TRYPSIN ENTRAPPED WITHIN LIPOSOMES. PARTITION OF A LOW-MOLECULAR-MASS SUBSTRATE AS THE MAIN FACTOR IN KINETIC CONTROL OF HYDROLYSIS

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Trypsin has been entrapped within liposomes prepared from egg yolk phospholipides by the method of controlled dialysis, and the hydrolysis kinetics of N^{α}-benzoyl-DL-arginine *p*-nitroanilide catalyzed by the liposome-entrapped trypsin has been studied by monitoring the flux of substrate and product across the liposomal membrane. The partitioning of the substrate and product between liposomal and extraliposomal environment has been found to represent the main factor in the kinetic control of the hydrolysis.

Reactions catalyzed by intracellular enzymes whose substrates are transported from extracellular to intracellular medium by diffusion can be simulated by application of the liposome-entrapped enzymes. Enzymes such as lysozyme[1], peroxidase[2], hexosaminidase[3], or alkaline phosphatase[4] have been entrapped in liposomes of different phospholipid composition. For the liposome-entrapped enzymes the substrate was inaccessible if the latter was incapable of diffusing across the lipid bilayers[5]. The activity of the entrapped enzymes becomes manifested if the liposomal membranes are partially solubilized with various detergents[4]. Thus, in order to describe transport and subsequent hydrolysis of a substrate catalyzed with a liposome-entrapped enzyme, it is necessary to choose enzymes with substrates diffusable across liposomal membranes. From this standpoint advantageous are enzymes which catalyze hydrolysis of synthetic artificial hydrophobic substrates.

The aim of the present paper was: (i) to prepare liposomes with entrapped trypsin and (ii) to characterize the process of transport of BANA** across liposomal membrane and its subsequent hydrolysis catalyzed by the entrapped trypsin.

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** The abbreviations used in the paper: BANA, N^{α}-benzoyl-DL-arginine 4-nitroanilide; 4-NA, 4-nitroaniline; SDS, sodium dodecyl sulfate.

EXPERIMENTAL

Materials

The phospholipids isolated from egg yolk containing choline (72·5%), lysophosphotidylcholine (4·6%), sphingomyelin (3·8%), phosphatidylethanolamine (19·1%) were obtained from Research Institute of Pharmacy and Biochemistry (Prague). Trypsin (EC 3.4.21.4) crystallized from bovine pancreas and BANA were obtained from Spofa (Prague), and Sigma (St. Louis), respectively. Sodium deoxycholate was obtained from Merck (Darmstadt); all other chemicals and reagents were of analytical grade and were obtained from Lachema (Brno).

Liposomes with Entrapped Trypsin

The liposomes with entrapped trypsin were prepared by controlled dialysis on Lipoprep Dianorm (Diachema AG, Zurich). Phospholipids and 0·02 mmol l^{-1} trypsin were solubilized with 30·2 mmol l^{-1} sodium deoxycholate in 1 mmol l^{-1} phosphate buffer pH 7·5 containing 129·8 mmol l^{-1} NaCl. The phospholipids/detergent molar ratio was 0·6. The solution was shaken 1 h in dark at room temperature and then dialyzed at 30°C with constant stirring (75 r.p.m.) 24 h. Membranes of pure cellulose with a cut-off of 10 000 daltons were used for the dialysis. The liposomes were rid of trypsin by means of size-exclusion chromatography on Sephadex G-50 (Pharmacia, Uppsala). The protein content in liposomes was determined[6] after solubilization in 1·25% SDS. The size and shape of liposomes were checked by electron microscopy analyzing an ultrathin section of epon-embeded liposomes.

Estimation of Enzyme Activity

The enzyme activity was estimated as the amount of 4-NA liberated by hydrolysis of BANA (0·05—0·50 mmol l^{-1} solution) catalyzed with free[7] or liposome-entrapped trypsin at 37°C. The activity of the entrapped trypsin was measured in a reaction medium containing 1 mmol l^{-1} phosphate buffer pH 7·6 and 160 mmol l^{-1} NaCl. The enzyme reaction lasting 20—150 min was started by addition of BANA solution in the same phosphate buffer and stopped by addition of 30% acetic acid in 5% SDS. The amount of the 4-NA liberated was determined spectrophotometrically at 405 nm (using 4-NA solution as a standard), that of 4-NA in extraliposomal medium was determined after rapid gel filtration using the same procedure. The specific activity of free and entrapped trypsin was expressed as the amount (in mmol) of the 4-NA liberated during the enzyme reaction (with 1 mg of trypsin) lasting 1 h.

Data Processing

The hyperbolic relationships were fitted by Eq. (*1*) and the sigmoidal ones were plotted according to Eq. (*2*).

$$Y = K_1 x(x + K_2)^{-1} \qquad (1)$$

$$Y = K_1^* x^2(x^2 + K_2^*)^{-1} \qquad (2)$$

The K_1, K_2, K_1^*, K_2^* parameters were computed by means of non-linear regression along with their respective S.E.M. values[8]. When describing the kinetic relationships of the 4-NA formation catalyzed by free or liposome-entrapped trypsin, the parameters K_1 and K_1^* represent the respective equilibrium values of the liberated 4-NA ($t \to \infty$), and K_2 and K_2^* represent the respective

half-lives. When describing the enzyme kinetics, the K_1 and K_1^* parameters represent the respective v_{max} values and K_2 or K_2^* the respective $K_{0.5}$ values.

RESULTS AND DISCUSSION

A homogeneous population of liposomes with entrapped trypsin was obtained by the method of controlled dialysis. The quality of the liposomes was characterized

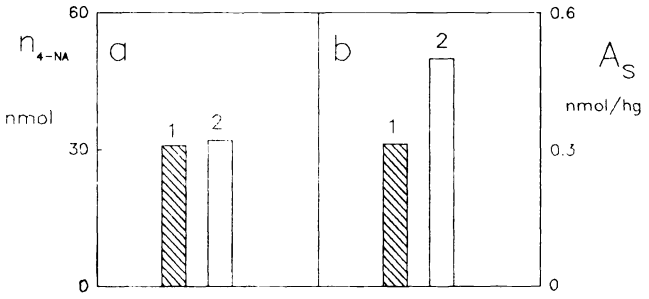

FIG. 1

The activity of liposome-entrapped trypsin a liberation of 4-NA during BANA splitting; b specific activity of entrapped trypsin. Trypsin content in intraliposomal medium (mg/ml): 1 0·50, 2 0·32

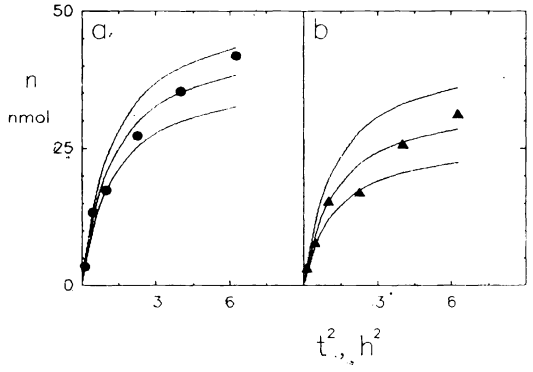

FIG. 2

Time dependence of 4-NA formation catalyzed by entrapped trypsin. The experimental data were fitted by means of Eq. (2). Panel a: Time dependence of 4NA formation. Parameters $K_1^* =$ $= (45{\cdot}75 \pm 1{\cdot}56)$ nmol 4-NA and $K_2^* = (1{\cdot}1 \pm 0{\cdot}1)$ h are the equilibrium amount of 4-NA and the reaction half-time, respectively, which were calculated by nonlinear regression. Panel b: Time dependence of increase in extraliposomal amount of 4-NA. For the parameters $K_1^* =$ $= (34{\cdot}13 \pm 1{\cdot}07)$ nmol 4-NA and $K_2^* = (1{\cdot}1 \pm 0{\cdot}1)$ h holds the same as above in Panel a

by means of electron microscopy. The mean liposome diameter was 125 ± 30 nm. The liposome preparation consisted of approximately 98% unilamellar phospholipid vesicles.

The liposomes with entrapped trypsin were separated from the free enzyme by means of size-exclusion chromatography[5]. The catalytic activities of entrapped trypsin were 0·32 and 0·5 mmol 4-NA g^{-1} h^{-1} for preparation with 0·5 and 0·32 mg trypsin per ml of intraliposomal medium (Fig. 1).

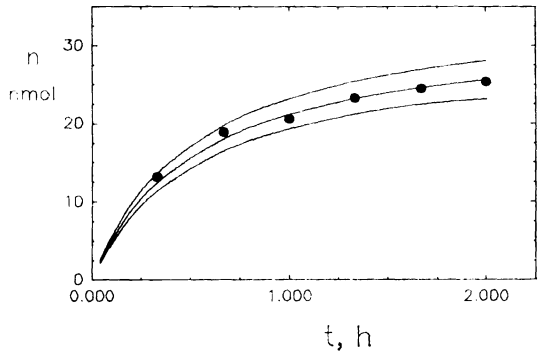

FIG. 3

Time dependence of 4-NA formation catalyzed by free trypsin. The experimental data were fitted by means of Eq. (*1*). The parameters K_1 = (32·36 ± 0·63) nmol 4-NA and K_2 = (0·53 ± 0·03) h represent the equilibrium 4-NA amount and the reaction half-time, respectively, which were calculated by non-linear regression

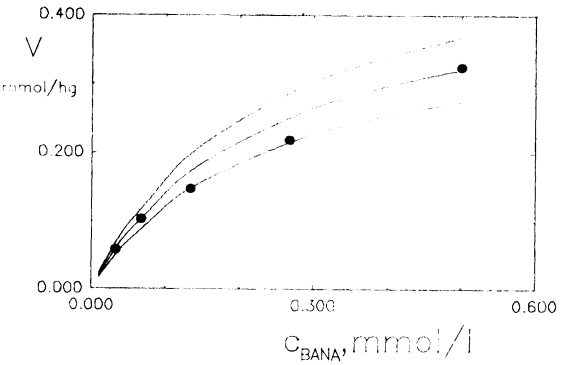

FIG. 4

Free-trypsin-catalyzed hydrolysis of BANA. The experimental kinetic data were fitted by means of Eq. (*2*). The parameters v_{max} = (0·92 ± 0·05) nmol 4-Na g^{-1} h^{-1} and $K_{0.5}$ = = (0·26 ± 0·03) mmol l^{-1} were calculated by nonlinear regression

On the other hand, the rate of formation of 4-NA in the presence of equal amounts of liposomes was equivalent for both preparations. The activity of the liposome-entrapped trypsin exposed to repeated freezing and melting increased to 1·2 nmol . . 4-NA g^{-1} h^{-1}. Hence, it can be supposed that the enzyme reaction only insignificantly controlled the process of BANA hydrolysis. On the other hand, very probably the process of BANA transport across the liposomal membrane plays the main role in the kinetic control of formation of 4-NA catalyzed by the entrapped trypsin.

The kinetics of formation of 4-NA catalyzed by the entrapped trypsin can be described by Eq. (*2*). The same relationship was observed for the time dependence of the enhancement of extraliposomal amount of 4-NA (Fig. 2). Moreover, almost identical half-lives (1·1 ± 0·1 h) were observed for both relationships. This suggests that always the ratio of the 4-NA formed to that in extraliposomal medium is constant. Thus the "partition law" for the concentration of 4-NA in both liposomal and extraliposomal media is the main factor in the kinetic control of the BANA hydrolysis catalyzed by the entrapped trypsin. The enzyme reaction equilibrium was observed at 34·13 ± 1·07 nmol extraliposomal 4-NA. Almost the same equilibrium value (32·36 ± 0·63 nmol 4-NA) was obtained for the hydrolysis of BANA catalyzed by free trypsin (Fig. 3). This suggests that during the hydrolytic reaction catalyzed by the entrapped trypsin the accumulation of 4-NA in the liposomal membrane within the respective range of the partition coefficient of 4-NA between the phospholipid membrane and water system represents the main factor responsible for establishing of the equilibrium.

The kinetic equation of hydrolysis of various substrates catalyzed by chymotrypsin was found to be exponential[9]. In accordance therewith is the fact that the

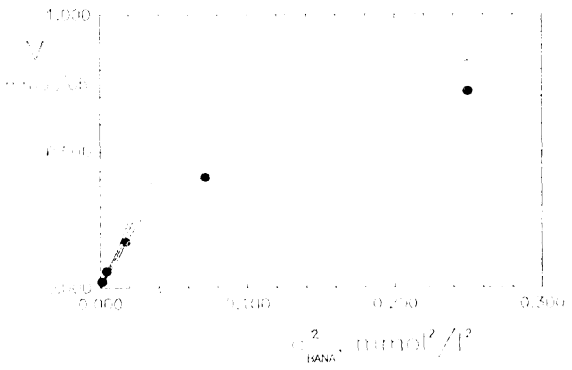

FIG. 5

Kinetics of BANA hydrolysis catalyzed with the entrapped trypsin. The experimental data were fitted by means of Eq. (*1*). The parameters r_{max} = (0·47 ± 0·02) nmol 4-Na g^{-1} h^{-1} and $K_{0.5}$ = (0·23 ± 0·02) mmol l^{-1} were calculated by nonlinear regression

relationship between the initial rate of the BANA hydrolysis catalyzed with free trypsin and the bulk concentration of BANA follow Eq. (*2*) (Fig. 4). On the other hand, the kinetic dependence of the BANA hydrolysis catalyzed with entrapped trypsin exhibits a different shape and can be described by the classical Michaelis–Menten equation (Fig. 5). This change is due to the mass transfer effect of both substrates and products across the liposomal membrane, this effect being the predominant factor in the hydrolysis of BANA catalyzed by the trypsin encapsulated in liposomes.

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