ENZYME KINETICS AT A LIPID PROTEIN MONOLAYER, INDUCED SUBSTRATE INHIBITION OF TRYPSIN

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1. Introduction

The catalytical properties of an enzyme interacting with a lipid film are tested in the case of trypsin adsorbed to a negatively charged methylstearate/eicosylsulfate monolayer. The hydrolysis of benzoyl-L-arginine-*p*-nitroanilide by the adsorbed trypsin is studied. Compared to the hydrolysis by free trypsin, it is found that, 1) the maximum rate is reduced by a factor of 2.2, 2) that the Michaelis constant is lowered by a factor of 170 (the actual rate being enhanced considerably over a wide range of substrate concentration), 3) that a substrate inhibition constant is detectable not observed for free trypsin, and 4) that the adsorbed trypsin exhibits higher stability against autohydrolysis.

2. Experimental [1–3]

A monolayer of methylstearate (Merck) and eicosylsodiumsulfate (Schuchardt) (molar ratio 4:1) is spread at the air/water interface from a 5 mM solution in chloroform/ethanol/water (vol. ratio 4:5:1) [3,4] and compressed to 30 dyn/cm. Bovine trypsin (Boehringer Mannheim) is adsorbed from a solution (150 μg/ml) in 10 mM NaCl at pH 2.3 (optimal stability of trypsin [5]) within 15 min using the multicompartment trough technique [1]. The lipid protein film formed is transferred onto a hydrophobic glass slide from a dest. water subphase [2]. The slide is separated from the preparation trough by means of the cuvette-minus-one-window technique [3]. The cuvette with the lipid enzyme layer on the inside of one window (fig.1) is mounted in a spectrophotometer (modified Gilford 2400-S).

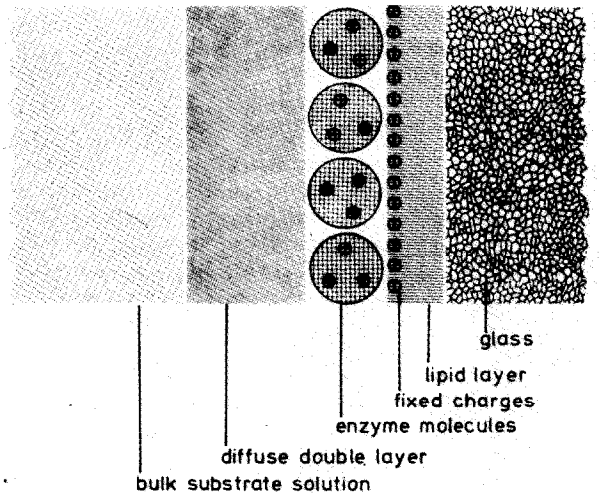

Fig.1. Schematic drawing of a lipid enzyme lamella in contact to a substrate solution. The glass slide forms one window of a cuvette. The lipid film consists of methylstearate and eicosylsulfate. The protein layer consists of trypsin molecules. The electrical double layer is caused by the fixed charges of the lipid film and of the adsorbed protein layer. The substrate solution may be exchanged without bringing the lamella out of the solution.

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Substrate solutions (benzoyl-L-arginine-*p*-nitroanilide (L-BAPNA, Merck) [6] in 0.5 mM Tris buffer at pH 8, 25°C) of various concentrations are applied and stirred by means of a magnetic propeller. The increase of absorbance at 410 nm due to the L-BAPNA hydrolysis is recorded continuously. The quantity of adsorbed protein is determined by the Lowry method (cf. [7]).

3. Results

The quantity of trypsin adsorbed (E_o) is found to be $E_o = 0.51 \pm 0.03\ \mu g/cm^2 = 21 \cdot 10^{-12}$ mol/cm^2 (MW23'746 [5]).
Desorption of trypsin under reaction conditions is not observed within one hr.

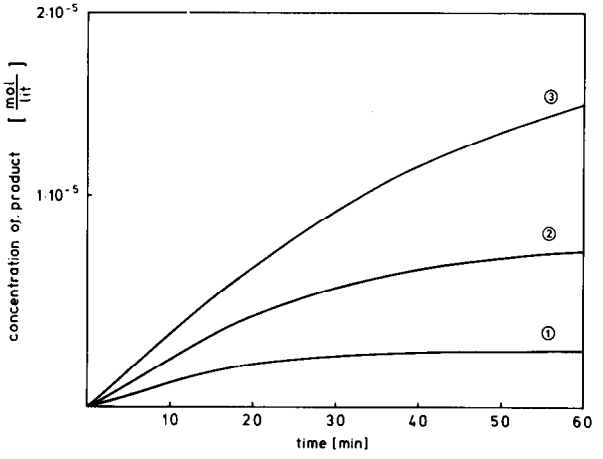

Fig.2. Concentration of product versus time during hydrolysis of L-BAPNA by trypsin adsorbed to a methylstearate/eicosylsulfate film (molar ratio 4:1). The initial concentrations of the substrate in 0.5 mM Tris, pH 8 are 1) $2 \cdot 10^{-6}$ M, 2) $1 \cdot 10^{-5}$ M and 3) $2 \cdot 10^{-5}$ M.

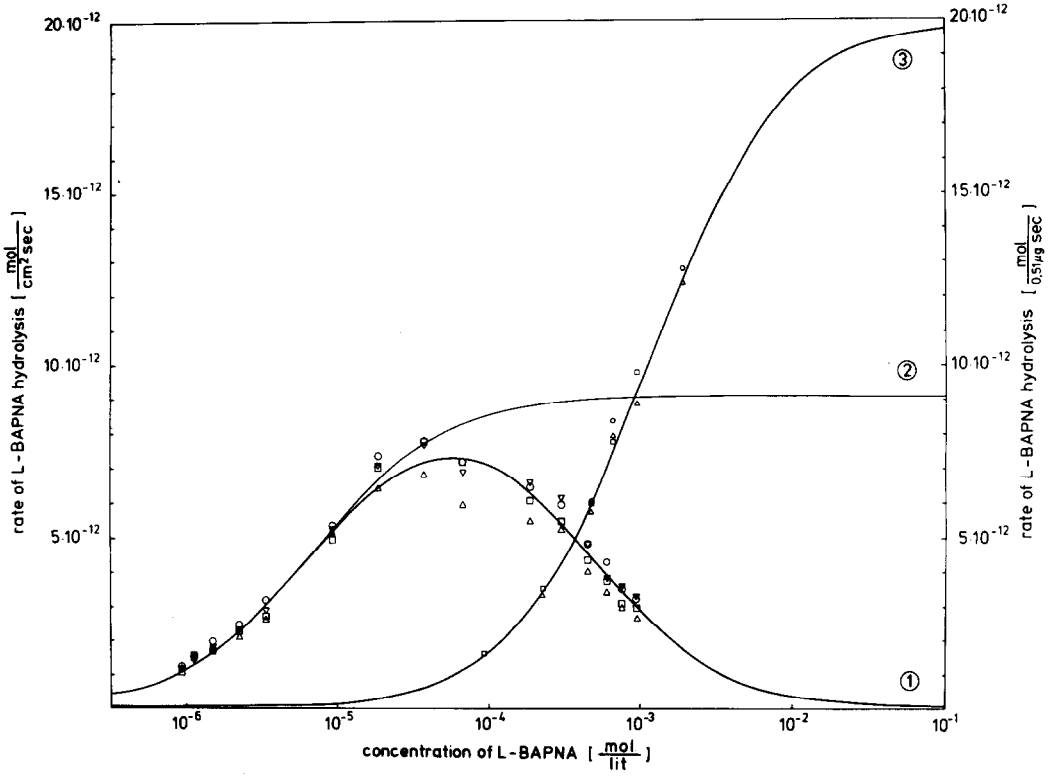

Fig.3. Initial rate of L-BAPNA hydrolysis versus logarithm of substrate concentration for trypsin adsorbed to a methylstearate/eicosylsulfate film and for an equivalent quantity of free trypsin in 0.5 mM Tris pH 8. The curves are calculated from equation 3 using the kinetic parameters of table 1. 1) adsorbed trypsin with inhibition term, 2) adsorbed trypsin without inhibition term, 3) free trypsin without inhibition term.

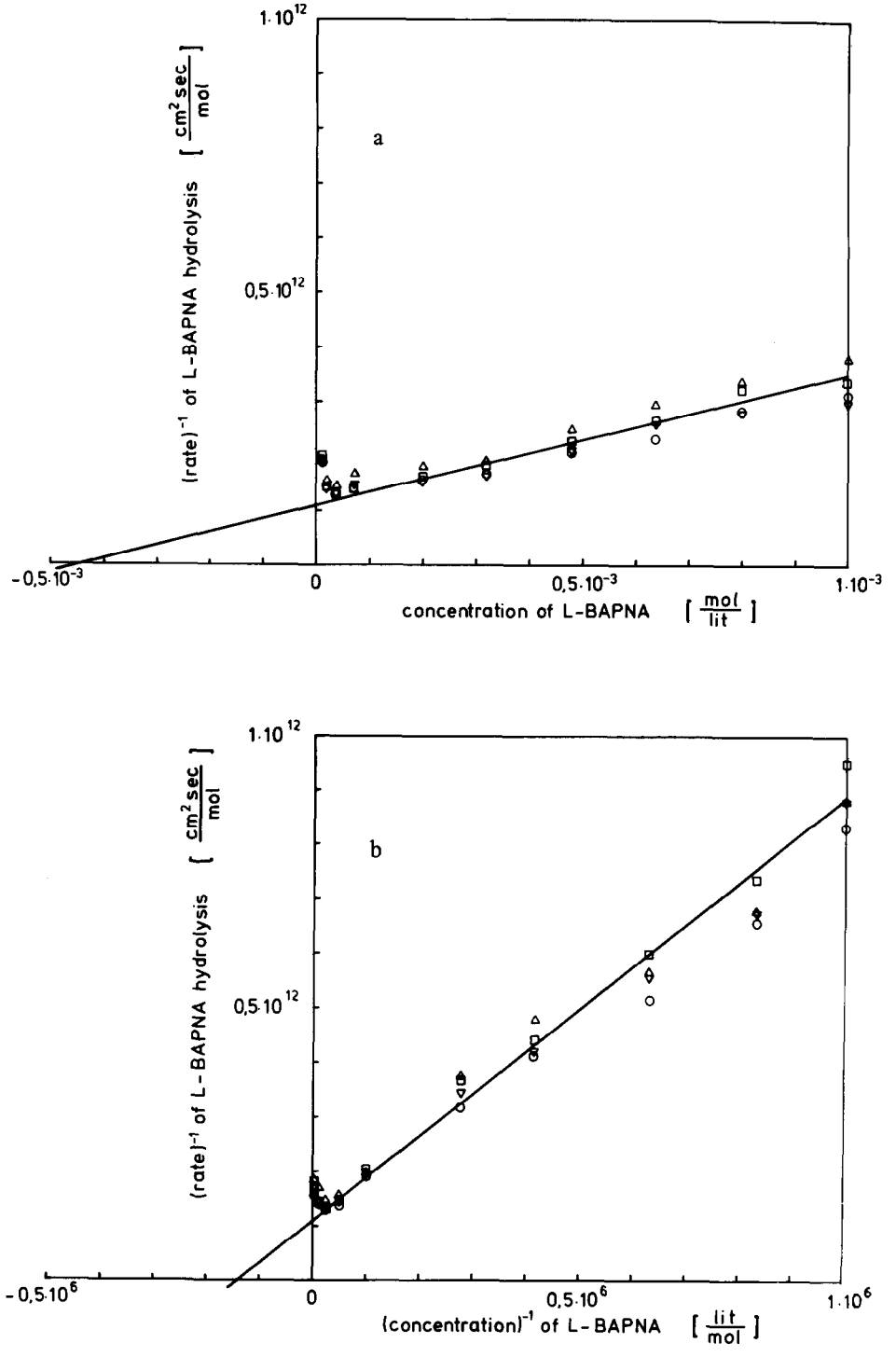

Fig.4. a) Reciprocal rate of L-BAPNA hydrolysis (1/v) versus concentration of substrate (c_S). b) Reciprocal rate versus reciprocal substrate concentration ($1/c_S$) for trypsin adsorbed to a methylstearate/eicosylsulfate film in 0.5 mM Tris pH 8.

The time course of product formation is shown in fig.2 for three substrate concentrations. The records are taken from one lipid-enzyme film preparation. The activity is not reduced within three hr under reaction conditions, in contrast to free trypsin under similar conditions where considerable loss of activity is observed within a few minutes.

The initial rates (taken from four lipid-enzyme film preparations) are depicted in fig.3 in dependence of the substrate concentrations. In addition, fig.3 shows the initial rates of the reaction catalysed by an equivalent quantity of trypsin in homogeneous solution under identical reaction conditions.

4. Discussion

The results may be described by a Michaelis-Menten kinetics together with a substrate inhibition equilibrium (equations 1 and 2), i.e. by the rate law given by equation 3 [8] with the initial rate v, the maximum rate $V = k_3 E_0$, the substrate concentration c_s, the Michaelis constant $K_m = (k_2 + k_3)/k_1$ and the inhibition constant $K_i = k_5/k_4$, where the k's are rate constants.

equation 1: $$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P$$

equation 2: $$ES + S \underset{k_5}{\overset{k_4}{\rightleftharpoons}} SES$$

equation 3: $$v = \frac{k_3 E_0}{1 + K_m/c_S + c_S/K_i}$$

The values of the constants V, K_m and K_i are taken from the $1/v$–c_s and $1/v$–$1/c_s$ plots (fig.4). k_3, K_m and K_i of adsorbed and free trypsin are compared in table 1.

At low concentrations the overall rate of an interfacial reaction becomes diffusion controlled, whereas at high concentrations the transport rate may be neglected and the rate law coincides with that of the bulk reaction. The critical concentration c_s^{rt} where the reaction control changes to transport control, is estimated by equating the maximum rates of reaction and diffusion. For a Michaelis-Menten kinetics one obtains with the diffusion coefficient of the substrate D_s and the thickness of the Nernst diffusion layer d_N equation 4.

Table 1
Kinetic parameters of BAPNA hydrolysis by free and adsorbed trypsin

	trypsin at lipid monolayer	trypsin in homogeneous solution
k_3	0.41 1/sec	0.91 1/sec
K_M	$7.1 \cdot 10^{-6}$ mol/lit	$1.2 \cdot 10^{-3}$ mol/lit
K_I	$4.5 \cdot 10^{-4}$ mol/lit	

equation 4: $$k_3 E_0 = D_S c_s^{rt}/d_N$$

Inserting $k_3 E_0 = 10^{-11}$ mol/cm^2 · sec, $D_s = 10^{-5}$ cm^2/sec and $d_N = 10^{-3}$ cm [9] into equation (4) one finds $c_s^{rt} = 10^{-6}$ mol/l. Thus the interfacial reaction rates measured (range of substrate concentration 10^{-6} to 10^{-3} mol/l) may be discussed in terms of the bulk rate law.

Two general schemes are conceivable to interprete the differences of k_3, K_m and K_i between the interfacial and the bulk reaction (cf. table 1). 1) The intrinsic reactive properties of each enzyme molecule are modified in the adsorbed state, and 2) the intrinsic properties are unchanged, but the number of operative enzyme molecules and the effective substrate concentration differ from the total number of adsorbed enzyme molecules and from the bulk substrate concentration respectively. For lack of any information on possible mechanisms concerning scheme (1), the discussion is restricted to some remarks concerning scheme (2).

The difference in k_3 indicates that $6 \cdot 10^{12}$ out of the $13 \cdot 10^{12}$ enzyme molecules/cm^2 i.e. approx. 50% are catalytically active. It is conceivable that within the adsorbed closely packed layer of enzyme molecules only a fraction has the proper orientation for effective catalysis.

The difference in K_m indicates an increase of the effective substrate concentration by a factor 170 compared to the bulk. This shift of the substrate concentration scale (fig.3) leads to the appearance of substrate inhibition i.e. of K_i, which is not detect-

able in the case of bulk reaction because the concentrations required are not attainable due to the low solubility of the substrate. The enhanced interfacial concentration of the positively charged L-BAPNA may be due to the electrostatic effect of the negative charges of the lipid film (7 electronic charges per adsorbed enzyme molecule). They reduce the repulsive effect of the intrinsic positive charges of the trypsin molecule itself (16 positively, 7 negatively charged aminoacid residues [10]), which is included in the bulk Michaelis constant.

Further investigations on the relations between rate of hydrolysis and quantity of adsorbed enzyme and electrical interfacial potential are in progress in order to obtain a detailed explanation of the kinetic properties of a lipid bound enzyme.

References

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