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INCORPORATION OF HYDROPHILIC PROTEIN MODIFIED WITH HYDROPHOBIC AGENT INTO LIPOSOME MEMBRANE

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Summary

The hydrophilic protein-enzyme, α-chymotrypsin, can be bound to the liposomal membrane after the preliminary increase in hydrophobicity induced by acylation of protein amino groups with palmitic chloroanhydride.

The efficacy of binding depends on the degree of modification. The bound enzyme almost completely preserves its catalytic properties and the ability to interact with a high molecular weight inhibitor. Binding can be performed during both the process of liposome formation and the incubation of a modified enzyme with preformed liposomes. According to ESR and fluorescence spectroscopy, the hydrophobic tail of the modified enzyme is incorporated into the membrane and the protein globule is located on the surface of the membrane. Protein incorporation causes an increase in the amorphous nature of the membrane, and the bound protein is not as mobile as the free protein. The approach discussed can be useful in binding soluble hydrophilic proteins to artificial membranes.

Introduction

Recently, the problem of protein binding to liposomes has been drawing increasing attention for at least two reasons. Firstly, the study of protein-liposome interactions can contribute to the knowledge of some processes occurring in natural membranes; secondly, active specific proteins firmly bound to a liposomal membrane can help in creating drug-targeting systems [1,2].

Unfortunately, non-membrane hydrophilic proteins cannot be firmly bound

to the liposome membrane in sufficient quantities either by adsorption on the surface of preformed liposomes, or by incorporation into the membrane during liposome formation [3—5]. Several approaches have already been suggested to solve the problem. A hydrophilic protein was, for example, bound to a membrane after its preliminary denaturation, which caused baring of the hydrophobic regions of the protein globule, facilitating protein binding to a liposome [1,6]. Nevertheless, it is evident that such denaturation should cause a noticeable loss of protein specific properties. We have developed a method of binding covalently hydrophilic proteins to the liposome surface via a spacer group [7]. This method provides effective binding of proteins with almost complete preservation of their specific properties, which was proved by experiments with animal [8].

Nevertheless, none of these methods permits more than $7 \cdot 10^{-5}$ mol of protein to be bound per mol of lipid.

At the same time, it is well known that integral membrane proteins are incorporated into natural or artificial membranes in high quantities [9—12].

As demonstrated in many experiments on reconstitution of natural membranes, integral membrane proteins unlike the non-membrane proteins contain long hydrophobic portions in the polypeptide chain or the long hydrophobic tails covalently bound to the polypeptide chain and can be firmly bound to membranes due to hydrophobic interaction [13].

It is quite reasonable then to expect the artificial increase in hydrophobicity of hydrophilic proteins to increase their affinity for the phospholipid membrane. Thus, the new phospholipid-containing alkylating agent for modification of immunoglobulins was shown to be the 'anchor' for attaching proteins to liposomes during joint incubation [14]. A more simple method of increasing hydrophobicity — modification of protein amino groups with reactive derivatives of long chain fatty acids — also leads to a sharp increase in the quantity of liposome-bound hydrophilic protein [15]. In the latter case, the degree of increase in hydrophobicity can be controlled by the type of fatty acid and by the degree of modification.

Here we present the data of a detailed study on the interaction between liposomes and the hydrophilic protein-enzyme, α-chymotrypsin, the amino groups of which have been preliminarily acylated by the action of palmitic chloroanhydride.

Materials and Methods

Materials.

α-Chymotrypsin, egg yolk lecithin, cholesterol, cholic acid, trinitrobenzenesulfonic acid and pancreatic trypsin inhibitor were the products of Sigma Chemical Co., U.S.A. Chloroanhydride of palmitic acid was obtained from Supelco, U.S.A., α-chymotrypsin-specific low molecular weight subtrate, *N*-acetyl-L-tyrosine ethyl ester from Koch-Light, U.K. [^{14}C]Cholesterol oleate was obtained from the Radiochemical Centre Amersham, U.K. Sepharose 4B was the product of Pharmacia, Sweden. All other reagents, salts and components of buffer solutions were analytical grade preparations from Reakhim, U.S.S.R.

Methods.

Preparation of palmitoyl chymotrypsin. To obtain palmitoyl chymotrypsin, a solution containing 0.145 M NaCl, 10^{-3} M phosphate buffer and 1% cholate, pH 8, was added with an acetone solution of palmitoyl chloride and the mixture was sonicated using an ultrasound disintegrator UZDN-1 (U.S.S.R.). The mixture obtained was immediately added with α-chymotrypsin solution up to a final enzyme concentration of $5 \cdot 10^{-5}$ M and incubated for 2 h at 4°C and pH 8. The precipitate was separated by 1 h of centrifugation at 20 000 × *g*. Where necessary, the detergent was removed by dialysis. The degree of modification can be controlled by varying the palmitoyl chloride : protein ratio.

The degree of modification was determined by spectrophotometric titration of protein-free amino groups with trinitrobenzenesulfonic acid [16]. Depending on the reaction conditions, the degree of modification varied from 10 to 45%, that implies introduction of approximately one to six fatty acid residues into a single enzyme molecule (there are 14—15 titrated amino groups in an α-chymotrypsin molecule [17]).

Preparation of protein-containing liposomes. Protein-containing liposomes were obtained by two different methods. In the first case, the enzyme was incorporated into the liposome membrane during liposome formation according to the cholate-dialysis method [18,19]. For this purpose, a $5 \cdot 10^{-5}$ M solution of modified enzyme in 1% cholate was mixed with a solution of egg yolk lecithin in the same cholate buffer containing trace amounts of [^{14}C]cholesterol oleate. The final concentration of lipid was 10 mg/ml and that of the protein $2.5 \cdot 10^{-5}$ M. Then cholate was removed by dialysis against 0.145 M NaCl in 10^{-3} M phosphate buffer, pH 7.4, at 4°C for 15 h. The liposomes formed as a result of detergent removal were separated from the free enzyme by gel chromatography on a Sepharose 4B column (1.5 × 30 cm). Experiments with [^{14}C]-cholate have shown that after gel chromatography and subsequent dialysis, the liposomes formed contain no more than 0.4% (of the initial) of non-separated detergent.

In the second case, pure liposomes were obtained by sonication of a vacuum-dried lecithin film in 0.145 M NaCl, 10^{-3} M phosphate buffer, pH 7.4, at a lipid concentration of 10 mg/ml [7]. Liposomes were separated from lipid droplets and metal particles by centrifugation. Then the liposomes were thoroughly mixed with a protein solution in the same buffer at a protein : lipid molar ratio of about 1 : 100. The mixture was incubated at 4°C for up to 72 h. The free protein was separated by gel chromatography on a Sepharose 4B column (1.5 × 30 cm).

Samples for ESR spectroscopy were obtained at a protein : lipid molar ratio of 1 : 1000 or less, and the free protein was not separated. For spectroscopic studies, aliquots were taken from the incubation mixture at different time-points of incubation.

The concentration of liposomes was determined according to the radioactivity associated with liposomal [^{14}C]cholesterol oleate using the Liquid Scintillation System Mark III 6880 (U.S.A.). Protein concentrations were calculated according to a modification [20] of the method of Lowry et al. [34] or according to enzymatic activity.

Determination of α-chymotrypsin enzymatic activity. Enzymatic activity of

native α-chymotrypsin and α-chymotrypsin immobilized on liposomes was determined following the initial rates of catalytic hydrolysis of the specific low molecular weight substrate, *N*-acetyl-L-tyrosine ethyl ester, in the pH-stat TTld Radiometer, Denmark, under standard conditions [21]. The measurements were made using 0.01 M substrate solution in 0.145 M NaCl at 20°C and pH 7.5, cell volume 10 ml.

When enzyme inhibition with a high molecular weight inhibitor was studied, a solution of pancreatic trypsin inhibitor of known concentration was added to the enzyme solution and enzymatic activity preserved was determined in the pH-stat [7].

Fluorescence spectroscopy of liposome-bound α-chymotrypsin. Fluorescence spectra of the free and liposome-bound modified enzymes were studied using the Aminco-Bowman spectrofluorimeter (U.S.A.) at the excitation wavelength of 280 nm [22]. For this purpose, 3 ml of pure liposome suspension containing up to 0.05 mg/ml of the modified protein were placed into a 1 cm cuvette, and the fluorescence spectra were recorded at different time-points of incubation. The fluorescence of the native protein, pure palmitoyl chymotrypsin, and the mixture of the non-modified enzyme with liposomes was determined at the same protein concentrations and incubation time-points as a control.

ESR spectroscopic studies of the processes of modified α-chymotrypsin incorporation into the liposome membrane. A new method of ESR spectroscopy, the free radical recombination method [23—25], was used in our studies. This method is based on the investigation of the recombination kinetics of a free radical label arising as a result of indirect radiolysis of biological compounds at a low temperature (77 K), or during ultraviolet irradiation of their aqueous solutions or dispersions. In this reaction, hydrogen atoms split off the molecule of water, the main component of the system, and interact with the hydrocarbon fragments of solubilized molecules, forming a free radical label. Upon the subsequent increase in temperature as a result of the transition mobility of the molecules in the free radical state, these free radicals can recombine at the temperature specific for each compound, thus decreasing the intensity of the ESR signal.

The biomolecular recombination reaction has a complex kinetic mechanism [26]. Under isothermal conditions, the reaction proceeds only to a certain degree of transformation, and only a temperature rise permits the process to be resumed.

For ESR spectroscopy measurements, samples of native or modified enzyme, pure and protein-containing liposomes were placed into ampules made of the special glass SK-4B (U.S.S.R.), that gives no ESR signal upon irradiation. O_2 was removed from the samples by aspiration with argon gas. After an appropriate incubation period, each sample was frozen at 77 K and subjected to irradiation by γ-rays with Co^{60} up to doses of 10 Mrad. In some cases, the samples were irradiated with ultraviolet light at 77 K for 2 h using the ultraviolet lamp DRS-1000 (U.S.S.R.) with a set of special filters. ESR spectra were registered on an ESR-2M-spectrometer (U.S.S.R.), Institute of Chemical Physics. Free radical concentration was determined by the double-integration method according to a special nomogram [27].

Results and Discussion

Incorporation of palmitoyl chymotrypsin into the membrane during the process of liposome formation

We have studied the dependence of the quantity of the incorporated protein on the degree of protein amino groups modification with palmitoyl chloride. The data of Table I show that with the increase in modification degree, i.e., the 'hydrophobicity' of the protein (from none to approximately three palmitic acid residues per protein molecule), a more than 10-fold increase in the binding of the protein with liposomes can be observed. This points to the decisive role of hydrophobic interaction in the binding. As a result, up to $3 \cdot 10^{-4}$ mol protein can be bound per mol of lipid, which noticeably exceeds the value achieved in the case of covalent binding [7]. At the same time, it was shown that a moderate degree of modification slightly affects the catalytic properties of α-chymotrypsin (see Table I).

After destroying enzyme-containing liposomes by the addition of a small quantity of Triton X-100, the enzymatic activity of the system increased 5-fold for liposomes with the native enzyme and only 2-fold for those with the modified protein. This means that native α-chymotrypsin is mainly entrapped in the inner water space of a liposome. At the same time, the enzyme with greater hydrophobicity is incorporated into the membrane and can be more or less evenly distributed between the inner and the outer membrane monolayers.

We have studied the ability of the liposome-incorporated modified enzyme to interact with a high molecular weight protein inhibitor (a model of interaction between a bound specific molecule and a target). The results presented in Fig. 1 show that the inhibition pattern of the liposome-bound modified enzyme differs only slightly from that of the native free enzyme.

So, artificially increasing the hydrophobicity of a non-membrane hydrophilic protein (after which the protein, to a certain extent, resembles a membrane hydrophilic protein) can increase its binding with liposomes without affecting its specific activity.

TABLE I

INCORPORATION OF THE NATIVE AND INCREASED HYDROPHOBICITY-CONTAINING α-CHYMOTRYPSIN INTO THE LIPOSOME MEMBRANE DURING THE PROCESS OF LIPOSOME FORMATION BY THE CHOLATE-DIALYSIS METHOD

The protein content was determined following α-chymotrypsin activity. After modification, the enzyme solution formed some precipitate due to partial aggregation caused by the increase in hydrophobicity. The precipitate was in all cases removed by centrifugation. The clear supernatant contained enzyme with the above-mentioned average modification degree (as was determined by free NH_2-group titration), with catalytic activity of about 40 and 30% of the initial for the samples with an average modification degree of 6 and 22%, respectively. Only clear supernatant was used in all further experiments.

The average degree of protein amino group modification with palmitoyl chloride (%)	Preservation of enzymatic activity after modification (%)	Bound protein (mol/mol lipid)
0 (native enzyme)	100	$1.3 \cdot 10^{-5}$
6	85	$1.4 \cdot 10^{-4}$
22	60	$3.0 \cdot 10^{-4}$

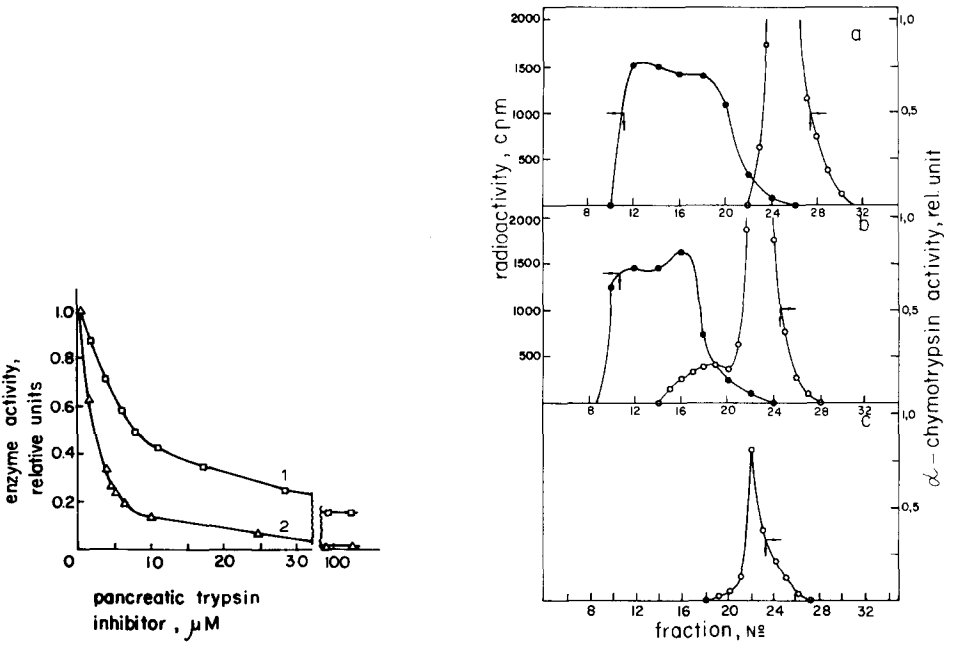


Fig. 1. Inhibition of the native α-chymotrypsin (2) and the liposome membrane-bound palmitoyl chymotrypsin (1) with the pancreatic trypsin inhibitor. (For experimental conditions see Materials and Methods).

Fig. 2. Gel chromatography on a Sepharose 4B column of the liposome/non-modified α-chymotrypsin mixture (a); liposome/palmitoyl chymotrypsin mixture (b) and pure modified enzyme (c). (In samples a and b the incubation time was 48 h; the modified enzyme contained approximately one fatty acid residue per molecule. For experimental conditions see Materials and Methods). ●———●, radioactivity of [^{14}C]-cholesterol oleate-labelled liposomes; ○———○, catalytic activity of α-chymotrypsin.

Incorporation of modified chymotrypsin into preformed liposomes during joint incubation.

Can artificial increasing of hydrophobicity increase the binding of a hydrophilic protein during its incubation with preformed liposomes? The data on gel chromatography of different protein and protein-liposome systems show that incubation of the native enzyme with preformed liposomes gives no noticeable binding effect, even after 50 h (Fig. 2a). At the same time, incubation of the modified enzyme (with a modification degree of about 20%) with preformed liposomes leads to the appearance of protein in the liposome fraction (see Fig. 2b). According to the kinetic data, the quantity of the bound enzyme is about $4.7 \cdot 10^{-5}$ mol per mol lipid, which corresponds — even in case of monolamellar liposomes [28] — to the binding of four to six protein molecules per liposome molecule with a diameter of about 800—1000 Å (such liposomes are formed under our experimental conditions [7]).

The small shift of the free modified enzyme peak (Fig. 2c) in comparison with the free non-modified enzyme can be explained by the formation of modified enzyme aggregates of moderate size as was observed under similar conditions [14]. Thus, artificial increase in hydrophobicity causes effective binding of a hydrophilic protein during its incubation with preformed liposomes. We

have studied the incorporation process and the properties of the protein-liposome system using two different methods: to study changes in protein properties, fluorescence spectra were registered [29] and to study changes in molecular dynamic properties of the membrane we used ESR spectroscopy, the free radical recombination method [23—25].

Changes in the fluorescence spectra of the modified protein during its incubation with preformed liposomes

It was recently shown [29] that incorporation of a membrane protein into the lipid bilayer causes an increase in protein fluorescence at 330 nm as a result of an increase in tryptophan residue fluorescence in the lipid phase. Studying the fluorescence spectra of palmitoyl chymotrypsin incubated with preformed liposomes for different periods of time, we, in contrast with the data of Ref. 29, observed a significant decrease in protein fluorescence and a shift of the fluorescence maximum towards a higher wavelength (Fig. 3a). This phenomenon is only characteristic of a system consisting of liposomes and modified enzymes. Fig. 3b shows that no change of fluorescence intensity or maximum position was observed for pure native or modified proteins during their incubation. A small decrease in protein fluorescence, independent of the incubation time for a system containing liposomes and a non-modified enzyme, was also observed [30], and can be explained by some polarity changes in the vicinity of the lipid membrane.

The fluorescence extinguishing observed cannot be explained by the presence of solubilized O_2 in the system [31], for the samples completely devoid of O_2 gave the same results. On the other hand, some conformational changes can occur in a protein molecule under the action of the lipid phase that increase aromatic amino acid residue accessibility towards water molecules. At the same time, the constancy of enzyme catalytic properties during all manipulations makes the occurrence of great conformational changes doubtful. The action of a heavy element atom located in the vicinity of the protein globule is more probable [32]. The phosphorus of the polar head of a phospholipid molecule may serve as the heavy element. In this case, the conformational changes in the protein molecule are not necessary, for during extinguishing the energy

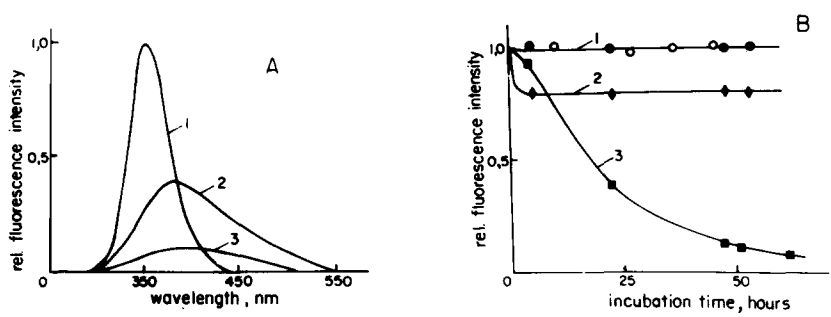


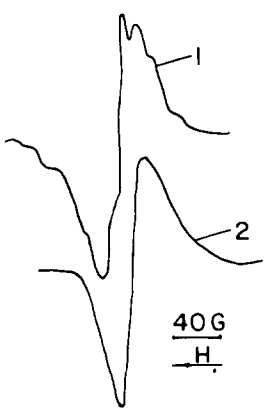
Fig. 3. Fluorescence spectra of the modified enzyme/liposome mixture. (A) The changes in the fluorescence spectra intensity and fluorescence maximum position during the incubation of the modified enzyme with preformed liposomes. 1, initial mixture; 2, 20 h of incubation; 3, 50 h of incubation. (B) The dependence of maximal protein fluorescence on the incubation time. 1, pure native α-chymotrypsin or pure palmitoyl chymotrypsin with the degree of modification of about 20%; 2, the native enzyme with preformed liposomes; 3, palmitoyl chymotrypsin with preformed liposomes.

can be transferred over a distance greater than the diameter of the α-chymotrypsin globule [33]. A joint action of both factors is also possible because the shift of the fluorescence maximum position often points to a transfer of the fluorescence group into more polar surroundings [30].

The difference between our results and the results of Vaz et al. [29] is quite natural, for in their experiments the whole protein molecule was incorporated inside the lipid bilayer. In our case, only hydrophobic modifier residues are incorporated into the lipid phase and the protein molecule itself is 'spread' on the surface of the lipid membrane.

ESR spectroscopic studies of interaction between a protein of increased hydrophobicity and liposomes

What are the changes in the dynamic properties of the fixed protein molecule and its lipid surrounding? The typical ESR spectra of protein and lipid free radicals induced by a low-temperature γ-ray or ultraviolet irradiation of liposomes, protein or their mixtures are shown in Fig. 4. Radiolysis of water dispersions or solutions of biomolecules at 77 K mainly provoke the appearance of two different radicals — $OH^{\cdot}$ radicals and biomolecule radicals ($R^{\cdot}$). During the warming of the system, the $OH^{\cdot}$ radicals recombine very quickly and already at 120 K, only the more stable $R^{\cdot}$ radicals are present in the system.



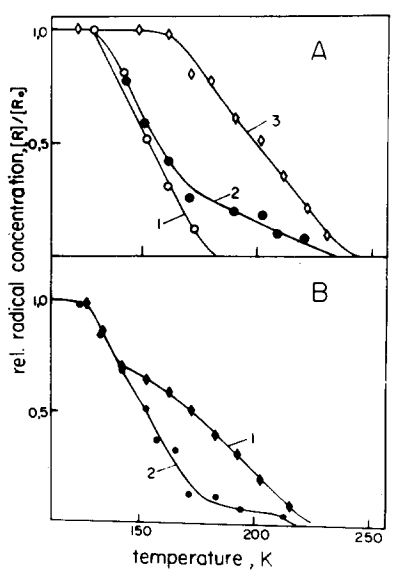


Fig. 4. The typical ESR spectra of the lipid (1) and protein (2) free radicals (for experimental conditions see Materials and Methods).

Fig. 5. (A) Temperature-dependent recombination of the free radicals induced by γ-irradiation with γ-rays. 1, pure native or pure modified α-chymotrypsin; 2, palmitoyl chymotrypsin-containing liposomes, obtained by the cholate-dialysis method; 3, pure liposomes. (B) Temperature-dependent recombination of ultraviolet irradiation-induced protein free radicals in protein-containing liposomes, obtained by the cholate-dialysis method. 1, liposomes with the modified enzyme; 2, liposomes with the native enzyme. (The degree of protein modification is about 20%. For experimental conditions see Materials and Methods).

The recombination of γ-irradiation-induced free radicals during the stepwise warming of the cholate-method obtained protein-containing liposomes is shown in Fig. 5a. The curves presented demonstrate that the radical recombination in the samples of pure modified or native protein, pure liposome suspension and the suspension of protein-containing liposome proceeds at different temperature intervals. Thus, the initial part of curve 2 (protein-containing liposomes) reflects the recombination of protein radicals which can diffuse in the water matrix like the radicals of the pure protein (curve 1). The final part of curve 2 (170—230 K) resembles rather the recombination of the free radicals in the pure liposome suspension (curve 3). These data show that in protein-containing liposomes, processes characteristic of both pure protein and pure liposomes are observed.

In Fig. 5b, data are presented showing the temperature-dependent recombination of the protein free radicals induced by ultraviolet irradiation for the proteoliposomes obtained by the cholate-dialysis method in the presence of palmitoyl chymotrypsin and native α-chymotrypsin. It can be seen that the recombination of the modified protein free radicals is shifted to higher temperatures in comparison with the recombination of the native protein free radicals. These data show that the molecules of palmitoyl chymotrypsin are located in more 'rigid' surroundings, i.e., in or on the lipid membrane, and the native protein is included into the water phase of liposomes.

ESR spectroscopy renders important information on the process of modified protein incorporation into the preformed liposomes during joint incubation. The temperature recombination curves of γ-irradiation-induced free radical in liposome/palmitoyl chymotrypsin mixtures with different incubation times are presented in Fig. 6. In a freshly obtained mixture or in a mixture with a relatively short incubation time, some radicals belong to the modified protein molecules (the initial part of curves 1 and 2) and some to the lipid components

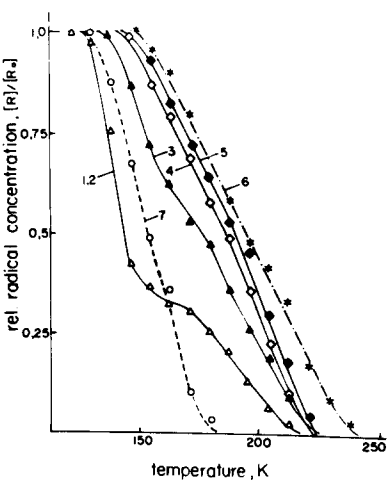


Fig. 6. Temperature-dependent recombination of the free radicals induced by γ-irradiation with γ-rays in the mixture of the modified enzyme and preformed liposomes. 1, 2, the initial moment of incubation and 6 h of incubation; 3, 20 h of incubation; 4, 35 h of incubation; 5, 50 h of incubation; 6, recombination of the pure liposome radicals; 7, recombination of the pure native or modified α-chymotrypsin radicals. (The degree of protein modification is about 20%. For experimental conditions see Materials and Methods).

of liposomes (the final part of the same curves). These radicals disappear at different temperature intervals, which causes a break on the recombination curve. With an increase in incubation time this break gradually disappears, pointing to the gradual transition of the modified protein from the water into the lipid bilayer and after a 50 h incubation only lipid radicals are found in the system (numerous lipid radicals mask protein radicals in the liposomes). An important conclusion was drawn from the study of the form of the recombination curves: the greater steepness of the lipid radical recombination curves during protein/liposome mixture incubation (compare the incline angle of curves 3—6 on Fig. 6) shows that protein incorporation causes some increase in amorphous character of the lipid bilayer [25].

Concluding remarks

(1) Artificial increase in hydrophobic content permits non-membrane hydrophilic proteins to be effectively incorporated, both passively and actively, into model membranes. This process can be controlled by varying the structure and quantity of the hydrophobic content-increasing groups in the protein molecule.

(2) The processes of modified hydrophilic protein incorporation into the lipid bilayers can serve as a model of natural biological membrane organization.

(3) The approach discussed can be useful for incorporation of transporting molecules into liposomal systems for drug targeting.

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