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A KINETIC STUDY OF α-CHYMOTRYPSIN INHIBITION IN MICELLAR AND LIQUID-CRYSTALLINE PHASES OF THE WATER—CTABr—HEXANOL SYSTEM

(*Presented by E. Lippmaa*)

In [1] it is shown that mixtures of cetyltrimethylammonium bromide (CTABr), hexanol and water can form, at room temperature, a number of structurally different liquid-crystalline (LC) phases. In their phase diagram the region with a lamellar structure is of special interest, having a typical bilayer structure: the LC regions consist of layers of amphiphilic materials, separated by layers of water. The polar head groups of the amphiphilic substances are in contact with the water phase, while the hydrocarbon tails stick together to form a hydrophobic core. The thickness of a bilayer, as determined by X-ray crystallography, amounts to 22—24 Å. The spacing between two adjacent double layers depends very much on the water content of the mixtures and varies from 7 to 80 Å. We have chosen lamellar mesophase as a model system for biological membranes. This model system, as compared with the lipid bilayer matrix, has the following advantages: (a) the chemical composition of the system is simple and well defined; (b) the geometric structure is well-known from X-ray analysis; (c) it is possible to obtain a homogeneous orientation of the bilayer system which, in turn, leads to a homogeneous ordering of the incorporated molecules. Spin labelling technique reveals the similarity of phospholipid dispersion and the LC model systems.

The physical properties of proteins in the presence of micelles and in LC environment are of interest for both membrane-bound and water-soluble proteins. Although the use of pure soluble proteins, most of which are normally not associated with membranes, is debatable, the approach has the advantage of a better-defined system and it has already provided some insight into the mechanism of phospholipid—protein interactions. Detergent—protein complexes may be good models for membrane—protein complexes [2, 3]. In addition, detergents have often profound effects on the structure of water-soluble globular proteins, which allow a study of protein-stabilizing forces [4, 5] (cf. ref. [5]).

The classical studies on the effect of surface charge on enzyme kinetics were made by Katchalski et al. (cf. ref. [6]), who immobilized soluble enzymes, mostly trypsin and chymotrypsin, on artificial solid matrices of polyanionic and polycationic character. In the present study, in contrast to [6], we investigated soluble enzyme kinetics in biological membrane models. The antichymotrypsin activity of organophosphorus inhibitors (OPI) was studied in various phases of the CTABr—hexanol—water system. This study is closely related to α-chymotrypsin-catalyzed hydrolysis of *p*-nitrophenylacetate in micellar and LC phases of the CTABr—hexanol—water system [7]. Earlier investigations have provided information on the effects of controlled phase-dependent rate changes in the enzymatic reaction.

The antichymotrypsin activity of O-alkyl-*p*-nitrophenylmethylphosphonates $(C_nH_{2n+1}O)(CH_3)P(O)OC_6H_4{-}NO_2{-}p$, $n=3$ to 8, was measured in micellar and LC phases. The kinetic mechanism of the reaction of α-chymotrypsin with organophosphorus quasisubstrates is the same as with substrates [8, 9]:

$$E+S \overset{K_S}{\rightleftharpoons} ES,$$

$$ES \overset{k_2}{\rightarrow} ES'+P_1,$$

$$ES'+H_2O \overset{k_3}{\rightarrow} E+P_2,$$

where K_S is the dissociation constant of ES, and ES' is the acylenzyme. In the reaction of chymotrypsin with OPI $k_3=0$.

Experimental

Sample preparation and experimental procedures were as described in [7, 10]. The reaction rate of α-chymotrypsin with O-*n*-alkyl-*p*-nitrophenylmethylphosphonates (25°C, pH 7.10, in 0.2 M phosphate buffer, containing 1 vol.% ethanol or dioxane) was measured in micellar and LC phases of CTABr—hexanol—H_2O system. All materials have been described previously [7, 11].

The reaction rate of α-chymotrypsin with OPI was determined in the pseudo first-order kinetics conditions ($[I]_0 \gg [E]_0$):

$$E+I \overset{k_{II}}{\rightarrow} EI'+P_1,$$

$$k_{II}=\frac{k_I}{[I]}=\frac{2.303}{t[I]}\log\frac{[E]_0}{[E]_t}.$$

The reaction was followed by measuring the absorption of *p*-nitrophenolate ions at 400 nm as described previously [11].

Results and discussion

Antichymotrypsin activity measurements of O-*n*-alkyl-*p*-nitrophenylmethylphosphonates ($n=3$ to 8) were determined in buffer solution, in micellar phases with CTABr alone, in hexanol—CTABr and in LC lamellar neat phases (the hexanol—CTABr ratio was held constant within the micellar phases with hexanol and the corresponding LC phases). In several systems the environment exerts a major influence on enzyme kinetics in the new local conditions which are very different from experiments performed in a homogeneous solution. In such studies, two factors play an important role in enzyme kinetics: (1) the chemical composition and physical state of the environment and hydrophobic properties, nature and density of fixed charges modulation of enzyme properties in these conditions; (2) the local concentration distribution of the reactants in the micellar and LC phases.

The reaction rate constants in buffer solution and in micellar phases are shown in Table 1 and for the LC phase in Table 2. Micellar effects of CTABr are largest in the reaction of O-*n*-propyl-*p*-nitrophenylmethylphosphonate with α-chymotrypsin (reaction rate increases 18 times). The rate of hydrolysis of O-*n*-hexyl-, O-*n*-heptyl- and O-*n*-octyl-*p*-nitrophenylmethylphosphonate is enhanced only slightly in micellar CTABr

Table 1

Reaction of O-alkyl-*p*-nitrophenylmethylphosphonates with α-chymotrypsin in buffer and micellar solutions (25 °C, pH 7.10, in 0.2 M phosphate buffer)

R	k_{II}, $M^{-1} \cdot sec^{-1}$					
	Buffer	Micellar phases, wt.%				
		0.09% CTABr	0.09% CTABr 0.06% hexanol	0.9% CTABr	0.9% CTABr 0.6% hexanol	9% CTABr
n-C_3H_7	23.9	433	366	230	364	—
n-C_4H_9	58.4	401	347	200	320	28.8
n-C_5H_{11}	175.2	400	470	—	450	—
n-C_6H_{13}	628.0	464	850	—	860	79.4
n-C_7H_{15}	501.0	1041	1300	520	1280	—
n-C_8H_{17}	165.6	768	809	390	800	—

Table 2

Reaction of O-alkyl-*p*-nitrophenylmethylphosphonates with α-chymotrypsin in LC phases of the CTABr—hexanol—H_2O system (25 °C, pH 7.10, 85 wt.% 0.2 M phosphate buffer, 9% CTABr, 6% hexanol)

R	*n*-C_3H_7	*n*-C_4H_9	*n*-C_5H_{11}	*n*-C_6H_{13}	*n*-C_7H_{15}	*n*-C_8H_{17}
k_{II}^{LC}, $M^{-1} \cdot sec^{-1}$	31	30	45	70	125	89
k_{II}^{Mi*}/k_{II}^{LC}, %	1174	1066	1000	1229	1024	899

* Mi — 0.9% CTABr, 0.6% hexanol, 98.5% buffer.

phases. The presence of hexanol retards micellar CTABr effects on the hydrolysis of phosphonates with $n=3$ to 5. The rate of hydrolysis of O-hexyl-, O-heptyl- and O-octyl-*p*-nitrophenylmethylphosphonate increases in the presence of hexanol in the micellar CTABr phases. No difference in the rate constants of hydrolysis of phosphonates by α-chymotrypsin was found at a constant hexanol—CTABr ratio in weight in micellar phases. This fact reveals that at the same hexanol—CTABr ratio the catalytic activity of α-chymotrypsin upon phosphonates does not change in micellar phases. An examination of the reaction rate in micellar phases with CTABr alone indicates a pronounced dependence on the variation of the micellar structure. An increase of the detergent concentration causes a small decrease in the values of k_{II}. At 9 wt. % CTABr concentration the hydrolysis of phosphonates is strongly retarded.

These results indicate that the rate constants k_{II} change differently for the reaction series of O-alkyl-*p*-nitrophenylmethylphosphonates with normal hydrocarbon radicals of different length in the O-alkylphosphonates. The number of carbon atoms in the radical and the hydrophobicity constants, as well as the molar volumes of phosphonates, are linearly related. The kinetics data of the reaction of α-chymotrypsin with the organophosphorus quasisubstrates log k_{II} *versus* n plots (number of carbon atoms in O-alkyl-*p*-nitrophenylmethylphosphonates) in micellar and LC phases and the buffer solution are shown in Fig. 1. The kinetic action of α-chymotrypsin upon phosphonates is different in the buffer solution and in the presence of CTABr micelles as well as in micellar

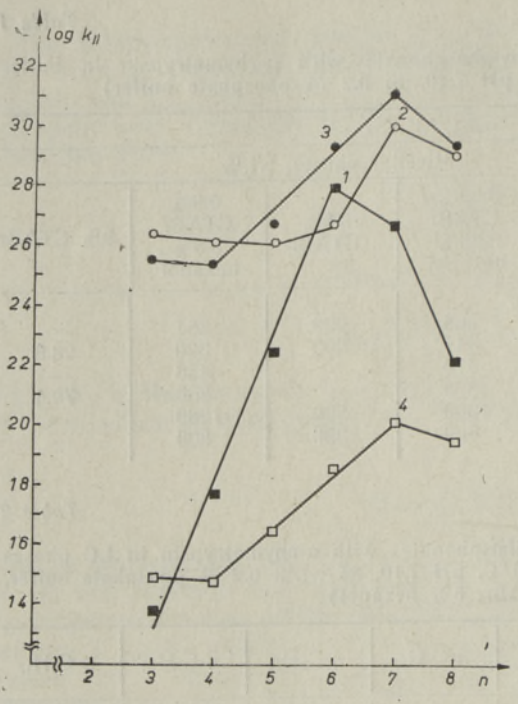

Fig. 1. Log k_{II} values for the series of OPI *versus* their n in the reaction with α-chymotrypsin (25 °C, pH 7.10): in 0.2 M phosphate buffer — *1*; in micelles with CTABr — *2*; in micelles with CTABr and hexanol — *3*; in LC phase — *4*. The compositions of different phases are given in Tables 1, 2.

phases with hexanol. The specificity of α-chymotrypsin is preserved at the same hexanol—CTABr ratio in micellar and LC phases. The specificity of the enzyme depends greatly on the composition of different phases. It should be noted that log k_{II} *versus* n plots has the same form in micellar and LC phases with the same hexanol—CTABr ratio. This may be caused by protein—detergent interaction, accompanied by conformational change of the enzyme. Supporting evidence for this suggestion can be obtained from UV differential spectra, as illustrated in [7]. Difference spectra of the micellar protein solution with respect to the protein water solution indicate a perturbation of the aromatic chromophores. It is known that the aromatic region of UV absorption spectrum of α-chymotrypsin is rather sensitive to the environment. Perturbations similar to those reported in [7] have also been obtained in micellar solutions of α-chymotrypsin [12]. The adsorbed protein molecules do not appear to be completely unfolded as judged by the area per molecule occupied at the interface. The CD-spectrum * of cytochrome C bound to phospholipid LC, although different from that of the native molecule in solution, does not indicate gross conformational changes [13]. In a 0.09% CTABr micellar solution the α-chymotrypsin specificity is changed, and these changes presume conformational changes in the micellar solution, in fact a change of «superactivity» in the micellar solution relative to the enzyme water solution.

In Fig. 2 the $\log k_{II}^{Mi}/k_{II}^{buffer}$ and $\log k_{II}^{buffer}/k_{II}^{LC}$ *versus* n plots are shown. In the CTABr micelles without hexanol the change in the length of the normal hydrocarbon radicals in the phosphoryl part of the organophosphorus quasisubstrate influences the antichymotrypsin efficiency of phosphonates differently. In the case of O-*n*-propylmethyl-*p*-nitrophenylphosphonate, the hydrolysis rate by α-chymotrypsin in micellar CTABr solution is highest as compared to the buffer solution in case of the phosphonate with $n=6$. In other words, it means that the influence of CTABr micelles consists not just in the protein activation. It depends upon the structure of the quasisubstrate, especially upon the length of the normal hydrocarbon radical. The $\log k_{II}^{Mi}/k_{II}^{buffer}$ *versus* n correlation plot has a break at $n=6$. Correlations of $\log k_{II}^{buffer}/k_{II}^{LC}$ and $\log k_{II}^{Mi}/k_{II}^{buffer}$ (micelles with hexanol) *versus* n plots also show a breaking point at

* CD, circular dichroism.

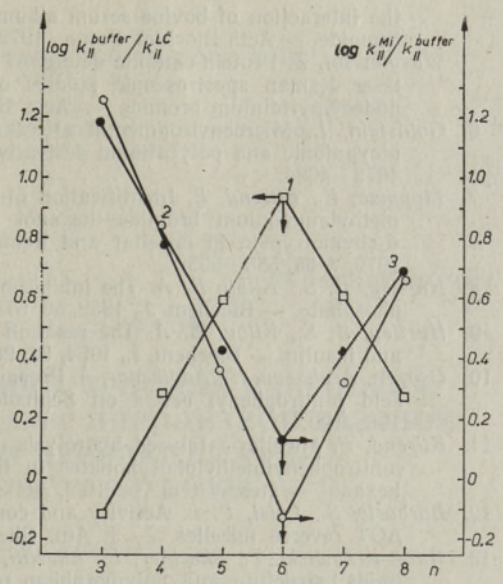

Fig. 2. Log $k_{II}^{buffer}/k_{II}^{LC}$ (*1*) and log $k_{II}^{Mi}/k_{II}^{buffer}$ (micelles with CTABr — *2*, micelles with CTABr and hexanol — *3*) values for the series of OPI *versus* their *n* in the reaction with α-chymotrypsin (25 °C, pH 7.10, 0.2 M phosphate buffer).

n=6. One should recognize that the reactivity of the enzyme in the micellar and LC systems depends upon the structure of the quasisubstrate, upon the length of the normal hydrocarbon radicals of phosphonate. The influence of micellar and LC phases relative to the reaction in a buffer solution depends upon the hydrophobicy of the quasisubstrate (Fig. 2). Antichymotrypsin activity of phosphonates changes differently in micellar and LC phases as compared to the buffer solution.

Conclusions

A kinetic analysis of the behaviour of α-chymotrypsin in micellar and LC phases reveals conformational modulation of the enzyme activity and specificity by structural effects exerted by the environment. Superactivity of the enzyme to phosphonates in micellar phases (except for the phosphonate with n=6, Fig. 2) was demonstrated. The specificity of α-chymotrypsin is the same at the same hexanol—CTABr ratios in micellar and LC phases (Fig. 1). It has been found that, for α-chymotrypsin in the LC phases, there are conditions under which all reactions with phosphonates are retarded in respect to enzyme reactions in micellar phases with the same hexanol—CTABr ratio. The retardation of enzyme reaction in LC phases is more pronounced as the alkaline hydrolysis of phosphonates in LC phases. The results show that even typical non-membrane enzymes are conformationally rather sensitive to structural influences exerted by the liquid-crystal membrane-like structures.

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α-KÜMOTRÜPSIINI INHIBEERIMINE MITSELLAARSES JA VEDELA KRISTALLI KESKKONNAS SÜSTEEMIS H_2O—HEKSADETSÜÜLTRIMETÜÜLAMMOONIUM-BROMIID—*n*-HEKSANOOL

On esitatud andmed fosfonaatide $(C_nH_{2n+1}O)CH_3P(O)OC_6H_4—NO_2$—*p* ($n$=3—8) fermentatiivse hüdrolüüsi kohta mitsellaarses (Mi) ja vedela kristalli (VK) keskkonnas. Katsete põhjal on konstateeritud, et mõlemas keskkonnas muutub kümotrüpsiini spetsiifilisus (võrreldes puhverlahuses toimuva reaktsiooniga). Mi-keskkonnas iseloomustab fermenti superaktiivsus, VK-keskkonnas on hüdrolüüs märksa aeglasem kui samasuguse massisuhtega *n*-heksanooli-detergendi mitsellides.

Эне КИЙРЕНД

КИНЕТИКА ИНГИБИРОВАНИЯ α-ХИМОТРИПСИНА В МИЦЕЛЛЯРНОЙ И ЖИДКОКРИСТАЛЛИЧЕСКОЙ ФАЗАХ СИСТЕМЫ ГЕКСАДЕЦИЛТРИМЕТИЛАММОНИЙБРОМИД—*н*-ГЕКСАНОЛ—ВОДА

Анализ кинетических данных указывает на возможность конформационного модулирования фермента, так как α-химотрипсин (α-ХТ) имеет разную специфичность по отношению к фосфонатам в мицеллярной и жидкокристаллической (ЖК) фазах в отличие от специфичности фермента в буферном растворе. Найдено, что реакция α-ХТ с ФОС заторможена в ЖК-фазе в отличие от реакций в мицеллярной фазе при одинаковом соотношении гексиловый спирт—детергент. Торможение ферментативной реакции в ЖК-фазе намного превышает торможение щелочного гидролиза ФОС в ЖК-среде. Результаты указывают на конформационную чувствительность типичного немембранного фермента к ЖК-структуре модельной мембраны.