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KINETICALLY CONTROLLED EQUILIBRIA

THE PERTURBATION OF HYDROLYSIS EQUILIBRIA IN REACTIONS CATALYZED BY α-CHYMOTRYPSIN IMMOBILIZED ON CHARGES SUPPORTS

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Abstract

The hydrolysis and synthesis of N-Acetyl-l-tyrosine-ethyl-ester catalyzed by α-chymotrypsin immobilized in polymeric supports (Sephadex), with positive or negative stationary charges has been studied. Charged matrices perturbed the equilibrium (at pH 9.o), so that no complete hydrolysis was observed in the bulk solution and ester could be synthetized from acid and alcohol. The change is due to dipole orientation energies in the electric double layer where the reactions are catalyzed. This represents a situation where the ***equilibrium in the system is kinetically controlled*** by the equilibrium in a sub-system (here the electric double layer).

Introduction

Hydrolytic enzymes hydrolyze substrates, whose synthesis *in vivo* normally is catalyzed by other enzymes. The synthesis and hydrolysis reactions are located in different compartments of living systems. The equilibrium concentrations of the hydrolysis reaction catalyzed by hydrolytic enzymes can *in vitro* be influenced by the addition of one of the products (alcohol) or amides and esters.
In the latter case coupled ester and peptide hydrolysis reactions are possible (3). Then hydrolytic enzymes can be used to catalyze the synthesis of the compounds that they normally hydrolyze. Under these conditions esters or oligopeptides were synthetized from acids and alcohol or amino acids, respectively, using free and immobilized α-chymotrypsin in homogeneous systems or inert enzyme supports (1-3). The microenvironment around the inert support did not influence the equilibrium (2).
We have previously shown that equilibrium constants, for a reaction with an immobilized ligand, can be perturbed by the microenvironment in the electric double layer around the support on which the ligand is immobilized (4). This is due to dipole orien-

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Abbreviations: AT = N-Acetyl-L-tyrosine; ATEE N-Acetyl-L-tyrosine-ethyl-ester; CT = α-chymotrypsin

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tation energies in the double layer.

The equilibrium constants for other reactions (ester or peptide hydrolysis) may also be perturbed by the electric field in an electric double layer (5). The volume of the double layer is, however, only a small fraction of the total volume of the system. When the equilibrium reactions in the bulk solution are very slow compared with the corresponding rates in the double layer, the observed equilibrium composition in the system may be kinetically controlled by the equilibrium constant in the double layer. Such a ***kinetic control*** should be possible to observe in systems where the reactions are catalyzed ***only*** in the double layer by immobilized enzymes or other groups on an insoluble support. Then synthesis of esters (or peptides) should be observed in systems where the equilibrium in the bulk solution favours the hydrolysis reaction.

The aim of this investiation was to study the existence of such kinetically controlled equilibrium systems.

THEORY

PERTURBATION OF STANDARD FREE ENERGIES FOR ESTERS HYDROLYSIS IN ELECTRIC FIELDS AROUND CHARGED POLYMER CHAINS

In the system studied here the ester hydrolysis reaction is catalyzed by the matrix, with or without enzyme. The rate of spontaneous ester hydrolysis is negligible in the bulk solution (Fig. 1).

The standard free energy change for the hydrolysis equilibrium in a double layer is (4)

$$\Delta G^{o}_{DL} = \Delta G^{o}_{hom} + \Delta(\Delta G^{o}_{el}) \qquad /1/$$

Where $\Delta(\Delta G^{o}_{el})$ is the perturbation due to the electric field in the double layer, and ΔG^{o}_{hom} is the standard free energy in an homogenous system. $\Delta(\Delta G^{o}_{el})$ is determined by concentration differences between the bulk solution and the electric double layer. For the charged solutes the contribution to $\Delta(\Delta G^{o}_{el})$ is given by the relation (6)

$$\Delta(\Delta G^{o}_{el})_{ch} = \sum_{\text{reactants}} z_i \cdot F \cdot d\Psi - \sum_{\text{products}} z_i \cdot F \cdot d\Psi \qquad /2/$$

where z_i is the charge of the solute, F is Faradays constant, and $d\Psi$ is the potential difference between the double layer and the bulk solution. For the dipolar solutes the contribution is (5)

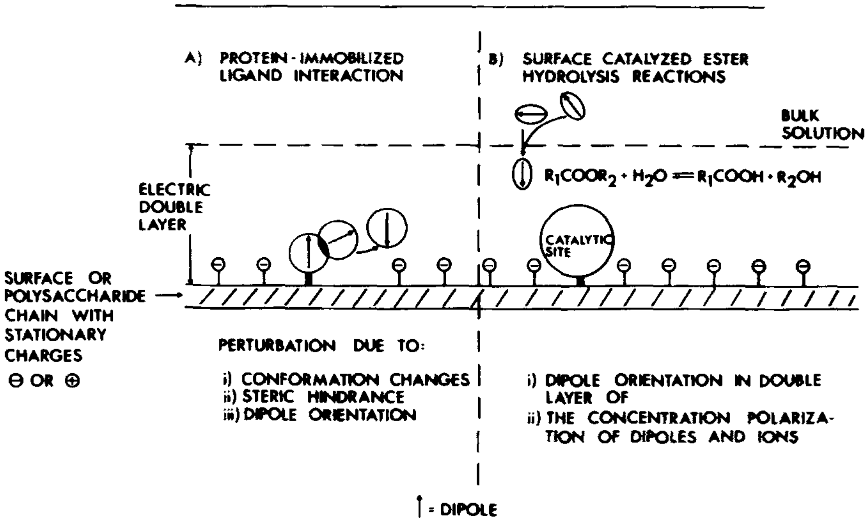

Fig. 1. Microenvironmental effects on equilibria at surfaces (polymer chains).

$$\Delta(\Delta G^{o}_{el})_{dip} = \frac{E^{2}\cdot A}{RT}\left[\sum_{\text{reactants}} \mu_i^{2} - \sum_{\text{products}} \mu_i^{2}\right] \qquad /3/$$

where μ_i^2 is the permanent and induced dipole moment, E the electric field and A a constant.

The hydrolysis equilibria studied here are:

$$\text{R-(CO)-O-CH}_2\text{-CH}_3 + \text{H}_2\text{O} \rightleftarrows \text{R-COOH} + \text{C}_2\text{H}_5\text{OH} \qquad /4/$$

$$\text{R-COOH} \rightleftarrows \text{R-COO}^{-} + \text{H}^{2} \qquad /5/$$

For these reactions $\Delta(\Delta G^o_{el})_{ch} = 0$, whereas

$$\Delta(\Delta G^{o}_{el})_{dip} = \mu^2_{\text{RCOOCH}_2\text{CH}_3} + \mu^2_{\text{H}_2\text{O}} - \mu^2_{\text{C}_2\text{H}_5\text{OH}} > 0 \qquad /6/$$

as $\mu^2_{H_2O} > \mu^2_{C_2H_5OH}$ (7). Thus $\Delta G^o_{DL} > \Delta G^o_{hom}$ i.e. the equilibrium is shifted to the left in /4/ (less hydrolysis) in the double layer. As $\Delta(\Delta G^o_{el})_{ch} = 0$ it also follows that no dependence of the sign of the charge on the support on the degree of ester hydrolysis should be observed. Thus for an uncharged ester (as ATEE) it should be possible to observe ester hydrolysis in systems where the reaction is only catalyzed in the electric double layer. The hydrolysis equilibrium is shifted in favour of the formation of molecules with larger dipole moments.

EXPERIMENTAL PROCEDURE

Materials: Different Sephadex® gels (Pharmacia Fine chemicals) were activated by the BrCN method, and α-chymotrypsin (Worthington CDI) immobilized in the activated gel particles as described in (8). N-acetyl-L-tyrosine-ethyl-ester (SERVA) and N-Acetyl-L-Tyrosine (SIGMA) were used as purchased. All other chemicals were reagent grade.

Determination of the degree of ester hydrolysis: The rate of H^+ production (ATEE Hydrolysis) or H^+ consumption (ATEE formation from AT) after addition of gel particles with (or without) immobilized CT to an ATEE- (or AT) solution in the titration vessel, kept at 25°C, of a pH-Stat (Radiometer) was recorded as a function of time. The amount of titrand ($NaOH_2$ or HCl) consumed when the rate was = 0 was used to calculate the degree of conversion. Triplicate determinations differed within 5 %. The following buffer was used to prepare ATEE- (or AT) -solutions: TRIC-HCl (I = 0.05), NaCl was used to adjust the ionic strength, 10 % Ethanol.

Thin layer chromatography: ATEE and AT-solutions were incubated in the presence of C-14-ethanol (composition as above) in closed and stirred scintillation vials for two days. The composition of the solution was determined by thin layer chromatography on Merck plates (DC-Fertigplatten Kieselgel 60 F_{254}). The following solvent was used: H_2O: Ethanol: Ammonia (25%) (8:1:1).

ATEE and AT were found to have R_f-values 0.5 and 1 respectively. Incorporation of C-14 in ATEE was determined by scintillation counting.

RESULTS AND DISCUSSION

For α-chymotrypsin immobilized in supports carrying different charged incomplete hydrolysis of ATEE was observed under conditions where quantitative hydrolysis is obtained in the presence of free CT (Fig.2). The data in Table 1 demonstrate that the observed change in ester hydrolysis equilibrium is mainly dependent on the charge

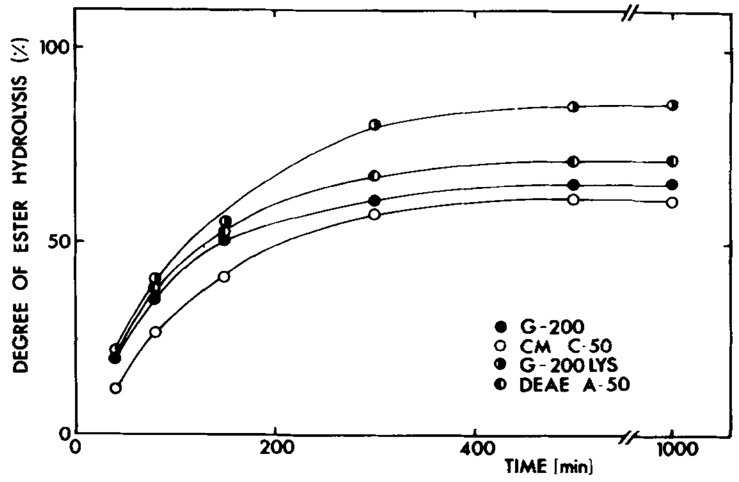

Fig. 2. Ester (ATEE) hydrolysis by α-chymotrypsin, immobilized in different spherical gel particles (Sephadex), in pH = 9.0 buffer (0.2 M NaCl, TRIS-CHl [I = 0.05], 10% Ethanol) at 25 C. The G-200 LYS Gel was treated with lysine after enzyme binding; all other gels were treated with ethanolamin.

Table 1. Degree of ester (ATEE) hydrolysis in the presence of free α-chymotryps immobilized in different supports at equilibrium for the systems in Fig. 1. The ionic strength and pH were varied by changing the NaCl- or TRIS-content, respectively.

SUPPORT	DEGREE OF ESTER HYDROLYSIS (%)*				STATIONARY CHARGE ON SUPPORT	CHARGE DENSITY AT pH 8-9 (9) (meq/g dry gel)
	pH = 8.0		pH = 9.0			
	I = 0.25	I = 0.25	I = 0.50	I = 1.0		
Sephadex® G-200	100	63	65	68	-	-
Sephadex® G-200 Lys.	100	82	-	87	+ and -	-
Sephadex® SP C-50	100	70	81	92	-	2
Sephadex® CM C-50	100	61	65	78	-	4.5
Sephadex® DEAE A-50	100	72	-	77	+	2
none	100	100	100	100	-	-

*experimental error ± 3 %.

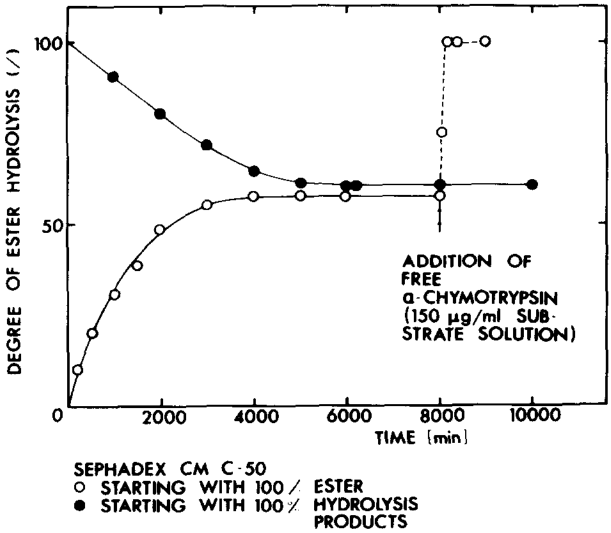

Fig. 3. *Kinetically controlled equilibrium:* Synthesis and hydrolysis of ATEE catalyzed by the ion-exchange gel Sephadex® CM C-50 at conditions given in Fig. 2. in the absence and presence of free chymotrypsin.
Starting with 100% ester (ATEE)
Starting with 100% hydrolysis products (AT and Ethanol).

density of the matrix. Then the ionic strength dependence indicates that the perturbation of the equilibrium constant decreases with *E*. The electric field in the double layer decreases with increasing ionic strength (6). This observation is in agreement with the behaviour predicted by Eq./6/. The same equilibrium state could be obtained in the absence of enzyme, where the matric acts as an ion-exchange catalyst. This is shown in Fig. 3. Ester synthesis was observed when 100% hydrolyzed ester was incubated with the matrix, with or without enzyme (Fig.3). When large amounts of free enzyme was added to the equilibrium mixture 100% ester hydrolysis is observed, as in the absence of matrix bound enzyme. With smaller amounts of free enzyme, intermediary equilibrium states were observed. This indicates that the overall equilibrium state is kinetically controlled by the rates of the equilibrium reactions in the bulk solution and the double layer, respectively.

Large absolute changes in hydrolysis equilibrium composition were only observed at pH 9.0. The perturbation energy $\Delta(\Delta G^o)_{el}$ at pH 8.0 should be approximately equal to the corresponding value at pH 9.0.

The free energy for ester hydrolysis, ΔG^o_{hom}, is a function of pH. The value at pH 8.0 is lower than at pH 9.0. Then the absolute change in equilibrium composition is to small compared to the experimental error in the determination of degrees of hydrolysis.

The data presented here show that the observed effects, in the absence of free CT, depend on the equilibrium conditions in the part of solution where the ester hydrolysis occurs, *i.e.* is catalyzed. This occurs in the microenvironement around the polysaccharide chains of the matric. As they carry stationary charges, the electric field in the electric double layer must cause the observed perturbation of the free energy of ester hydrolysis. The change in ester hydrolysis equilibria by a change in alcohol content of the reaction mixture cannot be of importance here, as the alcohol content should not be changed markedly in the double layer.

The synthesis of ATEE from AT and C-14 labelled ethanol at pH = 9.0 was verified by thin layer chromatography. The ATEE spots were found to contain C-14 labelled ester. The same applied for the ATEE spots after incubation of ATEE with immobilized enyzme at pH = 9.0 in the presence of C-14 labelled ethanol.

The findings presented here represent an equilibrium that is *under kinetic control*. The reactions /4/ are very slow in the absence of catalysts. The equilibrium composition of the systems observed in the bulk solutions differs markedly from equilibrium composition observed in the absence of immobilized catalysts. What we observe is the equilibrium composition in the subsystem where the rate of reactions /4/ is large *i.e.* in the double layer. This influences the overall equilibrium composition in the bulk solution, and may alone determine it when the catalyzed reaction rates are much larger than the spontaneous reaction rates.

The situation where equilibria in a system are kinetically controlled by the equilibrium conditions in a subsystem (as a charged surface) should be considered in technical applications of hydrolytic enzymes. It should also be of importance for processes *in vivo* that are catalyzed by enzymes bound to charged surfaces (10, 11).

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