A THEORETICAL MODEL DESCRIBING STEADY-STATE CATALYSIS BY ENZYMES IMMOBILIZED IN SPHERICAL GEL PARTICLES. EXPERIMENTAL STUDY OF α-CHYMOTRYPSIN-SEPHAROSE.

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Analytical expressions describing steady-state kinetics are given for systems with enzymes enclosed or covalently bound in spherical gel particles. In experiments using immobilized CT* the rate of product formation was found to be proportional to the square root of the enzyme content at low substrate concentration, and proportional to the enzyme content at high substrate content. These findings are in agreement with the results predicted from the theoretical model. The determination of the turnover number and Michaelis-Menten constant for immobilized enzymes is outlined. Some possible consequences for the description of enzyme kinetics in vivo are discussed.

The kinetics of reactions catalyzed by enzymes immobilized in membrane layers or spherical particles have been found to differ from the kinetics of the same reactions catalyzed by free enzymes (1-3). In the systems with immobilized enzymes the catalytic centers are non-uniformly distributed and the substrate- and product-distribution in the enzyme containing particles governed by both reaction and diffusion. General differential equations that describe the stationary state in such systems were derived by Rashevsky (4). For enzymes bound in membrane layers Katchalsky and his co-workers (1,5) have given a detailed description of enzyme kinetics for the case where substrate and products are uncharged.

For immobilized enzyme systems, however, we still lack information on:

(i) Whether the predictions derived from the theoretical treatment can be verified by experimental observations.

* Abbreviations: CT = α-chymotrypsin; ATEE = N-Acetyl-l-tyrosine-ethyl-ester.

(ii) How molecular properties as turnover numbers or Michaelis-Menten constants can be determined for the enzyme in these systems.

The aim of the present study is to give pertinent information on (i) and (ii) for enzymes immobilized in spherical gel particles. As a model system we use CT bound in Sepharose particles.

THEORY

The differential equations describing the stationary distribution of a substrate and products in spherical particles, neglecting coupling of flows of uncharged solutes, are (4):

$$\underline{D}'_S \nabla^2 f_S(\underline{r}) = A(\underline{r}) \; ; \quad \underline{D}'_{P_i} \nabla^2 f_{P_i}(\underline{r}) = - A(\underline{r}) \qquad (1)$$

where S and P_i are the substrate and one of the i products respectively; $\underline{r}$ is the distance from the center of the gel particle; $\underline{D}'$* the diffusion coefficient of the solute indicated by the subscript within the gel particle; $f(\underline{r})$ the concentration at distance $\underline{r}$ of the species given in the subscript; $\underline{A}(\underline{r})$ the rate of the enzyme catalyzed reaction at distance $\underline{r}$. For an enzyme that follows Michaelis-Menten kinetics we have:

$$\underline{A}(\underline{r}) = \frac{\underline{k}'_{cat}\, \underline{n}_E\, f_S(\underline{r})}{\underline{K}'_M + f_S(\underline{r})} \qquad (2)$$

where $\underline{k}'_{cat}$ and $\underline{K}'_M$* are the turnover number and the Michaelis-Menten constant respectively; $\underline{n}_E$ is the concentration of enzyme within the spherical particle assumed to be independent on $\underline{r}$. $\underline{K}'_M$ and $\underline{k}'_{cat}$ depend on pH. Consequently they depend on $\underline{r}$ when H^+ is a product as in ester hydrolysis catalyzed by CT. Then general analytical solutions to (1) do not exist. Analytical solutions can, however, be derived under the following conditions:

(i) A high ionic strength is maintained by solutes that do not participate in the enzyme catalyzed reactions. Coupling of flows of charged sub-

* Properties of the immobilized enzyme and diffusion coefficients within the gel particle will be primed throughout.

strate and product molecules may then be neglected.

(ii) The pH within the spherical gel particle is kept constant using a buffer of high buffer capacity.

With the boundary conditions: $f_S(\underline{R})$ = constant where $\underline{R}$ is the particle radius assuming that the thickness of the unstirred layer outside the particle is $\ll\underline{R}$, and a finite concentration of S at $\underline{r} = 0$ we obtain when $f_S(\underline{r}) \ll \underline{K}_M'$ (4):

$$f_S(\underline{r}) = \frac{f_S(\underline{R}) \cdot \underline{R}}{(e^{\underline{Ra}} - e^{-\underline{Ra}})} \cdot \frac{(e^{\underline{ra}} - e^{-\underline{ra}})}{\underline{r}} \qquad (3)$$

where $\underline{a} = \sqrt{(\underline{k}_{cat}' \underline{n}_E)/(\underline{K}_M' \underline{D}_S')}$, and for $f_S(\underline{r}) \gg \underline{K}_M'$:

$$f_S(\underline{r}) = f_S(\underline{R}) + \frac{\underline{k}_{cat}' \underline{n}_E}{6 \underline{D}_S'} (\underline{r}^2 - \underline{R}^2) \; . \qquad (4)$$

The steady-state rate $\underline{v}$ for the enzyme catalyzed reaction in these systems equals the inflow of substrate into the spherical particles (or the outflow of products) and is given by the sum over all particles:

$$\underline{v} = \sum \underline{D}_S' \, f_S'(\underline{R}) \, 4\pi \underline{R}^2 \; . \qquad (5)$$

Using equations (3) - (5) we obtain the rates given in Table I. It follows that at substrate content $\ll \underline{K}_M'$ the rate is proportional to $\underline{n}_E$ when the dimensionless parameter $\underline{Ra} \ll 1$, and proportional to $\sqrt{\underline{n}_E}$ when $\underline{Ra} \gg 1$. At these substrate contents the ratio of the rate in the system with immobilized enzyme to the corresponding rate for the free enzyme system decreases with $\underline{Ra}$. The above treatment is valid also when the gel particles differ in size. Then $\underline{R}$ in the case where $\underline{v}$ per unit gel volume is proportional to $\underline{R}^{-1}$ ($\underline{Ra} \gg 1$; $f_S(\underline{r}) \ll \underline{K}_M'$) is an average particle radius defined by $\underline{R}^2 = (\sum \underline{R}_i^2)/n$ where $\underline{R}_i$ is the radius of the i-th gel particle.

For enzymes immobilized in membrane layers a dimensionless parameter that is similar to $\underline{Ra}$ has been shown to be of importance (1). In this $\underline{R}$ is replaced with the thickness of the membrane layer.

EXPERIMENTAL PROCEDURE

Sepharose 4 B was activated by the CNBr-method as described earlier (3). Immediately after the activation known amounts of CT in 0.5 M $NaHCO_3$ were added to equal amounts of activated gel ($\approx$4 ml settled volume). These gel suspensions were left overnight in a shaking apparatus. They were then poured on glass-filters that retained the gel particles and washed with 2 ml aliquots of 0.1 M borate-buffer containing 1 M NaCl. The eluates were collected until no more CT appeared in the eluate. From the total amount of CT in the eluates as determined spectrophotometrically we obtained the amount of coupled CT and n_E for the different enzyme-gels. Finally the enzyme-gel was washed with different buffers as described in (3).

The pH-optimum for CT-Sepharose and free CT were found to be equal in a TRIS-HCl buffer of pH 8.0 (I = 0.05 due to the buffering components, 0.2 M NaCl and 10 % (v/v) MeOH). This result was considered to justify the assumption that in this buffer the pH within the gel during catalysis is constant. The measurements were performed as follows. Ten ml of buffer and 1 ml of ATEE in MeOH were equilibrated at pH 8.00, 25.0 ^{o}C in the thermostated titration vessel of a Radiometer pH-stat. Then known amounts of well stirred gel suspensions were added. The contents in the vessel were stirred at a speed where the rate of enzyme catalysis was practically independent on stirring speed. This was considered to minimize the thickness of the unstirred layer outside the gel particle. The steady-state production of H^+ per unit time was used as a measure for v in Eq. (5). Duplicate determinations were performed throughout. The maximum variation in the rate determinations was $\approx$10 %.

RESULTS AND DISCUSSION

From Table I follows that at constant substrate content and V_{gel} we have:

$$(v/v_{max}) = (n_E/n_{E,max})^m \qquad (6)$$

where v_{max} is the maximum rate observed for the enzyme-gel of the highest

Table I

Immobilized enzyme		Free enzyme
I $f_S(r) \gg K_M$		
$v = k'_{cat}\, n_E\, V_{gel}$		$v = k_{cat}\, n_E\, V_{gel}$
II $f_S(r) \ll K_M$		
$Ra = R\sqrt{\frac{k'_{cat}\, n_E}{K'_M\, D'_S}} \gg 1$	$Ra \ll 1$	$v = \frac{k_{cat}\, n_E}{K_M} V_{gel}\, f_s(R)$
$v = \frac{3}{R}\sqrt{\frac{k'_{cat}\, n_E\, D'_S}{K'_M}}\, f_s(R)\, V_{gel}$	$v = \frac{k'_{cat}\, n_E}{K'_M}\, f_s(R)\, V_{gel}$	

Table 1. Expressions giving the steady-state rate v in a system with an immobilized enzyme and the corresponding rate in a system with free enzyme at different substrate concentrations $f_S(R)$. V_{gel} is the volume of the spherical enzyme-containing particles. For other definitions see text.

enzyme content $n_{E,max}$. In Fig. 1 we have given the experimental data, at equal V_{gel} added to substrate, for $\log(v/v_{max})$ as a function of

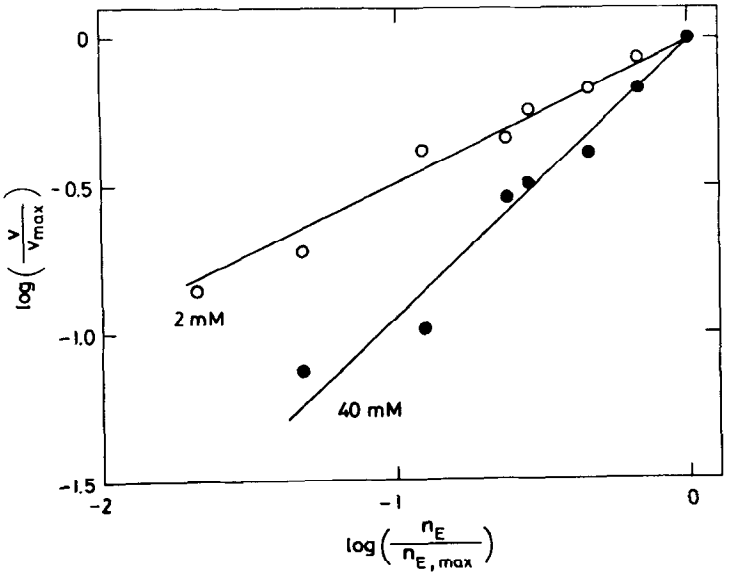

Figure 1. The logarithm of (v/v_{max}) for equal V_{gel} as a function of $\log (n_E/n_{E,max})$ at different substrate concentrations. The latter are given in the figure.

$\log(\underline{n}_E/\underline{n}_{E,max})$. At low substrate content the data fit a line with slope 0.48, at the high substrate content they fit a line with slope 0.94. From the data given in the figure we estimate the relative error in the slopes to be $\approx$10 %. These results agree within the experimental error with the behaviour expected for a system where $\underline{Ra} \gg 1$ and $f_S(\underline{r}) \ll \underline{K}_M'$ (m = 0.5) or $f_S(\underline{r}) \gg \underline{K}_M'$ (m = 1) (Table I, Eq. (6). In the experiments $\underline{n}_E$ was varied in the interval 10^{-5} - 4 x 10^{-4} M. For the system CT-ATEE in solution under similar experimental conditions we have $\underline{k}_{cat} \approx 10^2$ sec^{-1} (6), $\underline{K}_M \approx 4$ mM (6) and $\underline{D}_{ATEE}$ is estimated to be $\approx 10^{-6}$ cm^2 sec^{-1} (7). In the gel $\underline{K}_M'$ is expected to be larger than $\underline{K}_M$ (6) and $\underline{D}_{ATEE}' < \underline{D}_{ATEE}$. Assuming that the latter changes compensate each other and that $\underline{k}_{cat}' \approx \underline{k}_{cat}$, as is verified below, we obtain that with R $\approx 10^{-2}$ cm (8) $\underline{Ra} \gg 1$ in our experiments. From the close agreement between the results based on the theoretical model and the experimental observations we may conclude that the theoretical model gives an adequate description of our system.

When the system studied may be described by the above theoretical model $\underline{k}_{cat}'$ and $\underline{K}_M'$ can be determined as for the free enzyme. This can be done graphically by plotting $\underline{v}/(f_S(\underline{R})\ \underline{n}_E\ \underline{V}_{gel})$ as a function of $\underline{v}/(\underline{n}_E\ \underline{V}_{gel})$ (9). The abscissa intercept of this plot gives $\underline{k}_{cat}'$ as compared to $\underline{k}_{cat}$ for the free enzyme. From our data we obtain $\underline{k}_{cat}' = 60$ sec^{-1}. The ordinate intercept for the free enzyme gives $(\underline{k}_{cat}'/\underline{K}_M')$ and $(\underline{k}_{cat}'/\underline{K}_{M,app}')$ for the immobilized enzyme. From Table I follows that $\underline{K}_{M,app}' = \underline{Ra}(\underline{K}_M'/3)$ when $\underline{Ra} \gg 1$ and $= \underline{K}_M'$ when $\underline{Ra} \ll 1$. Thus when $\underline{k}_{cat}'$ and $\underline{K}_{M,app}'$ are used instead of $\underline{k}_{cat}$ and $\underline{K}_M$ in the expression for the rate in the free enzyme system we get the rate in the immobilized system. The coupling reaction-diffusion in the latter system does only influence the rate at low substrate content when $\underline{K}_M' \neq \underline{K}_{M,app}'$, i.e. $\underline{Ra} \gg 1$. Then $\underline{K}_{M,app}'$ depends on the kinetic parameters, diffusion coefficient $\underline{n}_E$, and the particle size. It also follows that methods to determine still active enzyme and $\underline{Ra}$ must be developed to allow more quantitative studies on $\underline{k}_{cat}'$ and $\underline{K}_M'$.

It has been shown that steady-state enzyme catalysis by immobilized enzymes differs markedly from the corresponding case with free enzyme when $f_S(\underline{r}) \ll \underline{K}_M'$ and the dimensionless parameter $\underline{Ra} \gg 1$. It is interesting to note that for many intracellular substrates the intracellular concentration is less than $\underline{K}_M'$ (10). Thus *in vitro*-data for enzymes that are immobilized *in vivo* can only be directly applied to describe the *in vivo*-kinetics when $\underline{Ra} \ll 1$. More data to estimate Ra are, however, required to discuss the consequences for the description of enzyme kinetics *in vivo*.

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