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STRUCTURE AND PROPERTIES OF COMPLEXES OF α-CHYMOTRYPSIN WITH HYDROXYL-CONTAINING GEMINI DICATIONIC SURFACTANTS WITH A SPACER MOIETY OF VARYING LENGTH

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The structure and properties of supramolecular complexes of α-chymotrypsin with hydroxyl-containing alkyl ammonium gemini surfactants (GSs) – α,ω-alkanedyl-bis(hydroxyethylmethylcetyl ammonium dibromides), with a polymethylene spacer of varying length have been studied. IR spectroscopy and tryptophan fluorescence data show that the interaction of GSs with α-chymotrypsin leads to changes of different intensity in the structural state of proteins. The most probable complexation mode of enzyme with GSs have been proposed by the molecular docking method. A correlation is found between the activity of α-chymotrypsin and the length of the GS spacer moiety. The enzyme activity correlates with the change in the substrate concentration in the aqueous phase of the surfactant micellar solution.

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In recent years, more and more attention has been paid to the complexation of proteins with various small molecules that modulate the structure and functional activity of biological macromolecules [1-6]. Interactions of proteins with surfactants are the subject of long-term interest since protein–surfactant complexes are used in a variety of areas such as analytical molecular biology [7], food, pharmacological, and cosmetic industries [8-10], drug delivery [11, 12], design of nanocapsules [13], development of catalytic [14] and sensor systems [15]. It has been established that the properties of supramolecular complexes of surfactant/proteins depend on various characteristics of surfactants, including the charge and size of the polar head group, the length of aliphatic radicals, and surfactant concentration [16-20].

Most papers on surfactant/protein complexes are devoted to classical single-chain surfactants. Dimer (gemini) surfactants (GSs), whose molecule contains two hydrophobic radicals linked by a spacer through the polar head groups or near them, allow extending the range of controlled properties of the complexes. GSs are far superior to their single-chain analogs in a number of characteristics. For example, they have lower Krafft temperatures and lower critical micelle concentrations (CMC) [21-25]. Another special feature of hydroxyl-containing GSs is the possibility of hydrogen bonding

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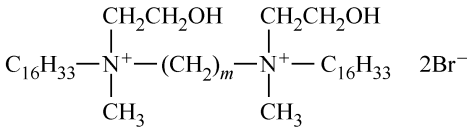
between GS molecules, which can further modify the structure and properties of micellar aggregates, such as the solubilization capacity of micelles. There have been relatively few studies of the interaction of GSs with proteins. It has been found that the spacer length affects the interaction of gemini surfactants with bovine serum albumin [26, 27], gelatin [27], ribonuclease A [28], and hemoglobin [29].

In this work, α-chymotrypsin (CT) is used as the protein component of the complex since its structure and mechanism of action are well understood. Serine proteases, which include chymotrypsin, are convenient model objects for the investigation of the protein–ligand interaction and the regulatory effect of different ligands on the structure and functional activity of enzymes. In this study, we investigated the influence of alkyl ammonium GSs with hydroxyl-containing fragments in the head group and polymethylene spacer of varying length on the structure and catalytic activity of chymotrypsin. Surfactant-protein complexes were characterized by dynamic light scattering, fluorescence, IR spectroscopy, and molecular docking. GSs were synthesized at the Laboratory of Highly Organized Media of the A. E. Arbuzov Institute of Organic and Physical Chemistry (Kazan Scientific Center of the Russian Academy of Sciences. This paper presents characterization of the surfactant-protein complexes and a comparative analysis of the structure and catalytic activity of α-chymotrypsin in the complexes. All studies were performed for micellar solutions of surfactants.

EXPERIMENTAL

We used alkyl ammonium gemini surfactants (GSs) – α,ω-alkanedyl-bis(hydroxyethylmethylcetyl) dibromides with a polymethylene spacer *n*-$(CH_2)_m$, where $m = 4, 6, 8, 10, 12$ (denoted in the text as 16-4(OH), 16-6(OH) etc, respectively), with the general formula



The single-chain analog – cetylhydroxyethyldimethyl ammonium bromide (CHAB) was used as a reference compound. GS samples were synthesized by the reaction of hydroxyethylmethylhexadecylamine with the corresponding α,β-dibromo-*n*-alkane in acetonitrile, followed by double recrystallization of the product from ethyl alcohol according to the procedure described in [30]. The identity of the obtained compounds was confirmed by elemental analysis and IR and NMR spectroscopy. A CTAG sample was synthesized by the reaction of 2-dimethylaminoethanol with cetyl bromide in ethanol. α-chymotrypsin (CT) isolated from bovine pancreas (EC 3.4.21.1) (Sigma) was used. The substrate for kinetic studies was *para*-nitroanilide *N*-benzoil-*L*-tyrosine (BTNA) (Sigma).

Investigation of the solubilization capacity of GS solutions was carried out using a published procedure [31]. BTNA was used as a probe. The solubilization capacity of GS solutions was determined as the ratio of the solubility of the BTNA substrates in the test solution of GS to its solubility in water. For this, GS solutions with an excess of BTNA were kept at 25 °C for 5 h under constant stirring. After this time, the solutions were filtered to remove undissolved probe. BTNA concentration was determined from its absorption at a wavelength of 323 nm.

The activity of CT was monitored by the hydrolysis of the specific substrate BTNA. Surfactant solutions were prepared using a 0.05 M solution of *tris*-HCl buffer (pH 7.6). The initial rate of the enzymatic reaction was measured at a temperature of 25 °C. The pH value was chosen in accordance with the maximum activity of the enzyme under these conditions. The reaction kinetics was studied on a Lambda 25 spectrophotometer (Perkin Elmer, USA). The solution was incubated at a predetermined temperature for 10 min. The reaction was initiated by introducing the substrate. The CT concentration in the reaction mixture was 1 μM, and the substrate concentration was 0.03-1.0 mM. The hydrolysis rate of BTNA was monitored by the change in the optical absorption at a wavelength of 390 nm. The initial reaction rate V_0 was determined from the slope of the linear part of the curve of the product accumulation for 1 min after the start of the reaction

and was calculated using the formula $V_0 = \Delta D/\Delta\varepsilon l\Delta t$. The enzyme concentration was determined from the absorbance at a wavelength of 283 nm using the absorption coefficient $E_{1\,\mathrm{cm}}^{1\,\%} = 20$ [32]. The values of the maximum reaction rate and the Michaelis constant were determined from the dependences of the initial hydrolysis rates on the substrate concentration in the Lineweaver–Burk coordinates.

Dynamic light scattering experiments for the investigated CT/GS systems were performed on a Zetasizer Nano ZS device (Malvern Instruments, UK). The solutions were pre- filtered and allowed to stay at a given temperature (25 °C) for 10 min. The surfactant concentration was 1 mM, and the protein concentration 0.2 mg/mL. Data were processed using the built-in software (Malvern DTS software, version 5.0). Each obtained value of the average hydrodynamic diameter of the particles was the average of ten measurements.

The fluorescence of the tryptophan residues contained in the protein was recorded on a Fluorat-02-Panorama spectrofluorimeter (LUMEX, Russia) in the wavelength range of 310-400 nm at an excitation wavelength of 295 nm. In all experiments, the background spectrum of the buffer without added protein was taken into account. The protein concentration in the samples was 20 μM. The surfactant concentration was varied in the range of 0.03–1.0 mM.

Before IR spectrum recording, exchangeable protons in CT and surfactants were replaced by deuterons. For this protein and a surfactant were dissolved in a 50 mM solution of tris-DCl buffer prepared in D_2O, (pD 7.6). After 3 h of incubation at room temperature, the protein and surfactant were lyophilized. After that, the protein, surfactant, and mixtures (CT GS) were dissolved in deuterated water, bringing the solutions to the required concentration: 0.1 mM for the protein and 4 mM for the surfactant. The weight concentration of the protein in solution was 2.5 mg/mL. IR spectra were recorded on a Tensor 27 spectrophotometer (Bruker, Germany) with a spectral resolution of 4 cm^{-1} and 128 scans. The solutions were placed in a CaF_2 temperature-maintained cuvette with a layer thickness of 100 μm or 10 μm. The spectra of the solvent and of atmospheric water vapor taken at the same temperatures were subtracted from the spectra of solutions. The spectra were not smoothed. The assignment of the components in the spectrum of native CT was made on the basis of published data [33, 34].

The probability of formation of protein–GS complexes was analyzed by molecular docking using the Autodock 4.2 computer program [35]. The total interaction energy ΔG was calculated taking into account the electrostatic (ΔG_{elec}) and van der Waals interactions (ΔG_{vdw}), hydrogen bonds ($\Delta G_{\mathrm{Hbond}}$), the solvation effect (ΔG_{sol}), and torsional entropy (ΔG_{tor}). The search for the configuration of the protein–ligand with the minimum free energy in the Autodock program was performed by a special algorithm (Lamarckian genetic algorithm). The protein was treated as a rigid structure, whereas rotation around single bonds was allowed for the surfactant. The structure and charge distribution for all surfactants were preliminarily optimized using the PM3 method, and the total charge of the ligand was set equal to zero. The protein structure (4CHA.PDB) was taken from the Protein Data Bank [36], and the charge distribution in the protein corresponded to a pH value of 7. It was assumed that the amino acid residue of the protein formed a complex with the GS molecule if the distance between them and any atom of the surfactant was less than 4 Å.

RESULTS AND DISCUSSION

One of the methods used in this work to evaluate the complexation of GS with protein was dynamic light scattering. Dynamic light scattering (photon correlation spectroscopy) is one of the simplest and most common methods for determining particle sizes in aqueous solutions that can be used for systems with particle sizes of several nanometers to several micrometers [37, 38] to determine the self-diffusion coefficient of particles and calculate their hydrodynamic diameter from fluctuations of the scattered radiation intensity. From the data presented in Fig. 1, it follows that GSs in aqueous solutions exist in the form of associations or micelles [25], whose size decreases with increasing length of the spacer [39]. In this case, the average hydrodynamic diameter of the GS–protein complexes is 15–25 % larger than the size of the micelles and one and a half times the size of the protein molecule.

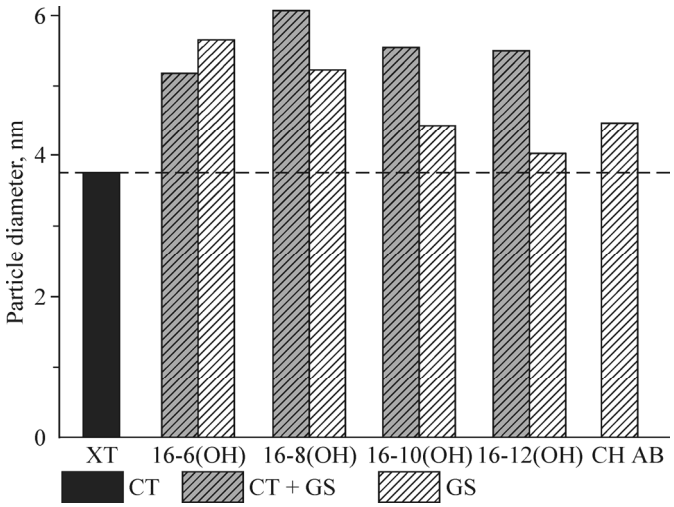


Fig. 1. Average hydrodynamic diameter of protein–surfactant aggregates.

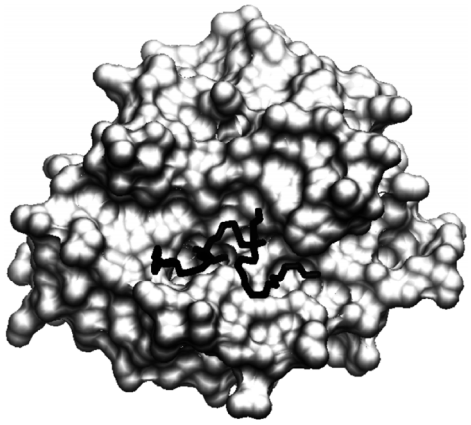


Fig. 2. 16-6(OH)-CT Complex according to molecular docking data.

TABLE 1. Energy of Interaction of GS with Chymotrypsin

Complex type	Average energy, kcal/mol
CT-16-6(OH)	–4.60±1.5
CT-16-10(OH)	–4.84±1.5

The molecular docking method was employed to study CT complexes with GS monomers. The Autodock program was used determine the geometry of the most energetically favorable complexes (Fig. 2) and calculate the energy of interaction between macromolecules (Table 1). Analysis of the probability of complexation of the surfactants with different amino acid residues of the protein showed that all the investigated GSs form energetically favorable complexes with the protein molecule, in which the hydrocarbon radicals of the GSs are located on the surface of the protein globule. The average energy of interaction of GSs with CT given in Table 1 indicates the formation of stable protein–surfactant complexes. The analysis was made for 50 most energetically favorable complexes. CT is characterized by a fairly uniform distribution of surfactant molecules on the surface of the protein, including the contacts with the amino acid residues of the active center His57 and Ser195 HT (Fig. 3).

The internal (tryptophan) fluorescence spectra were studied to determine the effect of GSs on the protein structure. Fig. 4 shows the relative change in the intensity of tryptophan fluorescence of CT in GS solutions. With increasing GS concentration, the tryptophan fluorescence intensity increases almost equally for all the investigated compounds. At the same time, a shift of the fluorescence maximum of tryptophan to higher wavelengths is observed (not shown). The CT molecule includes eight tryptophan residues, of which six are internal, and two are half exposed to the solvent [40]. The large number of tryptophan residues and their different accessibility to the solvent do not allow an unambiguous interpretation of the observed changes in the fluorescence parameters. Increase in the fluorescence intensity may be due to the partial unfolding and an increase in the distance between the chromophores and quencher groups as well as due to the passage of a part of the surface tryptophan residues to the more hydrophobic environment. Increase in the fluorescence wavelength may be due to the increased exposure of internal tryptophans to water during unfolding of the globule and a change in the state of the surface tryptophan residues. It should be noted that the simultaneous increase in the intensity and wavelength of tryptophan fluorescence is often associated with protein denaturation [40].

The CT structure in complexes with GS molecules was also studied by IR spectroscopy. Fig. 5 shows the absorption spectra in solutions of GSs with different length of the spacer moiety. For comparison, the figure shows the spectrum of the protein subjected to partial autolysis by a 24 h incubation in the buffer at 25 °C. The peak intensity of the amide 1 band of the protein denatured by autolysis is significantly reduced due to the broadening caused by a reduction in the content of the native β-structure (1636 cm^{-1}) and growth of disordered structures (1646 cm^{-1} and 1670 cm^{-1}). Simultaneously, there is an increase in the number of terminal COO groups, which is manifested in an increase in the absorption intensity at 1590 cm^{-1} [41]. Comparison of the absorption spectra of CT in the presence of 4 mM GS with the spectrum of the protein denatured by autolysis shows a qualitative similarity between the observed changes. At the same time, the denaturing action of the GSs is not very prononced.

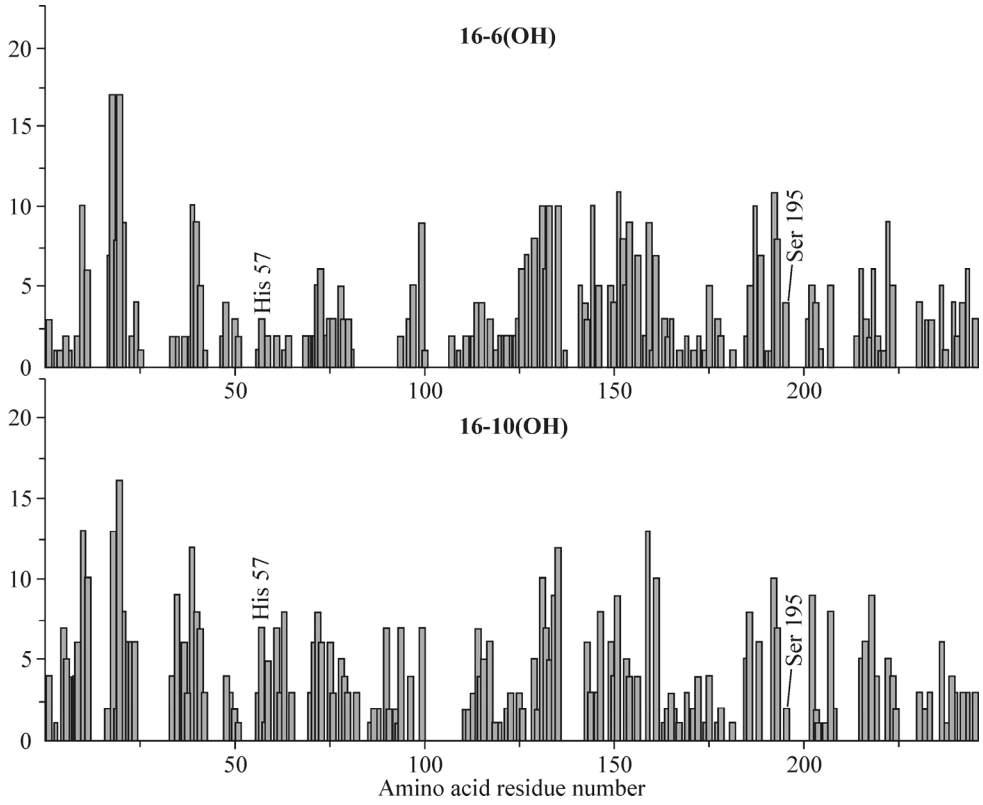


Fig. 3. Number of contacts formed by the amino acid residues of the CT molecules 6.16(OH) and 16-10(OH). The analysis was made for 50 energetically most favorable complexes.

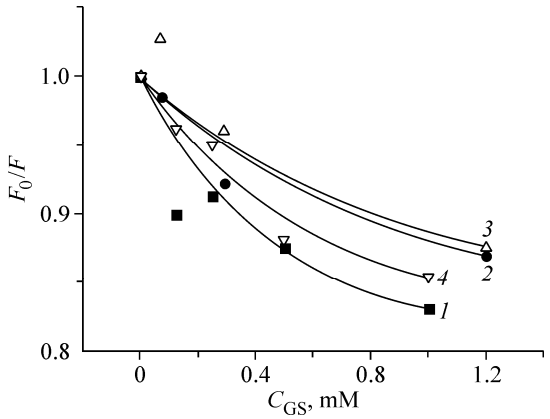


Fig. 4. Intensity of tryptophan fluorescence of CT versus ГS concentration: 16-4(OH) (*1*), 16-6(OH) (*2*), 16-10(OH) (*3*), 16-12(OH) (*4*). F_0 is the tryptophan fluorescence intensity in the buffer, and F is the same in the GS solution.

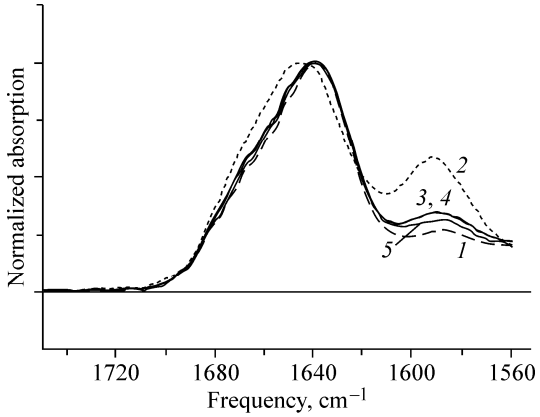


Fig. 5. Infrared absorption spectra of chymotrypsin in the buffer solution and in GS solutions: native CT (*1*), *C*T denatured by autolysis (*2*), 16-8(OH) (*3*), 16-10(OH) (*4*), 16-12(OH) (*5*). The spectra are normalized to the peak of the amide I band.

The study of the enzymatic activity of CT showed that a change in the activity of chymotrypsin is observed in the presence of the entire series of the investigated GSs (see Fig. 6). In this case, there is a correlation between the activity of CT and the length of the spacer moiety of the GS. It is interesting to note that for 16-10(OH) and 16-12(OH), the reaction rate is 5–8 higher than that in the buffer, whereas for GSs with shorter spacers, inhibition of the reaction is observed. Table 2 shows the kinetic parameters of the enzymatic hydrolysis of BTNA: V_{max} is the maximum reaction rate, K_M is the Michaelis constant, k_{cat} is the catalytic constant of the reaction, and k_{cat}/K_M is the efficiency of catalysis in the presence of 16-10(OH) and 6.16(OH), for which the activating and inhibitory effects, respectively, are observed. These data show that the affinity of the enzyme to the substrate, characterized by the quantity K_M, has similar values in the buffer and in the presence of GSs of diametrically opposite action. This suggests that we do not observe any significant disruption of the structure of the enzyme, nor the blocking of its active centre in the presence GSs. At the same time, compared with the control (buffer solution), there is a significant decrease (16-6(OH)) or increase (16-10(OH)) in the efficiency of catalysis.

It is known [42] that the enzymatic hydrolysis reaction involving serine proteases takes place in three stages: the formation of the enzyme-substrate complex, acylation of the active center of the enzyme and hydrolysis of the acyl-enzyme

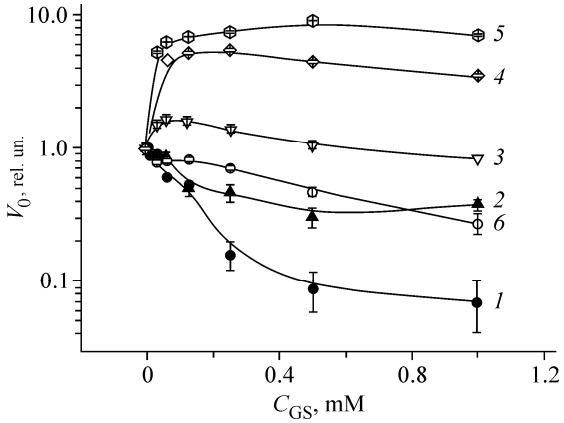


Fig. 6. Relative change in the rate of enzymatic hydrolysis of BTNA versus concentration of 16-4(OH) (*1*), 16-6(OH) (*2*), 16-8(OH) (*3*), 16-10(OH) (*4*), and 16-12(OH) (*5*) CHAB (*6*). Initial reaction rate in a buffer solution of $7 \cdot 10^{-8}$ M/s.

TABLE 2. Kinetic Parameters of the Enzyme Reaction*

System	$V_{max} \cdot 10^{-7}$, M·s^{-1}	$K_M \cdot 10^{-4}$, M	k_{cat}, s^{-1}	k_{cat}/K_M, M^{-1}·s^{-1}
CT	1.5±0.1	0.7	0.15	2143
CT+16-10(OH)	5±0.5	0.5	0.5	10000
CT+16-6(OH)	0.4±0.05	0.83	0.04	482

* CT concentration of 1 μM; GS concentration of 1 mM.

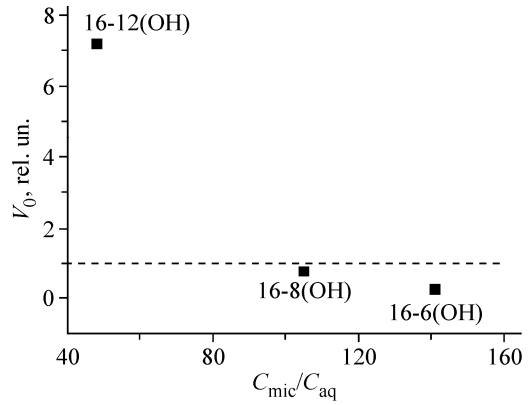


Fig. 7. Hydrolysis rate of BTNA in the CT/GS system with respect to the buffer solution versus the substrate distribution coefficient between the micellar and aqueous phases in 1 mM solutions of surfactants.

complex. Analysis of the kinetic data (Table 2) shows that the Michaelis constant K_M, characterizing the affinity of the substrate with the enzyme changes little in micellar systems compared with the buffer solution. At the same time, the catalytic constant k_{cat}, determined by the rate of formation and decay of the acyl-enzyme complex, decreases in the presence of 16-6(OH) and increases in the presence of 16-10(OH). In our opinion, the change in the rates of formation and decay of the acyl-enzyme complex may be caused by a change in the mobility of the amino acid residues of the active site of the protein, or by a change in the accessibility of the active site to water molecules as a result of modulation of the structure of the

enzyme by the interaction with the surfactant. A deeper understanding of the change in the activity of the enzyme in the presence of the investigated GSs requires a more detailed investigation.

Participation of surfactants in various physicochemical and biological processes largely depends on their state of aggregation, which is determined by the surfactant concentration in the solution. The CMC of the investigated surfactants are in the range of 1.8-3.7 μM [39], i.e., in the experiment, all GSs were micellized. It should be noted that the CMC of hydroxyethyl GSs are 1-2 orders of magnitude lower than that of their non-functionalized analogs. This may reflect possible specific interactions (hydrogen bonding) between the GS molecules that facilitate the micellization processes. Hydrogen bonding can affect the solubilization of the reactants and the rate of their interaction. The accessibility of the substrate to the enzyme can be reduced as a result of solubilization of the substrate in GS micelles. Our studies have shown that BTNA, which is a hydrophobic substrate, is actively solubilized in GS micelles, and the coefficient of its distribution between water and micellar solution decreases sharply with increasing length of the spacer. Fig. 7 compares the changes in the initial rate of the reaction catalyzed by CT with the coefficients of BTNA distribution between the micellar and aqueous phases of surfactant solutions. For the 16-6(OH) and 16-8(OH) systems, the redistribution of the substrate between aqueous and micellar phases is accompanied by a decrease in the reaction rate relative to its level in the buffer, whereas for the 16-12(OH) system, there is an increase in the reaction rate relative to the buffer. The given dependence implies that the substrate concentration in the aqueous phase of the system decreases sharply with decreasing length of the spacer in the investigated series of GSs, which is obviously the main factor leading to a reduction in the CT activity.

Thus, this study has shown that changing the length of the spacer moiety of alkyl ammonium hydroxyl-containing gemini surfactants significantly changes the structure of supramolecular surfactant/protein complexes and affects the catalytic activity of the supramolecular systems, which offers interesting prospects for the study of regulatory action of the gemini surfactants described here.

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