micelles [7, 8]. Using conductometric assay, it was shown that EG destabilized the structure of sodium bis-(2-ethylhexyl)sulfosuccinate (AOT) monolayer of the micelles [8]. Analogous conclusions were made based on results of ESR spectrometry demonstrating that EG molecules are incorporated between the polar heads of AOT groups [9], this decreasing the density of packing of the hydrocarbon chains of AOT molecules. There are data demonstrating the effect of polar organic liquids on the catalytic activity of enzymes included into AOT-based reverse micelles [10, 11]. Partial replacement of the water inside the reverse micelles with glycerol was shown to increase by several-fold the catalytic activity of α-chymotrypsin [12]. The authors explain this effect by a significant reduction in the conformational flexibility of the protein in the presence of glycerol under conditions of geometrical correspondence of the dimensions of the internal cavity of the reverse micelle and the protein molecule.

The goal of the present work was to study the influence of EG on the thermostability of trypsin solubilized in a reverse micelle system.

Abbreviations: EG) ethylene glycol; BAEE) N-α-benzoyl-L-arginine ethyl ether; AOT) sodium bis-(2-ethylhexyl)sulfosuccinate; ESR) electron spin resonance.

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MATERIALS AND METHODS

The following chemicals were used in this work. Trypsin from swine pancreas (EC 3.4.21.4, type IX-S, T-0303) and the specific hydrophilic substrate N-α-benzoyl-L-arginine ethyl ether were from Sigma (USA). The reverse microemulsion was prepared using the anionic surfactant AOT (MP Biomedicals, Inc., Germany), the final concentration in the microemulsion being 0.3 M. *n*-Decane was used as the dispersion medium, and 0.05 M Tris-HCl buffer, pH 8.5, was used as the aqueous phase. The hydration degree of the micelles was determined by the molar ratio W_0 = [water]/[AOT], varying from 12 to 20. In experiments with EG, part of the aqueous phase was replaced with EG (to 30% of the total volume).

Concentrations of the enzyme (E_0) and the substrate (S_0) are equal to 0.7-1.0 μM and 0.45-1.0 mM, respectively. Concentrations of the enzyme and the substrate were determined at 280 nm ($E^{1\%}_{1cm}$ = 14 [13]) and at 228 nm (ε = 10,700 $M^{-1}\cdot cm^{-1}$), respectively, and calculated for the total volume of the reaction medium. Kinetic measurements were performed using an M40 Specord spectrophotometer (Carl Zeiss, Germany) at 256 nm (absorption band of the product of N-α-benzoyl-L-arginine ethyl ether (BAEE) hydrolysis). The initial rate of the reaction V_0 was determined as the slope of the linear part of the kinetic curve of product accumulation (30-40 sec after the beginning of the reaction). The V_0 value was determined by the equation $V_0 = D/\Delta\varepsilon l\Delta t$, where l = 0.5 cm (cuvette pathlength), D is the optical density in the absorption maximum of the product of BAEE hydrolysis in the reverse micelles, and $\Delta\varepsilon = 1600\ M^{-1}\cdot cm^{-1}$ [14] (differential molar absorption coefficient). Before starting the reaction, the stock enzyme solution was kept in ice for 5 h, and then 5 μl of the solution was added into a cuvette and preincubated at a fixed temperature for 10 min. The reaction of hydrolysis was started by the addition of 5 μl of a stock substrate solution into the cuvette (total volume, 1 ml). The kinetics was measured at 15-45°C.

The secondary structure of trypsin was monitored using a Vector-22 FTIR spectrophotometer (Bruker) at 4 cm^{-1} resolution. Spectra were recorded using 64 accumulations. Measurements were made at 20-60°C in thermostatted CaF_2 cuvettes (10 and 100 μm pathlength). Data processing including calculation of the second derivative was performed using the OPUS program that was included into the software package of the spectrophotometer. Spectral components were identified as elements of secondary structure based on the known correlations [15]. In the spectroscopic experiments, protein concentration is equal to 0.25% (with respect to the microemulsion volume).

The dynamic structure of AOT monolayer was studied by spin probe ESR spectroscopy, using 4-(2-*n*-undecyl-3-oxyl-4,4-dimethyl-2-oxazolidin-2-yl)butyric acid as the spin probe. Due to the amphiphilic structure, the largest number of the probe molecules are localized in the monolayer of the AOT molecules (shell of the micelle), providing information on the packing order of the AOT molecules (maximal hyperfine splitting in the spectra, $2A_{max}$), on the mobility of the hydrocarbon chains of the AOT molecules (correlation time of rotational motion of the probe, τ), and the polarity of the microenvironment of the paramagnetic fragment of the probe (hyperfine splitting constant, a_N) [16]. The spectra were recorded using an RE 1306 radio spectrometer (USSR) at 10-60°C.

RESULTS AND DISCUSSION

The enzyme was chosen for the fact that for trypsin, as well as for α-chymotrypsin, laccase, and binase, the rate-limiting step of the catalysis is not connected with conformational transformations [17-21]. The rate constant of the reaction does not depend on the viscosity of the solution to almost 18 cP. Such enzymes catalyze the reaction by the tense substrate mechanism [22]. Therefore, in the case of partial replacement of the water with EG, we can neglect the change in the viscosity in the aqueous core of the micelles.

In microemulsions, the temperature maximum of enzymatic activity is shifted by 20-25°C towards lower temperatures compared to buffer solutions [23]. This is explained by a significant change in the physicochemical properties of the microenvironment of the enzyme. Increasing temperature increases the extent of dissociation of the head groups of AOT and enhances binding of the cationic substrate to the negatively charged surface of the micelles, this reducing the substrate concentration in the reaction zone [24]. Inhibiting action of Na^+ also cannot be excluded, since its concentration in the aqueous phase of the microemulsion grows with increasing dissociation extent of AOT under enhanced temperature. All these factors decrease significantly the reaction rate in the microemulsion compared to that in a buffer solution.

Figure 1 presents the temperature dependences of trypsin activity in two microemulsion media differing in the size of the reverse micelles. The level of the trypsin activity in the range before the temperature maximum in the micelles with W_0 = 12 is higher than that in micelles with W_0 = 20. This is in agreement with the "principle of geometrical correspondence" [6, 25], according to which the highest activity is observed when the diameter of the internal cavity of the reverse micelle is equal to the diameter of the included protein. Actually, considering that the average radius of the trypsin molecule is ~2.0 nm, the microemulsion with W_0 = 12 (the radius of the aqueous core of the micelles being ~2.2 nm) satisfies the principle of geometrical correspondence, in contrast to the system with W_0 = 20, where the radius is 3.4 nm [16].

As seen from Fig. 1, the presence of EG in the microemulsion medium shifts the high-temperature

decrease in enzyme activity to higher temperatures compared to the control sample. Such an increase in the enzyme activity can be explained by deceleration of the enzyme denaturation due to the interaction of EG with the hydrophobic groups of the protein.

Comparison of the IR spectra of trypsin in an aqueous solution and in the water–EG mixture reveals small but authentic differences in the region of the characteristic amide bands *1-3* (Fig. 2). The presence of EG in the solution enhances the intensity of the absorption of the components of α- and β-structures and diminishes the absorption intensity of disordered structures. The trypsin spectra in microemulsion are changed in a similar way, but the changes are more pronounced (Fig. 3). As compared to the native state, the secondary structure of the protein exhibits an enhanced content of ordered β-structures and α-helical structures. The introduction of EG into the microemulsion enhances helicity of the protein by reducing the content of disordered structures, the fraction of β-structures being reduced to the level characteristic for the native protein. This suggests that, in spite of the impact of the environment of microemulsion on the secondary structure of trypsin, EG interacts with the protein in the same way as in an aqueous solution and results in similar changes in its structure.

The secondary structure of the protein is destroyed with increasing temperature (cooperative melting, Fig. 4). The process starts at approximately 30°C and ceases at 45°C, the midpoint being at 38°C. The presence of EG in the system shifts the end of the transition, increasing the middle temperature of the transition by approximately 5°C without affecting significantly the shape and the slope of the curve. The initial region of the melting curve of the protein in the control microemulsion completely coincides with that in the microemulsion modified with EG. Thus, the stabilizing effect of EG on trypsin is revealed only on the terminal stage of melting, when the β-structure of the protein starts to be destroyed, i.e., in the range of temperatures where the catalytic activity of the enzyme in the control system starts to decrease sharply.

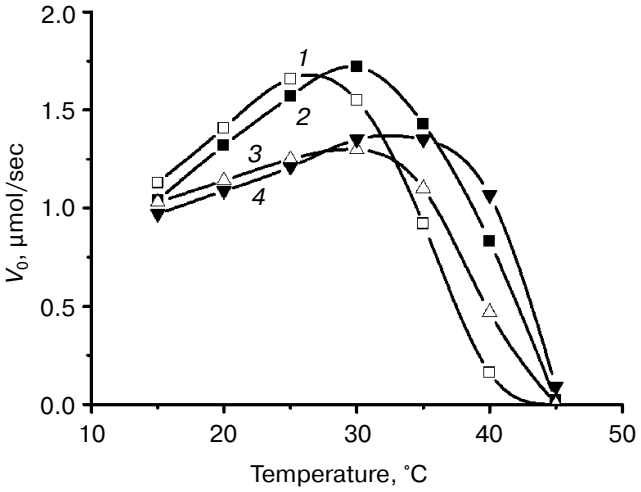

Fig. 1. Temperature dependence of the initial rate of BAEE hydrolysis in reverse micelles with different hydration degree and under partial replacement of aqueous phase with EG: W_0 = 12 (control sample (*1*) and in the presence of 15% (v/v) EG (*2*)) and W_0 = 20 (control sample (*3*) and in the presence of 15% (v/v) EG (*4*)). C_{AOT} = 0.3 M, C_E = 0.7 μM, and C_{BAEE} = 0.53 mM.

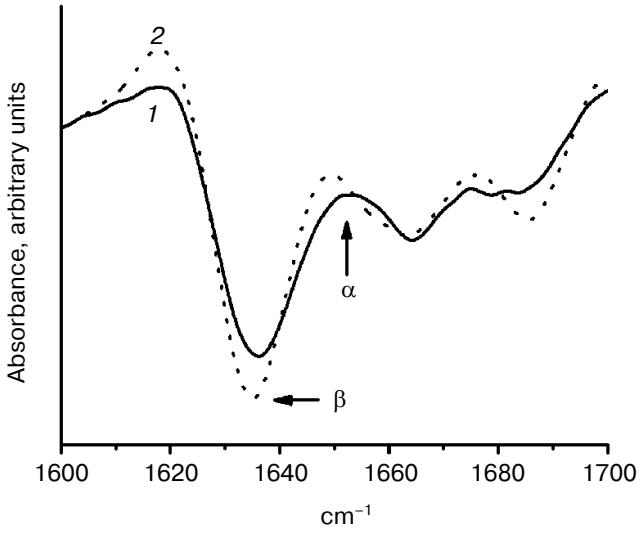

Fig. 2. Second derivative of trypsin absorption spectra in buffer solution without additions (*1*) and in the presence of 13% EG (*2*) at 20°C.

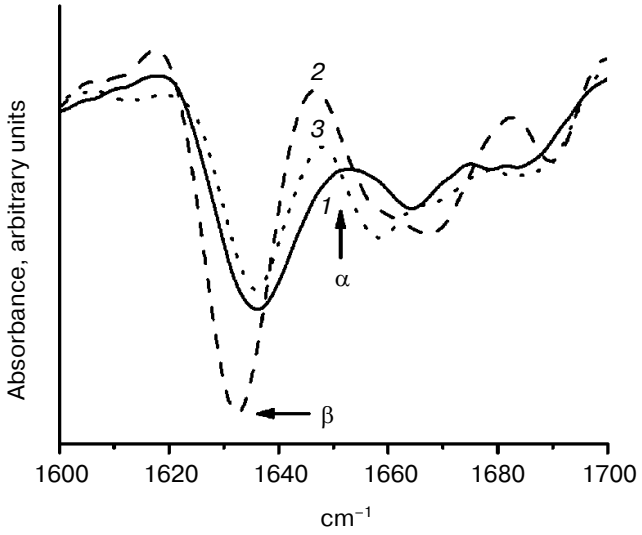

Fig. 3. Second derivative of trypsin absorption spectra in buffer solution (*1*), in microemulsion (C_{AOT} = 0.3 M, W_0 = 14) without additions (control) (*2*), and in the presence of 13% (v/v) EG in the aqueous phase of the microemulsion (*3*).

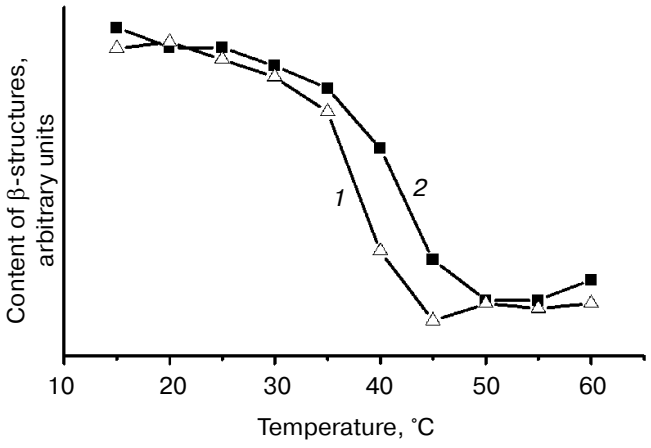

Fig. 4. Melting curves of the β-structure of trypsin in microemulsion (C_{AOT} = 0.3 M, W_0 = 14): *1*) control sample; *2*) 13% (v/v) EG in the aqueous phase of the microemulsion.

At temperatures exceeding 28°C, the ESR spectra exhibited three lines of hyperfine structure. The width and intensity of the lines indicated a relatively fast anisotropic motion of the paramagnetic fragment. In this case, the correlation time of the rotational motion of the nitroxyl fragment was determined by the spectral parameters according to the equation:

$$\tau = 6.65\ \Delta H_{+1}[(I_{+1}/I_{-1})^{1/2} - 1] \cdot 10^{-10}\ \text{sec},$$

where ΔH_{+1} is the width of the low-field component of the spectrum and I_{+1} and I_{-1} are the amplitudes of the low- and high-field components of the spectrum. Decreasing the temperature resulted in the appearance of outer wide peaks in the spectrum, this indicating the deceleration of the rotation of the spin fragment of the probe. In this case, to characterize the dynamic structure of the AOT monolayer of the micelle, we used the parameter $2A_{max}$ (maximal hyperfine splitting of the ESR spectrum). This parameter is known to depend linearly on the order parameter [16] and, consequently, characterizes the packing density of the surfactant molecules in the shell of the reverse micelles. The results obtained by ESR spectroscopy (Fig. 5) show that the introduction of EG into the system decreases the values of the rotational correlation time (τ) of the paramagnetic fragment of probe and the maximal hyperfine splitting of the spectrum ($2A_{max}$). This indicates reduction in the packing density of the surfactant molecules on the surface of the micelle. Consequently, the mobility of the spin fragment increases and its rotational correlation time decreases. It is known that EG molecules are incorporated between the polar head groups of AOT [8]. The changes in the packing density of the hydrocarbon chains of the AOT molecules provide a larger volume for the rotation of the spin fragment of the probe. Immobilization of the protein in the internal cavity of the micelle increases the τ and $2A_{max}$ parameters to values exceeding those in the control system. To explain these data, we analyzed the nitrogen hyperfine splitting constant of the spin probe a_N. The value of this constant depends on the polarity of the microenvironment of the spin fragment (the higher the polarity, the greater the a_N value).

Figure 6 shows that the partial replacement of the water with EG does not change the polarity in the regions containing the nitroxyl fragment of the probe (4-5 nm from the surface of the aqueous core of the micelle, where the sulfo-groups of AOT are localized), yielding the same a_N value as in the control sample. Solubilization of trypsin increases the polarity of the microenvironment of the nitroxyl groups, since some of the polar EG and water molecules are displaced from the water–AOT interface into the area of the hydrocarbon radicals of surfactants [6, 16, 25]. Increasing the polarity of the region adjacent to the surface of the reverse micelle can result in the formation of the additional hydrogen bonds between the water and EG molecules and the oxygen atoms of the carbonyl groups of AOT localized on the periphery of the polar head groups of the surfactant, this decreasing the mobility of the AOT molecules.

Thus, EG in the complex with the solubilized protein increases the rigidity of the micelles and enhances the solubilizing effect of the micellar matrix on the struc-

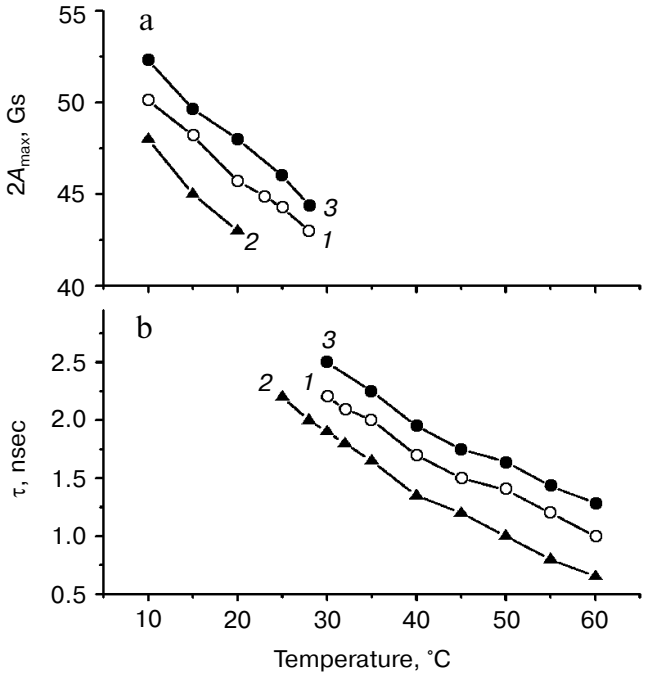

Fig. 5. Temperature dependence of maximal hyperfine splitting $2A_{max}$ (a) and rotational correlation time of the paramagnetic fragment τ (b) in microemulsion (C_{AOT} = 0.3 M, W_0 = 15): *1*) control sample; *2*) replacement of 30% of the aqueous phase with EG; *3*) 0.8% (w/w) trypsin and replacement of 30% of the aqueous phase with EG.

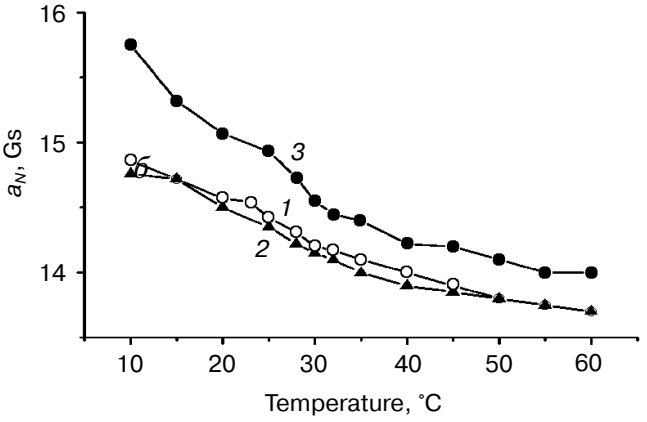

Fig. 6. Temperature dependence of nitrogen hyperfine splitting constant (a_N) in microemulsion (C_{AOT} = 0.3 M, W_0 = 15): *1*) control sample; *2*) 30% of the aqueous phase was replaced with EG; *3*) 0.8% (w/w) trypsin and replacement of 30% of the aqueous phase with EG.

ture of the included protein. This allows the trypsin–reverse micelle complex to exhibit its catalytic properties at higher temperatures compared to the control system. The results described in [16] support the idea concerning the increase in the rigidity of the micelles in the presence of EG. It was demonstrated that in the absence of EG, the displacement of the water by the protein into the hydrophobic region of the micelles resulted in the opposite effect (increase in the mobility of AOT molecules in the shell of the reverse micelle).

The results obtained indicate that in microemulsion media EG can enhance the thermostability of the solubilized enzyme by two mechanisms. First, as in aqueous solutions, EG stabilizes the enzyme structure against thermal denaturation. Second, the displacement of EG by the protein from the aqueous core of the reverse micelle into the region of hydrocarbon radicals of the surfactant enhances the rigidity of the micellar matrix that protects the protein from thermoinactivation.

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