Catalytic properties and conformation of hydrophobized α-chymotrypsin incorporated into a bilayer lipid membrane

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Abstract A set of artificially hydrophobized α-chymotrypsin derivatives, carrying 2–11 stearoyl residues per enzyme molecule, were synthesized and their catalytic parameters and conformation in water solution and in the liposome-bound state were investigated. Hydrophobization of α-chymotrypsin and its further incorporation into phosphatidylcholine (PC) liposomes have no effect on the rate constant of the *N*-acetyl-L-tyrosine ethyl ester (ATEE) ester bond hydrolysis (k_{cat}). At the same time, an increase in the number of stearoyl residues attached to the enzyme results in a drastic decrease of ATEE binding to the active center (K_M increase). Incorporation of the hydrophobized enzyme into the PC liposome membrane results in K_M recovery to nearly that of native α-chymotrypsin. The above changes are accompanied by partial unfolding of the enzyme molecules observed by fluorescence measurements. The obtained results are of interest to mimic the contribution of surface hydrophobic sites in the functioning of membrane proteins.

Key words: α-Chymotrypsin; Catalytic activity; Conformation; Hydrophobization; Protein-lipid interaction

1. Introduction

It is known that cell membrane proteins carry external hydrophobic sites, which play an important role in binding of these proteins to the lipid bilayer [1–4]. Interaction of protein sites with the hydrophobic inner part of the bilayer, formed by alkyl residues of lipid molecules, determines to a great extent the conformation and functioning of membrane proteins [4–6]. Extraction of proteins from a cell membrane to water solution usually results in a substantial disturbance of their conformation and a decrease in their biological activity. The functioning of isolated membrane proteins can be modulated by addition of lipids or synthetic detergents [1–4].

It has been shown recently that modification of a hydrophilic enzyme, α-chymotrypsin, with long-chain alkyl residues decreased its stability in water solution [7–9] and at the same time provided it with affinity to the lipid bilayer [10–14]. It has also been demonstrated that the catalytic activity of artificially hydrophobized α-chymotrypsin, placed into the three-component water-octane-surfactant system, displayed strong sensitivity towards the composition of the system [15] and a type of supramolecular organization [16]. At the same time, the influence of the lipid bilayer on the catalytic properties and conformation of artificially hydrophobized enzymes remains practically unstudied up to now.

In the present paper we investigated how an extent of hydrophobization affects the conformation and catalytic properties of α-chymotrypsin in water solution and incorporated into the lipid vesicle (liposome) membrane. The results obtained are of interest to mimic the contribution of hydrophobic sites in the functioning of membrane proteins.

2. Materials and methods

2.1. Modification of α-chymotrypsin by stearic acid residues

α-Chymotrypsin amino groups were chemically modified by the stearic acid *N*-hydroxysuccinimide ester (NHSS) in the deoxycholate micelle solution according to a modification of the procedure of Huang et al. [10]. 16 mg of α-chymotrypsin (EC 3.4.21.1) purchased from Sigma, USA, was dissolved in a mixture composed of 7 ml of 0.1 M borate buffer, pH 8.5, 10 mM *N*-acetyl-L-tyrosine and 3% (w/v) of deoxycholate. 700 μl of NHSS solution in dioxane was then added, and the mixture was stirred for 45 min at room temperature. After that, the mixture was centrifuged to remove free stearic acid. The stearoylated α-chymotrypsin was separated from the detergent and organic solvent by gel filtration on Sephadex G-50 and lyophilized. The number of stearoyl residues covalently attached to the protein was determined by spectrophotometric titration of free protein amino groups with trinitrobenzenesulfonic acid (Merck, Germany) [17] measuring optical density at $\lambda = 405$ nm in a Multiscan Plus microplate photometer (Titertek, Finland). Native α-chymotrypsin was used as a control, assuming each protein molecule carries 16 amino groups [18].

α-Chymotrypsin concentrations were measured either spectrophotometrically at $\lambda = 280$ nm ($\varepsilon = 50\,000$ M^{-1} cm^{-1}), or by the Lowry method [19]. Both approaches were found to give the same results.

2.2. Unilamellar liposomes

To prepare egg yolk phosphatidylcholine (PC) unilamellar liposomes the following procedure was used [20]. First, the corresponding amount of PC ethanol solution, purchased from Kharkov Bacterial Preparations Company (Ukraine), was put in a flask. Then the solvent was carefully evaporated under vacuum. A thin layer of lipid mixture was dispersed in 0.1 M borate buffer, pH 8.5, in a Cole-Parmer 4700 (USA) ultrasonic homogenizer (400 Hz, 2×200 s). Double-distilled water was used. Liposome samples thus obtained were separated from titanium dust by centrifugation ($8300 \times g$, 5 min) and used within 1 day. The diameter of liposomes, measured by quasi-elastic light scattering, was in the range of 50–70 nm.

2.3. Binding of modified α-chymotrypsin to liposomes

The stearoyl-α-chymotrypsin solution was mixed with the PC liposome suspension at a protein to lipid molar ratio of about 1:500 and a final protein concentration equal to 20 μM. The mixture was then sonicated (300 Hz) for 100 s at 0°C. Proteoliposomes thus prepared were separated from non-bound enzyme by gel filtration on Sepharose CL-4B; 10 mM borate buffer, pH 8.5, was used as eluent. Control experiments have shown that sonication of the stearoyl-α-chymotrypsin solution under these conditions did not affect the enzyme activity. Therefore, the amount of bound enzyme was determined from catalytic activity measurement (see below).

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Abbreviations: ATEE, *N*-acetyl-L-tyrosine ethyl ester; NHSS, stearic acid *N*-hydroxysuccinimide ester; PC, egg yolk phosphatidylcholine; STI, soybean trypsin inhibitor

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2.4. Enzyme catalytic activity

The catalytic activity of α-chymotrypsin, native and stearoylated dissolved in water and bound to PC liposomes, was determined using *N*-acetyl-L-tyrosine ethyl ester (ATEE) as a specific substrate. The rates of enzymatic ATEE hydrolysis were measured potentiometrically in 20 mM KCl solution at pH 8.0 using a RTS-822 pH-stat (Radiometer, Denmark).

2.5. Active site titration

The concentration of active sites in native and stearoylated α-chymotrypsin was determined spectrophotometrically with *N-trans*-cinnamoyl-imidazole (Sigma, USA) [21]. The concentration of active sites of stearoylated α-chymotrypsin bound to PC liposomes was determined using the soybean inhibitor titration procedure [22]. In the latter case, the proteoliposomes were incubated with soybean trypsin inhibitor (STI) for 30 min in 100 mM borate buffer solution at pH 8.5, the enzyme concentrations being much higher than the dissociation constant of the α-chymotrypsin-inhibitor complex. The residual enzyme activity was measured by potentiometric titration using ATEE. Concentrations of active sites were calculated from the initial secretions of the catalytic activity vs. STI concentration plot.

2.6. Equilibrium gel permeation chromatography

Possible interaction of ATEE with liposomes was analyzed with gel permeation chromatography using the Hummel-Dreyer technique [23]. A Sephadex G-50 (medium) column (1.1×10 cm) was equilibrated with 10 mM borate buffer additionally containing 20 mM KCl and 1 mM ATEE. The concentration of ATEE in the eluate was monitored with a flow-through UV-1740 detector (Bio-Rad, USA) at $\lambda = 280$ nm. PC liposomes were applied on the column and eluted with the same buffer. Binding of ATEE to liposomes was detected from the optical density value of a negative eluate peak following a positive liposome peak.

2.7. Fluorescence spectra

Fluorescence spectra of native and stearoylated α-chymotrypsin were measured using a Hitachi F-3000 spectrofluorometer (Japan) at $\lambda_{ex} = 280$ nm. The number of tryptophan residues in α-chymotrypsin samples accessible for quenching with iodide ions was determined using the procedure described by Lehrer et al. [24].

3. Results and discussion

Using a titration of enzyme active sites with *N-trans*-cinnamoyl-imidazole, it was found that their content in the native α-chymotrypsin was equal to 80%. Covalent attachment of stearoyl residues to α-chymotrypsin decreased the content of active sites to 55–60%, this value being practically constant for enzymes with an average number of stearoyl residues (*N*) of 2–12. The observed decrease was probably due to denaturation of some portion of the enzyme molecules (approx. 25–30%), resulting from their incubation in the presence of organic solvent during the modification procedure.

Hydrophobization of α-chymotrypsin led to aggregation of enzyme molecules in water solution. Using a gel permeation chromatography technique, the average number of hydrophobized molecules in such aggregates was found to be 4–8.

It was shown that an increase in the *N* value in modified α-chymotrypsin only slightly influenced the k_{cat} value of hydrolysis of the low molecular substrate ATEE in water solution (Fig. 1A, curve 1). This indicates that nearly all catalytic sites of aggregated α-chymotrypsin molecules were accessible for small ATEE molecules. In contrast to this, the K_M value of ATEE hydrolysis increased by two orders of magnitude (Fig. 1B, curve 1′).

Importantly, an increase in the *N* value was accompanied by a decrease of fluorescence intensity of the tryptophan residue of the enzyme molecule (Fig. 2A). This might result from denaturation of some portion of the enzyme molecules during

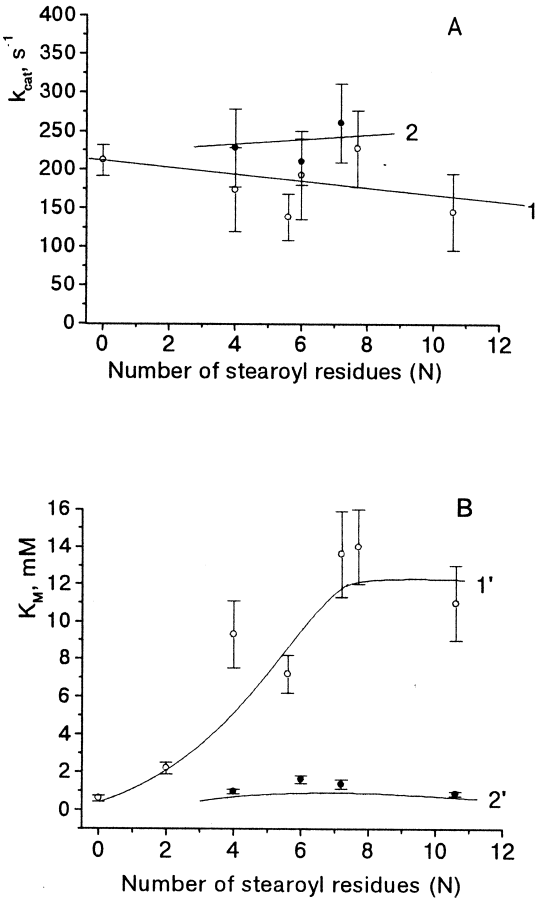

Fig. 1. Dependence of the catalytic constant, k_{cat} (A), and Michaelis constant, K_M (B), of ATEE hydrolysis induced by α-chymotrypsin in water solution (curves 1 and 1′) and after incorporation into the membrane of PC liposomes (curves 2 and 2′) on the number of stearoyl residues attached to the enzyme (*N*). Incubation mixture: 0.03 μM stearoyl-α-chymotrypsin active sites, 1.2 mM ATEE, 20 mM KCl; pH 8.0.

modification. In this case the decrease in tryptophan fluorescence should obviously follow the decrease in the content of active sites in the modified enzyme samples. However, as mentioned above the content of active sites in modified enzymes was practically independent of the *N* value, while the tryptophan fluorescence intensity decreased to 40% when increasing the *N* value from 2 to 12 (Fig. 2A).

At the same time, it is known that fluorescence quenching of tryptophan residues of water-soluble proteins in aqueous solutions can result from their interaction with a singlet form of water-dissolved oxygen [25]. In the light of this fact the above finding was likely caused by a partial unfolding of all hydrophobized α-chymotrypsin globules that increased the number of tryptophan residues available for oxygen molecules.

The partial unfolding of the enzyme globule after its modification by stearoyl residues was confirmed by another experiment. Fig. 2B represents the fluorescence intensity of tryptophan residues in the presence of potassium iodide, as an ionic quencher, plotted in modified Stern-Folmer graphs: $F_0/\Delta F - 1/[KI]$, where F_0 is fluorescence intensity without quencher, F is the intensity observed in the presence of a quencher and $\Delta F = F_0 - F$. According to Lehrer et al. [24], the $F_0/\Delta F$

value at 1/[KI] = 0 corresponds to the 1/*f* value, where *f* is the molar fraction of enzyme tryptophan residues quenched by I^- ions. As follows from the data in Fig. 2B, the *f* value increases from 38% for native α-chymotrypsin (curve 1) to 60% for α-chymotrypsin covalently modified with six stearoyl residues (curve 2). Taking into account that α-chymotrypsin molecule contains eight tryptophan residues [18], the above finding means that hydrophobization of α-chymotrypsin increases the number of fluorescent tryptophan residues, interacting with the quencher, from 3 to 5.

It should be especially emphasized that the conformational rearrangements, observed by fluorescence measurements, only partly affected the tertiary structure of the hydrophobized α-chymotrypsin globule. In fact, such rearrangements practically did not disturb the structure of the enzyme catalytic site, so that the k_{cat} value remained unchanged. In contrast, the substrate binding site of the modified enzyme might be affected by the same rearrangements, resulting in a K_M increase. In addition, stearoyl residues attached to either the same or neighboring enzyme molecules could compete with the substrate molecules for binding sites, increasing the apparent K_M value.

Binding of the hydrophobized enzymes to liposomes was achieved by sonication of enzyme water solutions with preformed PC liposome suspensions, followed by separation of proteoliposomes from non-bound enzyme by gel filtration. This procedure could result in different locations of bound enzymes within liposomes. Enzyme molecules could be adsorbed on the liposomal membrane, incorporated into the outer and/or inner leaflet of the liposome membrane and entrapped inside the inner water liposome cavity.

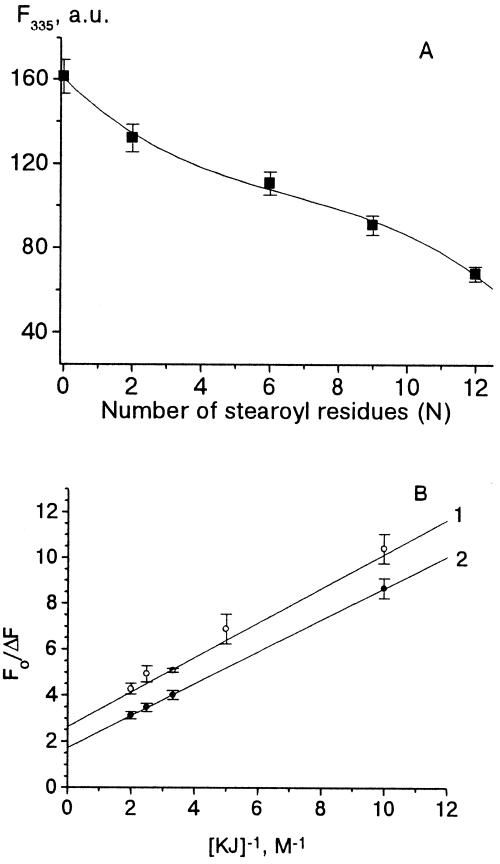

Fig. 2. A: Dependence of the fluorescence intensity of α-chymotrypsin on the number of stearoyl residues attached to the enzyme (*N*) at λ_{em} = 335 nm (λ_{ex} = 280 nm). Enzyme concentration 1 μM. B: Modified Stern-Folmer graph: quenching of native α-chymotrypsin (curve 1) and α-chymotrypsin modified with six stearoyl residues (curve 2) by iodide ions. Enzyme concentration 0.1 μM; 10 mM borate, pH 8.5, ionic strength was maintained at 0.5 M by addition of KCl up to 0.5 M.

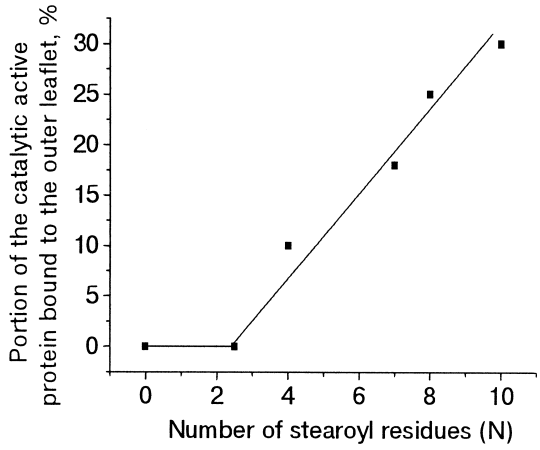

Fig. 3. Dependence of the extent of α-chymotrypsin bound to the outer leaflet of PC liposomes on the number of stearoyl residues attached to the enzyme (*N*). Enzyme to lipid ratio was approx. 1:2500. Enzyme concentration 12 μM; 10 mM borate, pH 8.5.

We titrated the enzyme active sites in proteoliposomes with STI. As the liposomal membrane is impermeable for hydrophilic proteins [26], STI could interact only with the active enzyme molecules adsorbed on and/or incorporated into the outer leaflet of the original proteoliposomes. To estimate the total amount of the enzyme bound to liposomes, proteoliposomes were destroyed by the addition of 1% Triton X-100 and the active sites were then titrated with STI. It was found that addition of Triton X-100 resulted in a nearly twofold increase in the number of enzyme catalytic sites accessible to STI. In addition, Triton-induced disruption of proteoliposomes was also accompanied by a twofold increase in the level of enzyme catalytic activity. Two important conclusions follow from these data. First, in the original proteoliposomes half of the active enzyme molecules were accessible to STI (they were located on the outer leaflet of the liposomal membrane) while the other half were not (these enzyme molecules were located on the inner leaflet of the liposomal membrane and/or entrapped inside liposomes). Second, only enzyme molecules bound to the outer leaflet of the proteoliposomal membrane contributed to the catalytic activity of the intact proteoliposomes, measured in the experiment.

It was shown that liposomes prepared by sonication of a mixture of native α-chymotrypsin with preformed neutral PC liposomes exhibited no catalytic activity towards ATEE. This means that under the chosen experimental conditions (20 mM KCl solution, pH 8.0) native α-chymotrypsin did not adsorb on the PC liposome surface (Fig. 3), i.e. it was neither adsorbed on nor incorporated into the outer liposomal leaflet. Obviously, native α-chymotrypsin was not able to bind to the inner liposomal leaflet. Some part of the enzyme molecules could be entrapped inside liposomes, however it did not work in ATEE hydrolysis.

Hydrophobization of α-chymotrypsin by stearoyl residues provided it with affinity to the liposomal membrane probably due to the increase in the enzyme surface hydrophobicity. Binding of the modified enzyme to PC liposomes, checked by ATEE hydrolysis measurements, became appreciable only when the N value exceeded 2 (Fig. 3), unlike short oligopeptide molecules for which two attached fatty acid groups were already enough for the peptide to be strongly bound [27]. However, the fraction of the enzyme molecules bound to the outer leaflet of the liposomal membrane did not exceed 30 molar percent even at $N = 10$ (Fig. 3) and was not enhanced by increasing the liposome content up to 5000-fold excess of the lipid relative to the enzyme. Therefore, it is reasonable to assume that not all hydrophobized protein molecules were able to bind effectively to the PC membrane but only those with some favorable location of stearoyl radicals, randomly attached to the protein amino groups. Taking into account that native α-chymotrypsin did not bind to the liposomal membrane at all, it is reasonable to assume that interactions between stearoyl residues and the hydrophobic part of the lipid bilayer played a key role in binding the hydrophobized enzyme to the liposomal membrane. The enzyme molecules could be incorporated into the outer and inner liposomal leaflets. In addition, some part of the enzyme molecules could be entrapped inside the liposomes. It should be emphasized that only enzyme molecules located on the outer leaflet contributed to the catalytic activity of proteoliposomes.

Incorporation of α-chymotrypsin, modified with different numbers of stearoyl radicals, into the liposomal membrane hardly influenced the k_{cat} value which still remained quite close to the k_{cat} value for the native enzyme (cf. points in curve 2 and the point at $N = 0$ in curve 1, Fig. 1A). At the same time, the hydrophobized enzyme, incorporated into the liposomal membrane, exhibited a significant decrease in K_M value to nearly that of the native enzyme (Fig. 1B, curve 2′). Using an equilibrium gel permeation chromatography technique (see Section 2) it was shown that ATEE molecules did not bind to PC liposomes. This indicates that the observed decrease in K_M was not due to the concentration of ATEE molecules within the liposomal membrane.

Importantly, the tryptophan fluorescence intensity of the hydrophobized α-chymotrypsin molecules that had decreased on hydrophobization did not recover after their binding to the liposomes, indicating that tryptophan residues remained available for environmental O_2 molecules. This means that structural rearrangements in α-chymotrypsin molecules, detected by fluorescence measurements, were apparently responsible neither for the increase in the K_M value nor for its recovery. Most likely, the decrease in the K_M value could be attributed to some local molecular rearrangements in the vicinity of the substrate binding site of the enzyme, not affecting tryptophan residues.

Thus, using a rather simple model system we have shown that hydrophobization of water-soluble non-membrane enzyme gives it membrane sensitivity typical for native membrane proteins.

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