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Noncovalent Adducts of Poly(ethylene glycols) with Proteins

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A new method of preparation of noncovalent complexes between poly(ethylene glycol) (PEG) and proteins (α-chymotrypsin (ChT), lysozyme, bovine serum albumine) under high pressure has been developed. The involvement of polymer in the complexes was proved using ^{3}H-labeled PEG. The composition of the complexes (the number of polymer chains per one ChT molecule) depends on the molecular mass of PEG and decreases with the increase in molecular mass from 300 to 4000, whereas the portion of the protein (wt %) in complexes does not depend on the molecular mass of incorporated PEG and corresponds to ∼70 wt %. The kinetic constants for enzymatic hydrolysis of *N*-benzoyl-L-tyrosine ethyl ester and azocasein catalyzed by the PEG−ChT complexes are identical with the corresponding values for the native ChT. According to the data obtained by the method of circular dichroism, the enzyme in the complexes fully retains its secondary structure. The steric availability of PEG polymer chains in the complexes was evaluated by their complexation with α-cyclodextrin (CyD) or polymer derivatives of *β*-CyD modified with PEG (PEG−*β*-CyD). In contrast to free PEG, only part of PEG polymer chains (∼10%) interact with α-CyD. Thus, the complexation of PEG with ChT proceeds by means of multipoint interaction with surface groups of the protein globule located far from the active site and results in the sufficient decrease in the availability of polymer chains. The complexes between PEG chains in PEG−protein adducts and PEG−*β*-CyD may be considered as a novel type of dendritic structures.

INTRODUCTION

It is known that poly(ethylene glycol) (PEG)[1] in aqueous solutions is inert in respect to biomacromolecules (proteins, nucleic acids, and dextrans) (*1*). Surface adsorption or grafting of PEG to sorbents, liposomes, proteases, and detector elements of biosensors is widely used for keeping them from undesirable nonspecific binding (*2*). Due to the incompatibility between proteins and PEG, the crystallization of a variety of proteins may be realized by the addition of PEG to aqueous solutions of proteins (*3*). At the same time, some properties of PEG conflict with the conception of its inertness in respect to proteins. For instance, the addition of PEG favors to the increase in the degree of folding of some proteins and inhibition of the process of aggregation of folding intermediates (*4*, *5*), that is possible only at the direct contact between protein molecules and PEG. In this case, PEG resembles chaperones—specific proteins responsible for the folding of synthesized polypeptide chains to native structures (*6*−*8*). Direct measurements of interaction between monolayers, one of which contains grafted PEG, and the other grafted protein, also demonstrate the existence of attractive forces between PEG and protein (*9*). That is why one may suppose that under certain conditions PEG is able to form noncovalent complexes with proteins. It is known that high pressure (∼400 MPa) induces denaturation of proteins (*10*). Moderate nondenaturing pressures exert reversible conformational changes related to intrinsic compressibility of proteins and hydration density effects (*11*). Besides, it is known that binding of proteins with specific ligands may be accompanied by a significant increase in packing density (*12*). In this case, the increase of pressure would favor the complexation process. Earlier we showed that the increase in pressure favors the formation of the complex between poly(ethylene oxide)−poly(propylene oxide) block copolymers (Proxanols, Pro) and ChT (*13*). In this work we first synthesized and isolated complexes based on PEG and a number of proteins prepared under elevated pressures. Their composition and some functional properties have been studied.

EXPERIMENTAL PROCEDURES

Chemicals. In this work, the following compounds were used: crystalline α-chymotrypsin from bovine pancreas with an activity of 40−60 IU/mg from Sigma (USA), a number of PEGs with number average molecular masses ($\bar{M}_n$) of 300, 750, 1500, 1900, 3000, and 4000 Da from Serva (Germany), monobutyl ether of block copolymers of ethylene oxide and propylene oxide (Pro) with number average molecular mass of 2000 Da containing 40 wt % propylene oxide from Scientific Manufacturing Association NIOPIK (Russia):

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[1] Abbreviations: BTEE, *N*-benzoyl-L-tyrosine ethyl ester; ChT, α-chymotrypsin; BSA, bovine serum albumin; CD, circular dichroism; CyD, cyclodextrin; PEG, poly(ethylene glycol); Pro, proxanol; TLC, thin-layer chromatography.

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$$C_4H_9O\text{-}[CH(CH_3)\text{-}CH_2O]_{14}\text{-}(CH_2\text{-}CH_2\text{-}O)_{25}\text{-}H$$

As substrates of α-ChT, the following compounds were used: *N*-benzoyl-L-tyrosine ethyl ether (BTEE) from Serva (Germany) and azocasein (casein, containing covalently bounded *p*-sulfodiazobenzene), kindly presented by Prof. V. V. Mozhaev (Lomonosov Moscow State University, Department of Chemistry, Chair of Chemical Enzymology). To prepare buffer solutions, Tris from Reanal (Hungary) was used.

Synthesis of PEG−*β*-CyD conjugate was performed by the polymerization of ethylene oxide initiated with hydroxyl groups of polyfunctional initiator, *β*-CyD, using the method described in ref *14*. Molecular mass of PEG−CyD derivative was found to be equal to 3500 Da.

As a support for affinity chromatography, Sepharose modified with classical soyabean Bauman-Birk inhibitor of trypsin from Sigma was used. The synthesis of the support was performed by mixing 5.0 g of BrCN−Sepharose (Pharmacia, Sweden) in 20 mL of 0.1 M borate buffer, pH 8.45, with 40 mg of soyabean inhibitor of trypsin. The reaction mixture was gently mixed at 0−4 °C during 24 h. After this, the support was incubated in 0.5 M Tris-HCl buffer, pH 8.9, during 6 h. Then the support was sequentially washed with 3 M KCl and 1 mM HCl (500 mL each solution) and finally repeatedly washed with 0.02 M phosphate buffer, pH 7.8.

Preparation of Isotope-Labeled PEG. The insertion of isotope label (tritium) in PEG 1900 was performed using the apparatus described by Shishkov et al. (*15*). The solution of the polymer in methanol (2 mg/mL) was evenly distributed over the inner surface of the reaction vessel, freezed in liquid nitrogen, and dried in a vacuum. A target was thermostated at the temperature of liquid nitrogen (77 K) in a deep vacuum. A reaction vessel was filled up to the point where pressure will reach 1.5−2.0 Pa. The production of tritium-reactive atoms was achieved due to the heating of tungsten spiral by variable current during 15 s at 2000 K. This procedure was repeated several times. To remove species containing free exchanged tritium incorporated into end groups of the polymer, the sample was dissolved in 50% ethanol solution and dried in a vacuum repeatedly. Then the polymer was purified by repeating precipitation from the saturated solution of the sample in ethanol to diethyl ether. The final product had specific radioactivity of 1.7×10^5 disintegration/min per 1 mg. Radioactivity was measured by liqiuid scintillator based on dioxane at the counter Delta (Tracor Analytic). The amount of [^{3}H]PEG bounded with protein was estimated by the value of specific radioactivity calculated from the ratio of radioactivity to mass of the sample normalized to specific radioactivity of the polymer.

Synthesis of PEG−Protein Complexes. To prepare PEG−protein complexes, the solution of protein (2 mg/mL) in 0.02 M phosphate buffer, pH 7.0, was mixed with 200-fold molar excess of the polymer. A mixture was stirred up to complete dissolution of components. Gauge pressure in the system was created by means of centrifuging during 1 h at 25 °C (37000−142000*g* and r_{av} = 5.1 cm) in a preparative ultracentrifuge MOM-3180. For every meaning of the speed, the corresponding value of maximal pressure in the system (without regard for the gradient from menisk to the bottom of the cell) was calculated:

$$P = \frac{dF}{dS} = \frac{\omega^2 \rho}{3 r_{max}} (r_{max}^3 - r_{min}^3)$$

where P is the maximal pressure in the system, dF is the centrifugal force, dS is the square under pressure, ρ is density of the solution (g/cm^3), ω is the angle rate (rad s^{-1}), and r_{min} and r_{max} are the minimum and maximum distances from the center of rotation correspondently.

Isolation of Complexes. The isolation of the complexes from the reaction mixture was performed by two methods. According to the first method, an aliquote of the reaction mixture was diluted up to 20 mL with 0.02 M phosphate buffer, pH 7.6−7.8. The preparative separation of the complexes from the excess of the polymer was made using affinity chromatography at the column (0.7 × 10.0 cm) with the support containing soyabean inhibitor of trypsin. Phosphate buffer, 0.02 M and pH 7.8, was used as an eluent. The composition of the eluate was analyzed by UV method at 206 nm. The elution of the complexes was performed using 1 mM HCl. The elution of protein fraction was detected at 280 nm. Complexes were isolated by means of dialysis of eluate and subsequent lyophilization.

According to the second method, the solution containing PEG−protein complex and the excess of the polymer was dialyzed against water during 6 h. The samples were concentrated by pouring the solution into dialyzing tube surrounded by dry PEG with molecular mass of 100 kDa. The complex was isolated by precipitation with cooled acetone.

The absence of free polymer impurity was monitored by the method of thin-layer chromatography (TLC) on Silufol plates (Kevalier, Czechia) in chloroform−ethanol−water (36:12:1 v/v). Iodine vapor was used to reveal the products. After the identification, plates were divided into horizontal strips of equal width and the support from every strip was placed into separate vessel with liquid scintillator based on dioxane. After the incubation of the solution during 12 h at 20 °C, radioactivity was measured at counter Delta (Tracor Analytic).

Characterization of Complexes. The protein content in the complexes was assayed either using UV spectrophotometry at 280 nm or by the biuret reaction using native ChT as a standard. The content of polymer (a number of polymer chains per one protein molecule), n, was calculated according to the following formula:

$$n = \frac{(100 - \text{wt \% ChT})/\bar{M}_n}{\text{wt \% ChT}/M}$$

where $\bar{M}_n$ is number average molecular mass of the polymer and M is molecular mass of the protein.

Enzymatic activity of the complexes was assayed by measuring the initial rate of hydrolysis of BTEE (*16*). Titration of active centers was performed using *trans*-cinnamoylimidazol as a titration agent (*17*).

The kinetics of thermoinactivation of ChT and its complexes with polymers were registered as follows. The solution of the enzyme or adduct with polymer (10^{-5} M) was incubated in thermostat at 45 ± 0.05 °C (0.2 M Tris-HCl buffer, pH 8.0). Aliquots were withdrawn at certain intervals, and their residual activities were assayed at 20 °C by measuring the initial rate of hydrolysis of BTEE.

The α-CyD content in the complexes PEG−ChT−α-CyD was assayed using IR spectrophotometry according to the intensity of the absorption at 1030 cm^{-1}, which corresponds to the valent fluctuations of C−O, O−C bonds in CyD. First, the essential calibration has been made (*18*).

Synthesis of Three-Component Complexes. Synthesis of three-component systems containing PEG, ChT, and PEG−*β*-CyD was performed using two methods.

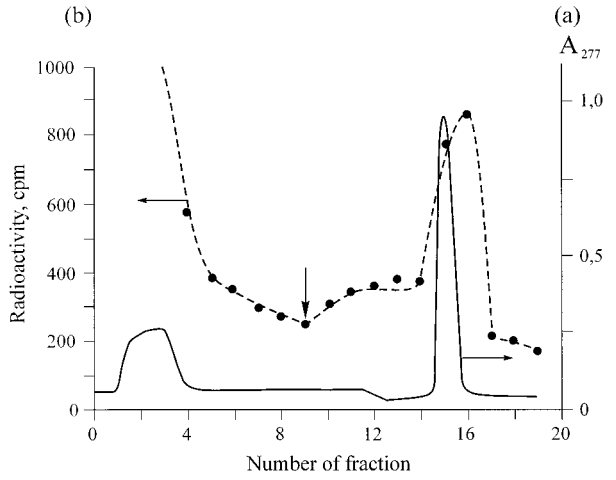

Figure 1. Profiles of elution of the reaction mixture containing complex between ChT and PEG1900. Elution was registered by the adsorption at 277 nm (a) and radioactivity of fractions (b). Vertical arrow points the beginning of elution of PEG−ChT complex with the solution of HCl.

Method 1 is based on the preparation of noncovalent PEG−ChT adducts and subsequent addition of PEG−β-CyD. Method 2 presents an one-step procedure based on the mixing of three components. To the solution of ChT (2 mg/mL) in 0.02 M phosphate buffer, pH 7.0, 200-fold molar excess of PEG and 400-fold molar excess of PEG−β-CyD were added. The mixture was stirred until complete dissolution of components and underwent to the action of elevated pressure in a preparative ultracentrifuge MOM-3180 (r_{av} = 5.1 cm) during 1 h at 25 °C and 37000−142000*g*. The product was isolated by the precipitation with cooled acetone.

RESULTS AND DISCUSSION

Synthesis and Characteristics of Complexes. In the present work, it was shown that the action of elevated pressure in the range 1.1−400 MPa on the systems containing proteins [ChT, lysozyme, and bovine serum albumine (BSA)] and PEG results in the formation of complexes. In control experiments under atmospheric pressure and room temperature, the complexation between PEG and protein does not occur.

Methods of isolation of complexes from the solution are worthy of particular attention. With the help of the affinity sorbent, Sepharose with covalently bound soyabean inhibitor, ChT containing fraction was isolated, and the excess of unreacted polymer was eliminated. Figure 1 shows profiles of elution of the reaction mixture containing ChT-PEG1900 complex. The elution of complexes was monitored by the adsorption at 277 nm (a) and radioactivity of fractions (b). From this figure, it is seen that fractions 14−16 contain both ChT and tritium-labeled PEG1900, whereas an excess of free polymer eluted at early stages (fractions 1−10). Precipitation of complexes with acetone is an alternative effective method of separation from the excess of polymer. In control experiments with [^{3}H]PEG, it was shown that the precipitation of the mixture of the polymer and the protein with acetone allows us to isolate pure protein, which does not contain coprecipitated [^{3}H]PEG.

Figure 2 demonstrates the distribution of radioactive label in the process of TLC of the complex isolated using acetone (curve 1), mixture of ChT and [^{3}H]PEG (curve 2), and initial labeled PEG (curve 3). Each point at the curve corresponds to radioactivity of the separate fraction. As it is seen from the Figure 2, complex of ChT with

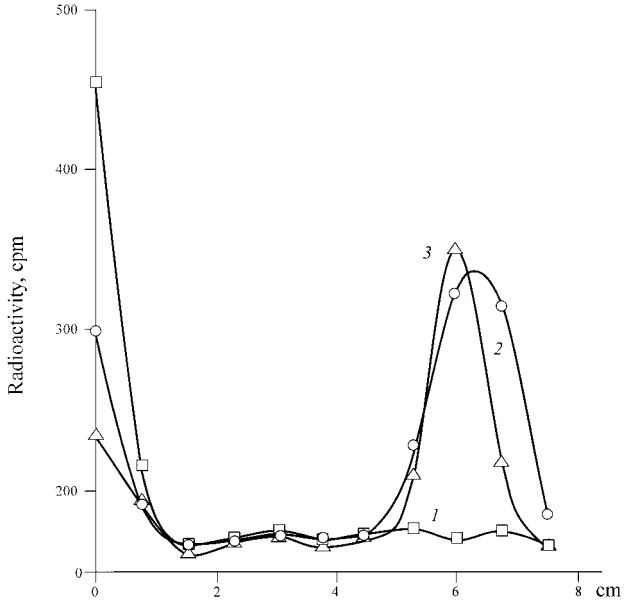

Figure 2. Distribution of radioactive label on TLC plates for [^{3}H]PEG1900-ChT complex isolated by the precipitation in acetone (curve 1), mixture of native ChT and [^{3}H]PEG 1900 (curve 2), and initial [^{3}H]PEG 1900 (curve 3). A total of 10 μL of the solution of the complex (15 mg/mL) was sprayed.

[^{3}H]PEG remained on the start as well as the original protein. R_f of free polymer in this system is equal to ∼0.6−0.65. Essential differences in R_f values between the complex and unbound polymer make possible using TLC as a control of the complex purity.

Data on the composition of complexes obtained from PEG of different molecular masses ($\bar{M}_n$) are presented in Table 1. It was found, that optimal molar ratio of polymer to protein for the synthesis of complexes is 200:1, and the content of polymer in complexes practically does not depend on the value of applied pressure in the above range and temperature 5−25 °C. In control experiments, it was shown that there were no polymer chains in the complexes produced by just mixing PEG and protein at room temperature and atmospheric pressure.

It is seen that the increase of $\bar{M}_n$ from 300 to 4000 Da results in the monotonic decrease in the number of polymer chains in the complex, whereas the content of the protein in all complexes practically does not change and comprises ∼70%. The content of PEG in the complex calculated from the measurement of [^{3}H]PEG1900 coincides with indirect data obtained by the protein assay in complexes. For reference, data on the content of the protein in temperature-induced complexes based on Pro and ChT (*19*) are also presented in this table. It is seen that the composition of complexes 4, 7, and 8 based on PEG and Pro of equal $\bar{M}_n$ but obtained under the action of different physical factors (elevated pressure or elevated temperature) is identical. Analysis of the data presented in Table 2 reveals the dependence between the number of polymer chains in complexes and dimensions of the protein. By the example of complexes based on PEG1900 and three different proteins with decreasing molecular mass (lysozyme < ChT < BSA), it was shown that the greater the radius of the protein, the greater the number of polymer chains in the complexes. The data presented in Tables 1 and 2 testify that the composition of polymer−protein complex primarily depends on the geometric fitness between the surface of protein globule and the length of polymer chain of PEG.

Table 1. Characteristics of Polymer−ChT Complexes

no.	complex	conditions for complex preparation	$\bar{M}_n$ of the polymer	no. of polymer chains in the complex	content of the protein in the complex (%)
	PEG1900 + ChT	atmospheric pressure	1900	0	98 ± 2
1	PEG300-ChT	16.0 MPa	300	31 ± 2	73 ± 4
2	PEG750-ChT	16.0 MPa	750	12 ± 1	74 ± 4
3	PEG1500-ChT	16.0 MPa	1500	7.8 ± 0.4	68 ± 3
4	PEG1900-ChT	16.0 MPa	1900	5.9 ± 0.3	69 ± 3
5	PEG3000-ChT	16.0 MPa	3000	2.9 ± 0.1	74 ± 4
6	PEG4000-ChT	16.0 MPa	4000	1.9 ± 0.1	76 ± 4
7	Pro-ChT	16.0 MPa	2000	6.2 ± 0.3	67 ± 3
8	Pro-ChT	48 °C	2000	7.0 ± 0.4	64 ± 3

Table 2. Characteristics of Complexes of PEG1900 with Different Proteins[a]

protein	molecular mass of protein (Da)	no. of polymer chains in the complex	content of the protein in the complex (%)
lysozyme	11 000	2.7 ± 0.1	68 ± 3
ChT	25 000	5.9 ± 0.3	69 ± 3
BSA	68 000	10 ± 1	77 ± 4

[a] Complexes were synthesized under the pressure 16.0 MPa.

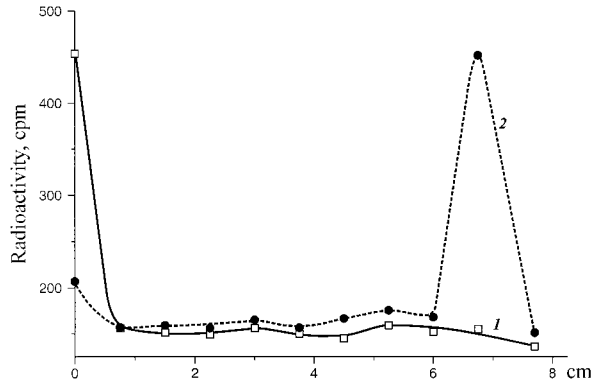

Figure 3. Distribution of radioactive label on TLC plates of [^{3}H]PEG−ChT complex, starting material (1) and incubated in water at 4 °C during 3 days (2). A total of 10 μL of the solution of the complex (15 mg/mL) was sprayed.

To study the stability of PEG−ChT complexes in the solution, [^{3}H]PEG1900-ChT complex was incubated in distilled water at 4 °C during 3 days. Aliquots were withdrawn at certain intervals, and the appearance of free polymer was assayed using TLC. It was shown that free polymer came to view already overnight. Figure 3 shows distribution of radioactive label on TLC plates for [^{3}H]PEG1900-ChT for initial complex (curve 1) and for the complex incubated during 3 days (curve 2). It is seen that after 3 days complex was fully decomposed. At the same time, it appears that the incubation of PEG−ChT complex at 45 °C during 3 h does not result in the appearance of free polymer that testifies its stability at elevated tempertature. These data suggest that the main driving force of complexation is hydrophobic interactions between PEG and protein globule. An analogous conclusion was made by Furness E. L. et al. studying complexes based on lysozyme and PEG (*20*).

Enzymatic Properties of PEG−ChT Complexes. Enzymatic properties of PEG−ChT complexes were assayed by measuring the hydrolysis of low molecular weight substrate, *N*-benzoyl-L-tyrosine ethyl ester (BTEE), and macromolecular substrate, azocaseine.

The values of the Michaelis constant (K_m) and catalytic constant (k_{cat}) of BTEE hydrolysis catalyzed by PEG−ChT complexes [$K_m = (5.6 \pm 0.4) \times 10^{-5}$ M and $k_{cat} = 24 \pm 2$ s^{-1}) in the limits of the experimental error coincide with the corresponding values for native ChT. The same picture was observed for the hydrolysis of the macromolecular substrate catalyzed with PEG−ChT adduct: $K_m = 0.026 \pm 0.003$ M and $k_{cat} = 4.8 \pm 0.4$ s^{-1}. The fact that PEG−ChT complexes retain high enzymatic activity in respect to both subtrates suggests that polymer chains in complexes are placed far from the active site of enzyme.

Earlier (*13*) we showed that noncovalent complexes based on Pro and ChT are characterized by higher thermostability than native ChT. The investigation of the kinetics of thermoinactivation of PEG−ChT complexes at 45 °C shows that their stability does not differ from the stability of ChT. Since, as was shown earlier, complexes are not destroyed under these conditions, one may suppose that noncovalent fixation of PEG in contrast to block copolymers (*13*) does not result in the screening of hydrophobic patches at the surface of the protein globule of ChT, that is necessary for the increase in thermostability of the protein (*21*, *22*).

Complexes Based on PEG−ChT Adducts and Cyclodextrins. The steric availability of polymer chains of PEG incorporating in complexes with proteins was characterized by the investigation of the ability of polymers to form inclusion complexes with α-cyclodextrin (CyD). We used this approach for the localization of polymer chains in conjugates of ChT with Pro (*23*) and PEG (*24*). It is known that PEG forms inclusion complexes, "molecular necklaces", with α-CyD (*25*, *26*). In these structures, dozens of CyD molecules are threaded onto polymer chains of PEG. Polymer chains in covalent PEG−ChT conjugates also form complexes with CyD of the same composition as complexes based on free polymers (*23*). Thus, one-end coupling of PEG at the surface of protein globule exert no sufficient influence on the availability of the second end of polymer chain that is responsible for the recognition of PEG by CyD molecules and subsequent formation of molecular necklaces.

Mixing of CyD with the solution of PEG1900-ChT complex results in the formation of insoluble complex too. The content of CyD in ternary complexes CyD−PEG−ChT is ∼10% from the amount which might be expected if all polymer chains of PEG would participate in the complexation process. In addition, on the basis of the analysis of the composition of the precipitate it was shown that the interaction of PEG−ChT complex with CyD did not accompany by the disruption of noncovalent bonds between PEG and ChT. Thus, most of polymer chains in complexes appears to be spatially inavailable for the interaction with CyD. The structure of ternary complex CyD−PEG−ChT is schematically drawn in Figure 4. Thus, these data suggests the following model of PEG−protein complexes. It is shown that only a part of polymer chains in PEG−ChT complex is saturated with CyD, whereas other chains remain free. This suggestion is based on the data of cooperative character of complexation (*18*, *24*).

Table 3. Constants of the Rate of Thermoinactivation of ChT and Its Complexes with PEG (45.0 °C; pH 8.0)

complex	molar ratio of PEG incorporated in adduct to PEG-β-CyD	A_1	k_1 (s^{-1})	A_2	k_2 (s^{-1})
native ChT		0.33 ± 0.11	0.15 ± 0.07	0.67 ± 0.11	0.0240 ± 0.0040
		PEG−ChT Complexes			
$(PEG300)_{31}$-ChT		0.46 ± 0.10	0.20 ± 0.09	0.54 ± 0.12	0.0230 ± 0.0050
$(PEG300)_{31}$-ChT-PEG-β-CyD	1:2	~0.01		0.99 ± 0.01	0.0059 ± 0.0003
$(PEG750)_{12}$-ChT		0.56 ± 0.10	0.10 ± 0.01	0.44 ± 0.08	0.0240 ± 0.0060
$(PEG750)_{12}$-ChT-PEG-β-CyD	1:2	0.25 ± 0.02	0.13 ± 0.05	0.75 ± 0.05	0.0097 ± 0.0012
$(PEG1500)_8$-ChT		0.54 ± 0.05	0.22 ± 0.01	0.46 ± 0.04	0.0230 ± 0.0030
$(PEG1900)_7$-ChT		0.58 ± 0.06	0.32 ± 0.01	0.42 ± 0.04	0.0230 ± 0.0030
	1:0.25	0.32 ± 0.02	0.20 ± 0.06	0.68 ± 0.05	0.0170 ± 0.0016
	1:0.4	0.29 ± 0.01	0.15 ± 0.04	0.71 ± 0.03	0.0090 ± 0.0009
	1:0.5	0.27 ± 0.01	0.19 ± 0.01	0.73 ± 0.02	0.0070 ± 0.0007
$(PEG1900)_7$-ChT-PEG-β-CyD	1:1	0.38 ± 0.02	0.14 ± 0.03	0.62 ± 0.03	0.0070 ± 0.0009
	1:2	0.35 ± 0.01	0.24 ± 0.04	0.65 ± 0.02	0.0060 ± 0.0005
	1:4	0.36 ± 0.01	0.29 ± 0.06	0.63 ± 0.02	0.0050 ± 0.0005

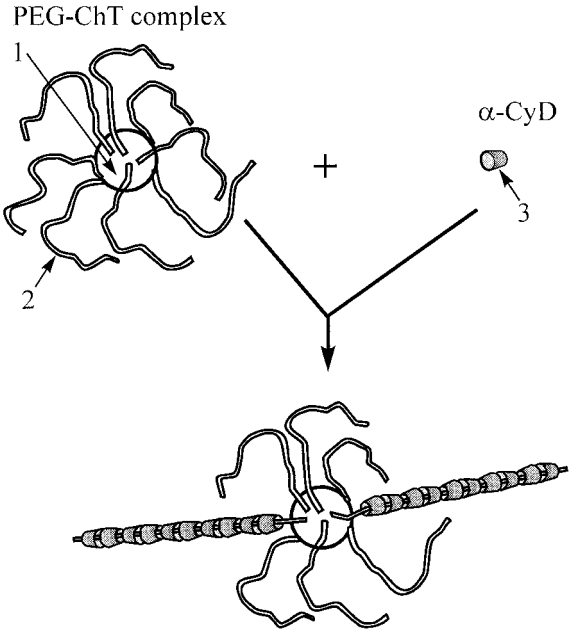

Figure 4. Structures of supramolecular ensemble on the basis of PEG−protein complexes and α-CyD. Abbreviations: *1*, ChT; *2*, PEG; *3*, α-CyD.

Thus, sorbtion of PEG occurs by means of multipoint interaction with the surface of protein globule resulting in sufficient decrease of the mobility of polymer chains. PEG is held on the protein globule by relatively few attached segments and the remaining segments form loops and tails. Polymer chains of PEG of different molecular mass are placed at the same sites of protein globule which are far from the active site. Total occupied surface in all cases is approximately the same.

One may expect that if we shall substitute CyD for its derivatives, the formation of molecular necklaces will be improbable because of inability of CyD derivatives of forming dense packing along the polymer chain. With this aim in mind, a branched polymer derivative of β-CyD, PEG−β-CyD (*14*), was used. Structure of this compound is presented in Figure 5. The choice of polymer derivative of β-CyD is caused by the following considerations. The modification of CyD by PEG chains results in the decrease of effective diameter of inner cavity of CyD. That is why PEG−α-CyD conjugate does not complex with PEG, whereas PEG−β-CyD does. The formation of inclusion complexes between PEG incorporated in PEG−protein complexes and single molecules of PEG−β-CyD seems to be quite probable. An analogous type of complexation was demonstrated by the example of the interaction between PEG-surfactants and PEG−β-CyD (*27*). Besides, using branched hydrophilic derivative of CyD would yield water-soluble inclusion complexes with PEG−protein adducts. One may anticipate that the change in microenvironment of protein globule due to the complexation with PEG−β-CyD would affect the functional properties of the protein.

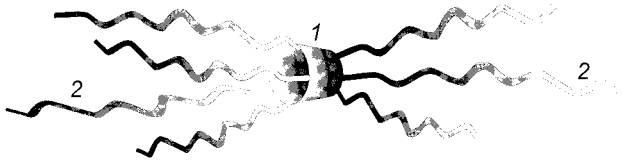

Figure 5. The structure of covalent adducts of β-cyclodextrin with PEG. 1 and 2 are cyclodextrin and PEG, respectively.

Three component complexes PEG−ChT−(PEG−β-CyD) were prepared using two methods: the addition of PEG−β-CyD to the solution of PEG−ChT complexes (stepwise method) and the action of elevated pressure on the mixture of three components with subsequent isolation of the product from the solution (one-step method). As the test for complexation between PEG−ChT complexes and PEG−β-CyD, we used the study of thermostability of ChT in three component complexes. The study of the kinetics of thermoinactivation of PEG−ChT complex shows that the addition of PEG−β-CyD results in the increase of thermal stability of the protein (Figure 6). The kinetic data are treated assuming that they can be described by the sum of two exponential terms:

$$A/A_0 = A_1 e^{-k_1 t} + A_2 e^{-k_2 t}$$

where A/A_0 is the relative enzyme activity, A_1 and A_2 are the relative contributions of each stage of the process, respectively ($A_1 + A_2 = 1$), and k_1 and k_2 are the rate constants of thermal inactivation for each stage. The results of this mathematical treatment of the curves are presented in Table 3.

As is seen from the Table 3, the increase in thermostability of the protein in ternary complexes based on PEG300, PEG750, and PEG1900 produced by stepwise method is first due to the decrease in the constant of the rate of thermoinactivation, k_2 (the change of k_1 is negligible). Figure 7 demonstrates the dependence of the k_2 value on molecular ratio of PEG−β-CyD to PEG incorporated in PEG−ChT complex. This plot looks like a titration curve with a breakpoint. Thus, the complexation between components takes place. The value of the breakpoint corresponds to a molar ratio of PEG−β-CyD to PEG

Table 4. Characteristics of Complexes Including ChT and PEG−*β*-CyD Prepared under the Pressure 16.0 MPa

no.	complex	content of the protein in complex (%)	number of molecules of branched component in the complex	A_1	k_1 (s^{-1})	A_2	k_2 (s^{-1})
1	ChT- PEG-*β*-CyD	81 ± 4	2	0.15 ± 0.01	0.12 ± 0.06	0.85 ± 0.04	0.0075 ± 0.0008
2	ChT-PEG-*β*-CyD-PEG1900[a]		2	0.35 ± 0.05	0.10 ± 0.03	0.65 ± 0.10	0.0078 ± 0.0014
3	ChT-PEG-*β*-CyD-PEG1900[b]	57 ± 3		0.45 ± 0.03	0.03 ± 0.01	0.55 ± 0.04	0.0062 ±0.0012

[a] Complex was prepared by two stages: (1) by the action of elevated pressure on the mixture of ChT and PEG-*β*-CyD and (2) by adding of PEG1900 to the two component complex. [b] Complex was synthesized by the action of elevated pressure on the mixture of three compounds: ChT, PEG1900, and PEG-*β*-CyD (method 2).

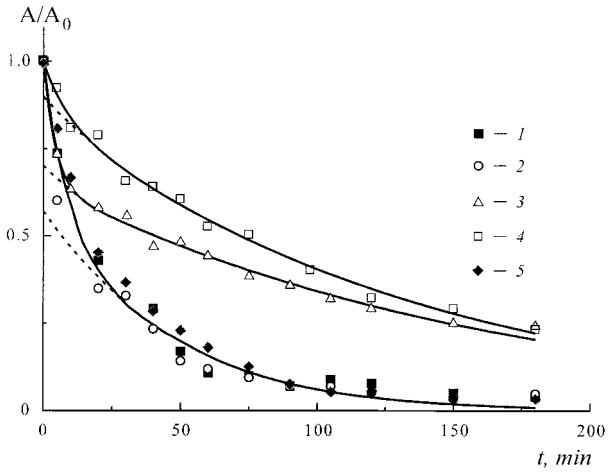

Figure 6. Kinetics of thermal inactivation of ChT (*1*) and its complexes: (*2*) $(PEG1900)_7$−ChT; (*3*) $(PEG1900)_7$−ChT-PEG−*β*-CyD complex was synthesized according to the method 1 at molar ratio of PEG−*β*-CyD to PEG incorporated in PEG-ChT complex equal to 1:2; (*4*) $(PEG1900)_7$−ChT-PEG−*β*-CyD, complex was synthesized according to the method 2; (*5*) a control mixture ChT and *β*-CyD. Conditions: 0.2 M Tris-HCl buffer, pH 8.0; 45 °C. Dotted lines correspond to the slow phases of thermal inactivation.

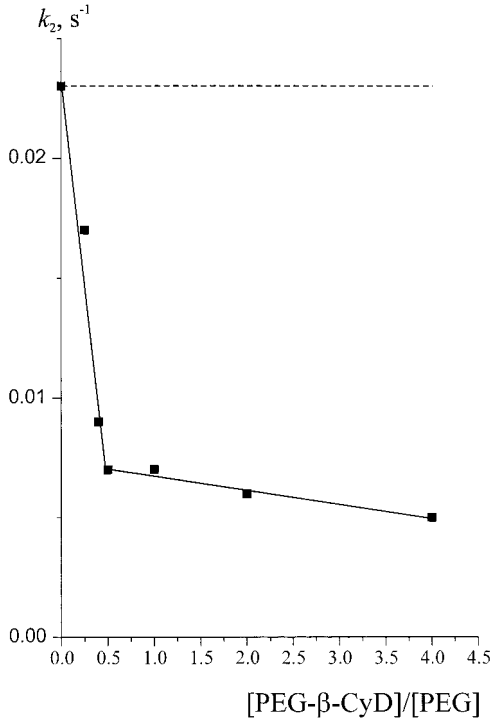

Figure 7. The dependence of the rate constant k_2 for thermoinactivation of ChT in three component complexes [$(PEG1900)_7$−ChT−PEG−*β*-CyD] on molecular ratio of PEG−*β*-CyD to PEG incorporated in PEG−ChT complex. Dotted line indicates the value of the k_2 for thermoinactivation of the native ChT and ChT in the presence of PEG−*β*-CyD.

incorporated in PEG−ChT complex equal to 0.5:1 (or 1:2). It means that only half the chains connected with a protein are available for the complexation with PEG−*β*-CyD. It should be noted that the same approach used for the study of steric availability of PEG chains incorporated in covalent PEG−ChT conjugates demonstrates that each chain of PEG participates in the complexation with PEG−*β*-CyD (*28*).

The increase in thermostability of the systems involving PEG−ChT complexes and PEG−*β*-CyD is probably caused by the fact that the complexation results in the "lengthening" and "branching" of polymer chains in polymer−protein complex. It is known that using polymer reagents with branched structure (e.g., di-PEG-reagents) for modification of proteins results in a sufficient increase in their stability compared with conjugates based on mono-PEG-reagents (*29*). Figure 8 shows the proposed structure of supramolecular ensemble containing all three components. This structure involving highly branched elements of PEG−*β*-CyD derivative, PEG, and protein is close by its architecture to dendrimers (*30*).

The formation of supramolecular structures based on PEG−protein complexes and PEG−*β*-CyD is a way for preparation of the protein samples with increased thermostability, which allows us to dispense with time-consuming stage of the chemical modification of the protein. It should be noted that the simplest approach for the formation of such supramolecular structures was realized when the pressure was applied to a mixture of three components (PEG, ChT, and PEG−*β*-CyD). As is seen from the Table 4 and Figure 6 (curve 3 and 4), constant k_2 for the system obtained by mixing of three components is close to this value for the complex obtained by stepwise synthesis.

When discussing the structure of the resulting ternary complex prepared under the action of elevated pressure on the mixture of three components, it was crucial to find out if PEG−*β*-CyD may form complexes with protein. A special experiment on the action of elevated pressure on the mixture of ChT and PEG−*β*-CyD conjugate verified the formation of the adduct containing two molecules of PEG−*β*-CyD per one molecule of the protein. It is of interest to compare the composition of complexes based on linear and branched PEG with equal length of the polymer chain. As was shown in ref *14*, an average molecular mass of the polymer chain in PEG−*β*-CyD is equal to 350 Da. Therefore, for comparison, we may use the data on the composition of the complex based on PEG300. As it is seen from the Table 1, the number of polymer chains in PEG300-ChT adducts equals to 31. This value exeeds the number of bound PEG−*β*-CyD in the complex with ChT in more than 1 order in magnitude. It seems to be quite reasonable from steric point of view and demonstrates that the complexation of linear PEG chains with proteins is favored over branched chains. It is of importance that in contrast to the PEG−ChT adduct,

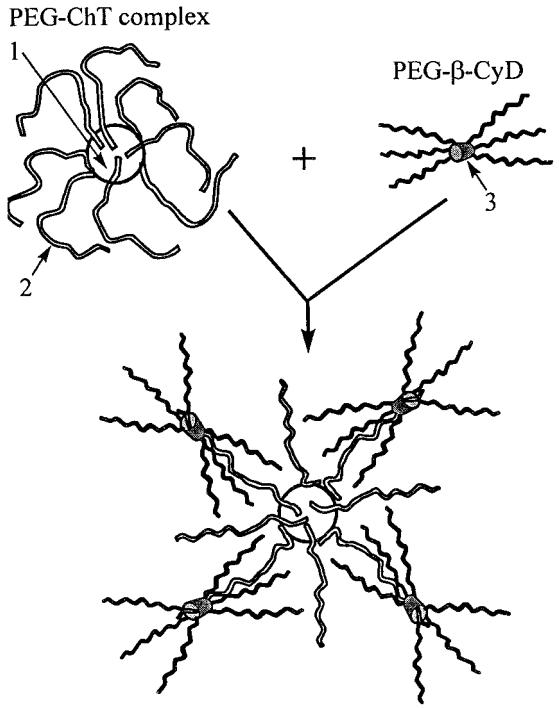

Figure 8. Structures of supramolecular ensemble on the basis of PEG−protein complexes and PEG−β-CyD. Abbreviations: *1*, ChT; *2*, PEG; *3*, PEG−β-CyD.

the adduct based on ChT and PEG−β-CyD is characterized by increased thermostability compared to the native ChT (Table 4). This fact demonstrates that the binding of branched PEG-macromolecules to the protein globule even at their less content produces a stabilizing effect. The addition of PEG to the adduct between PEG−β-CyD and ChT results in even more stabilizing effect (Table 4), which probably is indicative of the inclusion of PEG molecules in the adduct containing ChT and PEG−β-CyD.

Thus, the method of self-assembly which we used earlier for the synthesis of dendritic structures based on PEG-surfactants (*27*) was applied in this work for the preparation of adducts, which contain protein and are characterized by increased thermostability. These structures present unique example of supramolecular ensembles including native and synthetic macromolecules.

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