Vectorial product concentration obtained with a permeable immobilized enzyme membrane

A new approach to the analysis of biological transport systems

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The theoretical analysis of the distribution on both sides of a flat porous membrane of the product generated by an enzyme covalently bound only on to one side of the membrane separating two compartments of widely different volumes is presented. Contrary to what occurs with heterogeneous symmetric systems, the diffusional limitations at the enzyme level play a prominent role, not only on the apparent enzyme activity, but also on product flux-splitting. The mathematical model developed shows that it is possible to concentrate the reaction product in the compartment opposite to that where the reaction occurs. The influence of the parameters and of the physical characteristics of an asymmetrical system on product distribution is analysed. This theoretical analysis is in excellent agreement with experimental data obtained with glucose oxidase immobilized on a porous collagen membrane.

INTRODUCTION

The importance of microenvironment and metabolic compartmentation in biological systems is now well established (Dietschy, 1978).

With regard to the intracellular milieu, the diffusion barriers affecting the movement of solute molecules and the microenvironmental effects due to enzyme aggregates and their spatial organization when associated with intracellular membranes may play a prominent role in enzyme kinetics. Different approaches to studying these phenomena have been reported in the literature.

Some attempts have been made by different groups to simulate metabolite-transport processes with artificial membranes. Such approaches have been based mainly on theoretical and ideal considerations with enzyme molecules homogeneously embedded within the matrix (Goldman *et al.*, 1968). Only in a few cases have experiments been performed with inert layers associated with catalytic ones or with enzymes reticulated on one or both sides (Broun *et al.*, 1970; Bunow & Caplan, 1984). In the most elaborate approach, a structured multilayer bienzyme membrane was used to perform glucose active transport (Selegny *et al.*, 1971). Unless otherwise stated, the experiments reported were generally conducted with membranes separating identical compartments in uniformly stirred diffusion cells.

Bearing in mind the natural asymmetry of most biological compartments, and not forgetting that, *in vivo*, situations appear far more complex, we have developed a model with a covalent immobilization technique which makes it possible to obtain a surface binding of enzymes on only one side of a porous membrane. Furthermore, a specially designed diffusion cell with two compartments of widely different volumes and equipped with independent stirring systems, separated by the enzymic membrane, allowed us to study how the product of reaction

generated at the enzymic surface level is distributed on both sides (Maïsterrena *et al.*, 1985). With such an amplified asymmetry of the system, it was possible to obtain a high concentration of the reaction product in the compartment opposite to that where the reaction occurred. We now propose a mathematical model illustrating such an effect. Experimental data attesting to the validity of this model are provided.

EXPERIMENTAL

Enzyme and reagents

Collagen membranes (type F61). These were donated by the Centre Technique du Cuir, Lyon, France. Their thickness after enzyme binding, determined with 1234IC Millitron device with a MR 1301-type probe supplied by Mahr Feinprüf, Göttingen, Germany, was between 120 and 160 μm , depending on the collagen preparations.

The measuring device consists of a flat support on which the membrane is placed. An inductive gauging head in contact with the upper side of the membrane allows the determination of its thickness after calibration.

Glucose oxidase (GOD, EC 1.1.3.4). The grade 1 freeze-dried powder from the fungus *Aspergillus niger* (210 units/mg) was purchased from Boehringer. Other reagents of the highest grade available were supplied by Prolabo, Paris, France.

Enzyme immobilization. This was performed with discs of 5 cm diameter cut out of the dry membrane. They were activated by the acyl-azide procedure previously developed in our laboratory (Coulet *et al.*, 1974): carboxy groups of glutamate and aspartate residues are esterified by immersion of the discs in acidic methanol (0.2 M-HCl) for

Abbreviations used: symbols and their subscripts are listed and defined in Table 1; GOD, glucose oxidase.

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1 week at 20–22 °C. Then the discs are immersed overnight in an aq. 1% (v/v) hydrazine solution at the same temperature. The acyl-azide derivative is formed immediately before enzyme coupling by dipping the membrane into an extemporaneously prepared HNO_2 solution (0.5 M- NaNO_2 in 0.3 M- HCl), for 3 min. After a thorough washing in 0.05 M-glycine/ NaOH buffer, pH 9, glucose oxidase (1.5 mg/ml in the same buffer) was bound on to the surface of only one side of the membrane (Maïsterrena *et al.*, 1985).

Apparatus and methods

A previously designed asymmetric diffusion cell (Maïsterrena *et al.*, 1985) was used. This cell consists of two compartments, I and II, of 9 and 350 cm^3 respectively, separated by the collagen disc bearing immobilized GOD. The two compartments, with independent and variable stirring systems, were filled with 0.2 M-sodium acetate buffer/0.2 M- KCl /0.02% (w/v) NaN_3 , pH 5.5, 25 °C; glucose at the desired concentration was added to both compartments, and the appearance of H_2O_2 generated by the reaction

was monitored with time by an electrochemical method on samples withdrawn from both compartments.

The very sensitive amperometric measurement of H_2O_2 was performed with a PRGE-type polarograph from Solea-Tacussel, Villeurbanne, France, according to the following reaction:

The anodic current obtained was proportional to H_2O_2 concentration. The overall enzyme activity was expressed by the total amount of H_2O_2 produced in the two compartments per unit time for the total enzymic area ($=\pi \text{ cm}^2$).

THEORY

Classically, when a reaction due to a surface-bound enzyme occurs, the consumption of substrate and its transport from the bulk phase (also referred to as 'macroenvironment') to the microenvironment take place consecutively. At steady state, both proceed at the same rate. The resulting layer, in which a gradient for both substrate and product is established, is called the 'boundary layer' or 'diffusion layer'. The profile of gradients occurring in this boundary layer is dependent on the enzyme activity and on hydrodynamic conditions. The product tends to accumulate to a certain extent in the microenvironment and then is transported across the diffusion layer to reach the bulk phase.

Fig. 1 illustrates the system we have designed and used in the present study. The enzyme is bound on to the surface of only one side (Side II) of a flat porous membrane separating two compartments ($V_{II} > V_I$) equipped with two independent and variable stirring systems. In the compartment adjacent to the enzymic surface (volume V_{II}), i.e. the donor compartment, a low stirring rate allows the creation of a boundary layer of thickness δ , whereas in the compartment opposite to the reaction one (volume V_I), i.e. the receptor compartment, a high

Fig. 1. Scheme describing a permeable enzymic membrane separating two compartments of widely different volumes ($V_{II} > V_I$) non-uniformly stirred ($\omega_1 > \omega_2$)

The enzymic surface is adjacent to the donor compartment (volume V_{II}) where a boundary layer at the enzymic membrane/solution interface of thickness δ exists. In the receptor compartment (volume V_I) it is assumed that no unstirred layer exists at the membrane level. S is the substrate concentration in both compartments and S_0 its value at the enzyme membrane level; P_0 , P_I and P_{II} are the product concentrations at the enzyme level and in Compartment I and Compartment II respectively.

Table 1. Definitions of symbols and their subscripts

Symbol	Definition	Units
A	Membrane area	(cm^2)
A_w	Effective membrane pore area	(cm^2)
D	Diffusion coefficient	($\text{cm}^2 \cdot \text{min}^{-1}$)
d	Membrane thickness	(μm)
K_m	Michaelis constant	(mM)
δ	Boundary layer thickness	(cm)
P	Product concentration	(mM)
Q_p	Product quantity	(mmol)
R	Diffusion resistance	($\text{min} \cdot \text{cm}^{-3}$)
S	Substrate concentration	(mM)
t	Time	(min)
V	Volume compartment	(cm^3)
r	Apparent activity of the bound enzyme	($\text{nmol} \cdot \text{min}^{-1}$)
V_T	Total liquid volume of both compartments	(cm^3)

Subscripts	Definition
0	Membrane level
I	Compartment I, i.e., the receptor compartment
II	Compartment II, i.e., the donor compartment
δ	Relative to the boundary layer
m	Relative to the membrane
p	Relative to the product

stirring rate makes the unstirred layer negligible. Such conditions are easily realized, as previously reported (Maïsterrena *et al.*, 1985). (Note: symbols and their subscripts are listed and defined in Table 1.)

When the enzyme is working, the product flux generated at the enzyme level splits both through the diffusion layer to reach the bulk phase of Compartment

II and, simultaneously, through the membrane to reach Compartment I.

The present model differs from that previously published (Maisterrena *et al.*, 1985) in that the bound enzyme is here facing the larger compartment (side II). The role of the boundary layer is now explicitly defined and the diffusion resistance for the product is split into two terms, $R_{m,p}$ and $R_{\delta,p}$.

In order to simplify the mathematical analysis of the model system, the following assumptions were made: (a) the thickness of the enzyme layer is negligible compared with the membrane thickness; (b) the activity of the bound enzyme remains constant with time; (c) no product inhibition is exerted on the enzyme reaction; (d) the flow of substrate and product is perpendicular to the surface of the membrane; (e) the pH of the system does not vary with time. (f) the enzyme membrane is electrically neutral and no interaction exists between the membrane and the product; (g) at steady state the amount of product existing in the boundary layer and in the membrane is negligible compared with the total amount in Compartments I and II.

The apparent bound enzyme activity can be expressed as follows:

$$r = \frac{dQ_{P_I}}{dt} + \frac{dQ_{P_{II}}}{dt} \quad (1)$$

or

$$r = \frac{V_I P_{I,t} + V_{II} P_{II,t}}{t} \quad (2)$$

where V_I , V_{II} , P_I , P_{II} , Q_{P_I} and $Q_{P_{II}}$ are respectively the volumes, the product concentrations and the amounts of product in Compartments I and II.

Assuming that the product distribution conforms to Fick's first law, it follows that:

$$r = \frac{1}{R_{m,p}} (P_{0,t} - P_{I,t}) + \frac{1}{R_{\delta,p}} (P_{0,t} - P_{II,t}) \quad (3)$$

The new parameters introduced $P_{0,t}$ and $R_{m,p}$ are respectively the product concentration at the enzyme level and the membrane resistance to the product reaching the receptor compartment, i.e. $d/(D_p A_w)$, where d represents the membrane thickness, D_p the product diffusivity and A_w the effective membrane pore area.

$R_{\delta,p}$ is the unstirred layer resistance to the product reaching the donor compartment, i.e. $\delta/(D_p A)$, δ and A being respectively the thickness of the boundary layer and the membrane area.

Introducing $P_{I,t}$ from eqn. (2) into eqn. (3), we obtain:

$$P_{0,t} = \frac{r R_{\delta,p} (V_I R_{m,p} + t) + P_{II,t} (V_I R_{m,p} - V_{II} R_{\delta,p})}{V_I (R_{\delta,p} + R_{m,p})} \quad (4)$$

At any time, at steady state, the concentrations P_I and P_{II} in both compartments can be obtained from the previous equations. Considering first Compartment II, where the enzymic reaction takes place.

$$\frac{dP_{II}}{dt} = \frac{1}{V_{II} R_{\delta,p}} (P_{0,t} - P_{II,t}) \quad (5)$$

When substituting the $P_{0,t}$ value given in eqn. (4) into eqn. (5), the change in concentration P_{II} with time is given after integration by:

$$P_{II,t} = \frac{r}{V_T} \left\{ t + \frac{V_I (V_{II} R_{\delta,p} - V_I R_{m,p})}{V_T} \times \left[\exp - \left(\frac{V_T t}{V_I V_{II} (R_{\delta,p} + R_{m,p})} \right) - 1 \right] \right\} \quad (6)$$

Now substituting into eqn. (2) the value of $P_{II,t}$ given by eqn. (6), we obtain the change in concentration $P_{I,t}$ with time:

$$P_{I,t} = \frac{r}{V_T} \left\{ t - \frac{V_{II} (V_{II} R_{\delta,p} - V_I R_{m,p})}{V_T} \times \left[\exp - \left(\frac{V_T t}{V_I V_{II} (R_{\delta,p} + R_{m,p})} \right) - 1 \right] \right\} \quad (7)$$

Knowing all the parameters of the system (V_I , V_{II} , V_T , $R_{\delta,p}$, $R_{m,p}$), eqns. (6) and (7) make it possible to draw the theoretical curves of the product concentration versus time in both compartments for a given value of r .

RESULTS AND DISCUSSION

The validity of the model was tested experimentally by using a flat and porous collagen membrane bearing glucose oxidase covalently immobilized on only one side of the membrane.

This enzyme was chosen because one of the products (H_2O_2) is very easy to detect electrochemically at a very low level (≈ 1 nM), allowing its detection in the early stages of the reaction.

Two sets of experiments were conducted with the asymmetric diffusion cell previously described (see the Experimental section): the first one with a low substrate concentration of glucose (2.5 mM) compared with the K_m value for glucose (33 mM), and the second one with a high substrate concentration of glucose (380 mM). At the temperature of the experiments (25 °C), the concentration of the co-substrate O_2 can be considered as constant and equal to 0.2 mM (Hitchman, 1978).

The stirring rates in the two compartments were chosen so as to create different hydrodynamic conditions on both sides of the membrane. In Compartment I, a stirring rate (ω_1) equal to 450 rev./min in each experiment and obtained with a special impeller, made the unstirred layer negligible, as previously reported (Maisterrena *et al.*, 1985). By contrast, in Compartment II, lower and constant values of the stirring rate (ω_2) allow the creation of boundary layers of different thicknesses adjacent to the enzymic surface.

The experimental values of $P_{I,t}$ and $P_{II,t}$ were determined on samples withdrawn from both compartments and allowed the calculation of the overall apparent bound enzyme activity, r . The values of the thickness of the boundary layer in the different cases were estimated by plotting Q_{P_I} and $Q_{P_{II}}$ versus time (results not shown). The derivative curves obtained allowed calculation of $P_{0,t}$ and the boundary-layer thickness, δ . The parameters previously determined (Maisterrena *et al.*, 1985) were used, i.e. the membrane porosity A_w (20.5%) and the H_2O_2 diffusivity ($1.75 \times 10^{-3} \text{ cm}^2 \cdot \text{min}^{-1}$). For each experiment, the membrane thickness was measured (120 μm). Knowing A , A_w , D_p , d and δ , we can calculate the membrane resistance, $R_{m,p}$, to the product transfer towards Compartment I and the boundary-layer resistance, $R_{\delta,p}$, to the product transfer towards Compart-

Fig. 2. Product concentration pattern obtained versus time under first-order kinetics with respect to glucose (2.5 mM)

The volume of the receptor compartment, V_I , is equal to 9 cm³ and the volume of the donor compartment, V_{II} , is equal to 350 cm³. The stirring rates in Compartment I and Compartment II are respectively $\omega_1 = 450$ rev./min and $\omega_2 = 45$ rev./min. Under these conditions, the calculated value of boundary layer existing at the enzyme membrane level is equal to 280 μ m. The open circles (○) are the data for the receptor compartment (I). The closed circles (●) are the data for the donor compartment (II). The broken (---) and continuous (—) lines are obtained by using eqns. (6) and (7) respectively with the previous given parameters.

ment II. Finally, by using eqns. (6) and (7) with the experimental r values, the theoretical curves of P_I and P_{II} versus time were computed.

Figs. 2 and 3 show the experimental and theoretical plots of P_I and P_{II} versus time. The experimental conditions in Fig. 2 were: a low glucose concentration

(2.5 mM), a constant stirring rate in Compartment II equal to 45 rev./min, giving a boundary-layer value of 280 μ m. The r value is 54 nmol·min⁻¹ for the total enzymic area (π cm²). Fig. 3 shows the data obtained with a high glucose concentration (380 mM) for two different and constant stirring rates in Compartment II: (a) $\omega_2 = 250$ rev./min, giving a boundary layer of 80 μ m, $r = 105$ nmol·min⁻¹ (for π cm²); (b) $\omega_2 = 25$ rev./min, giving a δ value of 380 μ m, $r = 55.5$ nmol·min⁻¹ (for π cm²). The open and closed circles represent the experimental data. The continuous and broken lines represent the theoretical curves calculated according to the mathematical model outlined in the Theory section. Figs. 2 and 3 show an excellent agreement between the experimental data and the theoretical curves calculated from eqns. (6) and (7). The theoretical findings are verified by these experimental results and demonstrate the validity of our mathematical model. This theory, which takes into account the existence of a boundary layer at the level of a porous membrane bearing a catalytic activity, allows prediction of the product distribution in any compartmentalized system.

When the exponential term of eqns. (6) and (7) becomes negligible, the difference of product concentration in both compartments becomes constant and equal to:

$$P_{I,t} - P_{II,t} = \frac{r(V_{II}R_{\delta,p} - V_I R_{m,p})}{V_T} \quad (8)$$

Thus $P_{I,t} - P_{II,t}$ depends on the geometry of the system, the membrane's resistance to the product, the unstirred layer's resistance to the product and the apparent bound enzyme activity. It must be pointed out that, if $V_{II}R_{\delta,p} = V_I R_{m,p}$, $P_{I,t}$ would be equal to $P_{II,t}$. So, to obtain a hyperconcentration effect of the product in the receptor compartment, $V_{II}R_{\delta,p}$ should always be greater than $V_I R_{m,p}$: the greater the difference, the better the hyperconcentration effect.

The plots drawn in Fig. 4 make it possible to visualize

Fig. 3. Product-concentration pattern obtained versus time under zero-order kinetics with respect to glucose (380 mM)

(a) Compartment I, $\omega_1 = 450$ rev./min; Compartment II, $\omega_2 = 250$ rev./min, leading to a boundary layer δ value of 80 μ m. (b): Compartment I, $\omega_1 = 450$ rev./min; Compartment II, $\omega_2 = 25$ rev./min, leading to a boundary layer δ value of 380 μ m. Symbols are as in Fig. 2.

Fig. 4. Product concentration and amount plots corresponding to data of Figs. 3(a) and 3(b) at three selected times (60, 120 and 240 min)

The height and the area of the rectangles correspond respectively to the concentration and to the amount of product. P_0 is the product concentration at the enzyme level, P_I and P_{II} the product concentrations in the receptor and donor compartment respectively.

both product concentration and product quantity in the two compartments at three selected times and for two different thickness values of the diffusion layer.

The concentration of product at the enzyme level, P_0 , is also given. As expected, we can see that the diffusion layer plays a prominent role in the total amount of product in both compartments, i.e. the apparent enzyme activity. The thicker the unstirred layer, the lower the apparent enzyme activity. However, on the other hand, a thick diffusion layer leads to a preferential product flux toward the receptor compartment, thus amplifying the product hyperconcentration effect. Although in our study the product quantity in the receptor compartment was always lower than in the donor compartment, we must bear in mind that when considering situations occurring *in vivo*, enzyme kinetics and diffusional effects are strictly dependent on the molecular concentration of metabolites. Furthermore, the existence of sequential enzyme systems associated with membrane barriers pumping the intermediary species would certainly favour the hyperconcentration effect of the last sequential product. It must be stressed that as long as the enzyme works, the product concentration at the enzyme level, P_0 , acts as a barrier

preventing the retrodiffusion of the product from Compartment I to II.

Conclusions

When considering situations occurring *in vivo*, it must be borne in mind that cellular and subcellular biological membranes separate compartments of widely different volumes and that enzymes are not uniformly distributed within these membranes. Furthermore, diffusional barriers, playing a role in substrate or product diffusion and enzyme kinetics, may exist in the immediate vicinity of membranes (Dietschy, 1978).

In order to simulate these situations, we have developed a mathematical model and an experimental approach which have enabled us to explain and visualize a phenomenon which could not be obtained either with the enzyme free in solution (Bardetti *et al.*, 1985) or with an enzymic membrane placed in a diffusion cell equipped with identical stirred compartments of the same volume.

Even if such a macroscopic approach using artificial membranes and stirring systems is far from situations occurring *in vivo*, it does nevertheless demonstrate that a biochemical reaction can be made vectorial if it occurs

in an anisotropic medium with non-identical physical properties.

As a matter of fact, transport systems are known to be substrate-specific and substrate-saturable; in addition, they may be inhibited and are genetically determined. The model developed here, with an enzyme bound on one side of a membrane, adjacent to a large external compartment, porous to the product of reaction and developing in its immediate vicinity a high product level, can lead to a vectorial concentration pattern in a small and opposite internal compartment. This phenomenon appears similar to what could be expected with a carrier-mediated process. Such a hypothesis, compatible with the properties of transport systems, especially for group translocation, appears promising for sequential enzymes located on the two sides of the membrane.

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