Compartmentalized system with membrane-bound glycerol kinase

Activity and product distribution versus asymmetrical substrate supply

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An artificial-membrane-bound glycerokinase chosen as a membrane-bound two-substrate–enzyme model has been used to separate two unequal compartments of a specially designed diffusion cell. An interesting feature is the asymmetry of compartments and the existence of a diffusion layer adjacent to only one face of the enzymic membrane. In such a situation the apparent enzyme activity and the product distribution in the system have been studied versus all the possibilities of combination of ATP and glycerol supply. Our approach has lead us to differentiate two different roles played by a diffusion layer adjacent to a permeable enzymic membrane. Depending on the spatial origin of the enzymic substrates (i.e. from which compartment they derive), the diffusion layer can play either the role of a passive additional resistance to that of the membrane or the role of a third compartment in which the reaction product can partially accumulate before splitting on both parts of the membrane. Our results mainly demonstrate that a membrane-bound enzyme activity and the resulting product distribution occurring in a compartmentalized system may be regulated by the cumulative effect due to the asymmetry in volumes of the compartments, the presence of a diffusion layer and the different possibilities of substrate supply. With the topography studied, which is close to that reported for many ‘*in vivo*’ situations, the results of the conflict between substrate transformations and substrate diffusion associated with the direction in which the product may be diffused lead to vectorial metabolism processes.

INTRODUCTION

It has been recognized for many years that numerous enzymes of the living cell are localized on or within membranous structures (Welch, 1977) and consequently that their activities are strongly influenced by substrate supply (Barry & Diamond, 1984).

Enzyme immobilization is now a widespread technique which can be realized on various synthetic membranes of well-defined characteristics, and artificial enzyme membranes still remain an interesting experimental tool for studying the interplay between mass-transfer phenomena and enzyme reactions under well-defined conditions (Bunow & Caplan, 1984; Maïsterrena *et al.*, 1986; Vincent *et al.*, 1988).

In order to elucidate tentatively the mechanism of biological transport processes and metabolic compartmentation, numerous authors have used such a catalytic barrier to separate two compartments of a diffusion cell (Goldman *et al.*, 1968; Broun *et al.*, 1970; Thomas *et al.*, 1972; De Simone & Caplan, 1973).

Most of these studies were performed in symmetrical systems, i.e. involving an enzymic membrane separating two identical compartments under the same stirring conditions. In such a symmetrical system, anisotropy was created either by using inhomogeneous enzymic membrane (Goldman *et al.*, 1968; Bunow & Caplan, 1984) or by starting the reaction with different initial substrate concentrations in both compartments (Bunow & Caplan, 1984) or by inducing pH gradients on both sides of the membrane (Vincent *et al.*, 1988). In such an anisotropic medium it has been possible to observe a difference in the product build-up in both compartments of the system.

Bearing in mind that most of the enzymic biomembranes separate compartments of widely different volumes, it appears

more appropriate to use an asymmetrical system in order to mimic ‘*in vivo*’ situations.

Our purpose may be illustrated by the following example: when considering a two-substrate enzyme like glycerol kinase (GK), it is now an accepted fact that this enzyme can be bound to the porins of the outer membrane of liver mitochondrion (Fiek *et al.*, 1982; Östlund *et al.*, 1983; Benz *et al.*, 1988). With regard to the topography only of this ‘*in vivo*’ situation, the GK outer membrane delimits two asymmetrical compartments, i.e. the small intermembranous space of the mitochondrion and a larger compartment corresponding to the cytoplasmic medium. Regarding now the kinetic behaviour of GK, it has been postulated that its two substrates in the cell are flowing from different compartments, i.e. glycerol comes from the cytoplasmic medium, whereas ATP flows from the mitochondrion (Ludwig *et al.*, 1989). Concerning asymmetrical system, i.e. involving two compartments of widely different volumes, only a few results have been reported. The main conclusion of these studies was that the concentration level of the product appearing in the smaller compartment of the system was greatly favoured (Maïsterrena *et al.*, 1986), and this observation was corroborated in studies dealing with uphill transfer of metals (Uto *et al.*, 1986; Cox & Bhatnagar, 1988).

To our knowledge, there are no experimental data concerning the behaviour of a two-substrate enzyme membrane separating two compartments of widely different volumes as regards its two-substrate supply. Thus we decided that it would be worthwhile to study *in vitro* how two different substrates like glycerol and ATP, flowing from either identical or different compartments, could affect the activity of a GK membrane and might modulate the product distribution in an asymmetrical compartmentalized system.

Abbreviations used: GK, glycerol kinase; Gro3P, glycerol 3-phosphate; DH3P, dihydroxyacetone phosphate; PBIA, Pall Biotyne Immuno Affinity membrane(s); DH3P, glycero 3-phosphate.

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EXPERIMENTAL

Enzymes and reagents

GK (EC 2.7.1.30) from *Escherichia coli* (50–100 units·mg⁻¹; ref. G4509) was purchased from Sigma. L-Glycerol-3-phosphate (Gro3P) oxidase (EC 1.1.3.21) from different micro-organisms (50 units·mg⁻¹; ref. B775797) was from Boehringer. Other reagents of the highest grade available were supplied by Prolabo, Paris, France, and Sigma, St. Louis, MO, U.S.A.

Membranes

Pall Biotryne Immuno Affinity membranes (PBIA) were from Pall Industries, St. Germain-en-Laye, France. These polyamide-preactivated membranes (120 μm thickness; 3 μm size cut-off), initially devoted to antibody and antigen immobilization, also allowed enzyme coupling (Assolant-Vinet & Coulet, 1986).

Enzyme immobilization

Coupling of GK was performed on preactivated discs of 5 cm. diameter and 1.8 cm diameter cut out of the dry PBIA membrane by immersing at 4 °C for 20 h in 3 cm³ of the enzyme solution at 1.5 mg·cm⁻³ in 0.1 M-phosphate buffer, pH 7.

Coupling of Gro3P oxidase for the design of a Gro3P electrode was done as described above, but on preactivated discs of 0.8 cm diameter cut out of the PBIA membrane.

After these enzyme couplings, all the enzymic discs were washed twice in 1 M-KCl for 20 min and stored at 4 °C in a 0.1 M-glycine/NaOH buffer/0.1 M-KCl, pH 8.5.

Diffusion cell

The asymmetric diffusion cell previously designed was used (Maïsterrena *et al.*, 1985). This cell consists of two compartments of unequal volumes: compartment I of 9 cm³ consists of a 2 cm-internal-diameter tube obturated by the enzymic membrane; this compartment is immersed in a compartment II of 350 cm³, thermostatically controlled at 25 °C. Two independent and variable stirring systems were used in both compartments: in compartment I, a high stirring rate makes the unstirred layer negligible, whereas in compartment II, a low stirring rate allows the creation of a diffusion layer of thickness δ as previously reported (Maïsterrena *et al.*, 1985).

Measurements of product generated in a compartmentalized system separated by a PBIA-bound GK

The two substrates of GK, i.e. ATP/MgCl₂ and glycerol were added into the previously described diffusion cell at the desired concentration, taking into account all the combinations described in the insets to Fig. 1. For each case, experiments were conducted under fixed and identical physical conditions. The appearance of Gro3P, one of the reaction products generated by the following reaction:

was monitored with time on samples withdrawn from both

compartments by using a Gro3P electrode. This electrode is composed of an amperometric platinum probe associated with a Gro3P oxidase membrane coupled to a PRGE-type polarograph from Solea Tacussel, Villeurbanne, France. This biosensor allows the Gro3P concentration determinations according to the following reactions:

The anodic current thus obtained is proportional to the H₂O₂ concentration, which is itself proportional to the Gro3P concentration. Such a probe, specially designed for this study (Merchie, 1989), exhibits a detection limit below 10⁻⁷ M and a linear response to Gro3P up to 10⁻³ M at 25 °C in 0.1 M-glycine/NaOH/0.1 M-KCl buffer, pH 8.5, in the presence of 4 mM-Mg/ATP and 2 mM-glycerol.

Determination of the kinetic constants of the PBIA-bound GK

This study was realized, under fixed hydrodynamic conditions, in a measurement cell thermostatically controlled at 25 °C. The cell, in which the Gro3P biosensor was immersed, contained 20 cm³ of Mg/ATP at the desired concentration in a 0.1 M-glycine/KCl/0.1 M-buffer, pH 8.5, and a disc (1.8 cm diameter) of the PBIA-bound GK.

The enzymic reaction was initiated by adding glycerol at the desired concentration, and the appearance of product was monitored with time. Knowing the calibration curve of the biosensor, the slopes of the different curves obtained for various substrate concentrations permitted one to determine the corresponding membrane enzymic rates.

RESULTS AND DISCUSSION

First, the apparent K_m values for both substrates of the bound GK were determined and found to be 0.7 mM and 70 μM respectively for ATP and glycerol. These values are very close to those reported in the literature (Thorner & Paulus, 1973).

Nine cases were then considered which correspond to the different possibilities of the two-substrate supply in the compartmentalized system. These cases are schematically described in Table 1 and in the insets of Fig. 1, where the open circles (○) and the closed triangles (▲) represent respectively the two substrates Mg/ATP (4 mM) and glycerol (2 mM). The vertical double lines symbolize the enzymic membrane separating compartments I and II, whereas the vertical broken line represents the diffusion layer of thickness δ existing only in compartment II of the system.

At first it must be stressed that the identical coupling conditions used for all the membranes allow an excellent reproducibility of the coupling procedure with variations lower than 5%, as previously reported (Assolant-Vinet & Coulet, 1986). Secondly,

Fig. 1. Gro3P concentration levels versus time in both compartments of an asymmetrical compartmentalized cell separated by a membrane-bound GK with regard to the different possibilities of ATP and glycerol supply

In the insets a scheme describes the corresponding initial situation. The open circles (○) and the closed triangles (▲) represent respectively the initial concentrations of Mg/ATP (4 mM) and glycerol (2 mM). The vertical double lines symbolize the membrane-bound GK separating compartment I (9 cm³) and II (350 cm³), whereas the vertical broken line represents the diffusion layer of thickness δ existing only in compartment II of the system. For each study, the product concentrations measured in compartment I, i.e. P_I , are represented by open squares (□), whereas P_{II} , the product concentrations measured in compartment II, are represented by closed lozenges (◆). The resulting pattern of P_I and P_{II} versus time are represented by two corresponding continuous lines. Case 1, which corresponds to initial identical concentration of Mg/ATP (4 mM) and glycerol (2 mM) in both compartments, was chosen as a reference, and the corresponding P_I and P_{II} values versus time are represented for comparison by two broken lines in each study. All the experimentations were conducted under identical physical conditions in 0.1 M-glycine/0.1 M-KCl buffer, pH 8.5, at 25 °C. The stirring conditions in compartments I and II were respectively ($\omega_1 =$) 450 and ($\omega_2 =$) 100 rev./min.

it must be underlined that our experiments do not involve a biological system, but a system in which all the parameters, including mainly membrane properties and physical conditions, are strictly controlled. All the experiments were conducted in parallel and under strictly comparable conditions. The experimental errors, which are mainly due to the level of precision of the method used for the determination of product concentration and the precision of volume withdrawal, have been calculated very precisely and are given in Table 1. This allows us to compare the different cases, since the experimental conditions in all cases were highly reproducible.

Case number 1 was chosen as a reference, and corresponds to initial identical concentrations of the two substrates in both compartments. For this reference, we have obtained a linear relationship versus time for the total quantity of product generated in the system. Such a result demonstrates the stability of our enzymic membrane. The two substrate concentration values, i.e. $\text{Mg/ATP} = 4 \text{ mM}$ and $\text{glycerol} = 2 \text{ mM}$, were chosen so that the enzymic reaction follows, under initial conditions, zero-order kinetics. This choice is due to the fact that, when external diffusional limitations occur, and this applies in the case in compartment II of our system, the negative effect of these limitations disappears at high substrate concentrations (Engasser & Horvath, 1976).

Fig. 1 shows the product concentration P_I and P_{II} existing respectively in compartment I and II versus time for all the cases considered. Such P_I and P_{II} patterns can give an overview of what happens in our system during the time course of the enzymic reaction. On the other hand, it is difficult to see in Fig. 1 the differences between enzyme activities and product concentrations of the cases studied. For this reason we have also given in Table 1 the numerical values and the corresponding experimental errors of the total apparent enzyme activities and of the different product concentrations P_I and P_{II} existing in the system at 210 min for all the situations studied. When considering

Fig. 1, the reference product concentrations in compartment I and compartment II versus time (case no. 1) are represented by two corresponding broken lines on each graph of the eight other cases considered.

For the sake of clarity we have distinguished the three following series: a first series corresponding to cases 2, 6 and 8, where the reaction can start only on the compartment-I side, and where one or two of the substrates may diffuse from compartment I to compartment II, a second series (cases 3, 7 and 9) where the reaction can start only on the compartment-II side and where the substrates diffusion can only occur from compartment II to compartment I, and, lastly, cases 4 and 5, where a crossed diffusion of the two substrates must occur before the reaction can start. The following comments involve the cumulative effects of three different factors simultaneously, i.e. the asymmetry in volumes of the compartments, the presence of a diffusion layer and the different possibilities of substrate supply. Depending on the experimental conditions used, each factor will be involved to a greater or lesser extent.

(a) Comments on the first series (cases 2, 6 and 8)

First, it should be noted that, for cases 2 and 6, it has been impossible to detect any product in compartment II of the system, even at 210 min. These two cases correspond to situations where both substrates are initially present in compartment I, but where ATP can leave this compartment. The prominent role of this ATP diffusion from compartment I to compartment II can be explained by the dilution phenomenon of the initial ATP concentration value, which is only of about $6K_m$ and, consequently, by a time-dependent variation of the reaction-order kinetics of the membrane-bound enzyme. When comparing case 2, where the glycerol diffusion occurs, with case 6, where this diffusion cannot happen, the P_I profiles of these two cases appear quite similar. This can be explained by the initial glycerol concentration value, which is close to $30K_m$ and consequently by a weaker effect of the dilution phenomenon, due to the diffusion of glycerol from compartment I to compartment II, compared to the dilution effect for ATP.

The high P_I value of case 8 compared with the P_I value of the reference may be explained, in the presence of identical ATP concentrations, by the diffusion only of glycerol through the enzymic membrane, diffusion of which favours the collision of this substrate with the enzymic surface and then increases the enzymic reaction rate.

(b) Comments on the second series (cases 3, 7 and 9)

When looking at cases 3, 7 and 9 we can observe that the P_I and P_{II} profiles appear quite linear, leading to the conclusion of a rather constant enzymic rate which can be explained (in contrast with the cases of the first series) by a very low substrate-dilution phenomenon due to the diffusion of substrates from compartment II to compartment I.

The main conclusion for this series is that, even with a starting reaction only in the diffusion layer adjacent to compartment II, the product accumulates preferentially in the opposite compartment, I, leading to an apparent Gro3P translocation process. This observation corroborates a previous result concerning an apparent glucose 6-phosphate translocation process obtained with a membrane-bound hexokinase (Maïsterrena & Coulet, 1989). Such a phenomenon can be explained by the key role played by the diffusion layer existing at the membrane level in compartment II, which acts as a new compartment in which the product can build up before being distributed simultaneously into compartment I after crossing the membrane and through the diffusion layer to distribute in compartment II (Maïsterrena *et al.*, 1987).

Table 1. Asymmetrical compartmentalized system separated by a PBIA-bound GK: apparent activity and product distribution versus the two-substrate supply

The initial situations of the nine cases considered are given in the second column. The symbols are as in Fig. 1. The apparent activity ($\text{nmol} \cdot \text{min}^{-1}$) of the bound GK has been calculated for all the cases considered by the measurement of the Gro3P concentration level (μM) existing in compartment I (P_I) and in compartment II (P_{II}) at 210 min.

Case	Situations			Apparent bound enzyme activity ($\text{nmol} \cdot \text{min}^{-1}$) at 210 min	Product concentration (μM) at 210 min in compartments I and II	
	I	δ	II		P_I	P_{II}
1 (reference)	○ ▲		○ ▲	11.8 ± 1.5	55.9 ± 4.0	5.9 ± 0.8
2	○ ▲			< 2.0	30.6 ± 1.5	< 0.5
3			○ ▲	16.2 ± 0.7	80.3 ± 1.9	8.0 ± 0.4
4	○		▲	10.5 ± 1.2	49.9 ± 3.1	5.2 ± 0.7
5	▲		○	12.3 ± 1.4	42.9 ± 2.0	6.5 ± 0.8
6	○ ▲		○ ▲	< 2.7	40.1 ± 3.3	< 0.7
7	▲		○ ▲	16.0 ± 1.3	56.8 ± 3.4	8.4 ± 0.7
8	○ ▲		○	12.0 ± 1.0	83.1 ± 2.6	5.4 ± 0.6
9	○		○ ▲	13.1 ± 1.7	72.3 ± 4.3	6.3 ± 0.9

(c) Comments on the third series (cases 4 and 5)

In these two latter situations no initial reaction can occur in the first step of the experiment, either in compartment I or in compartment II: each substrate must first diffuse from one compartment to the other before reacting with the enzymic membrane. Case 4 corresponds to the situation reported to exist in the cell, i.e. ATP flowing from the small intermembranous mitochondrion compartment, whereas glycerol flows from the large cytoplasmic medium. Even with a strong dilution effect due to the ATP diffusion from compartment I to compartment II, an effect which greatly affected both the apparent bound enzyme activity and the product distribution of cases 2 and 6 (see Table 1), surprisingly we observe for this case, 4, an enzyme activity and product profiles very close to the reference data. When comparing cases 4, 2 and 6, which are the situations where ATP exists only in compartment I of the system (see Table 1), situation 4 appears as the most advantageous one, i.e. the situation involving the lowest quantity of ATP in the system and leading to data very close to the reference data.

When referring to case 5, where, as previously underlined, the dilution effect due to glycerol diffusion from compartment I to compartment II is not a rate-limiting factor, this case (5) should have been more favourable than case 4. In fact, when referring to Fig. 1 and the data given in Table 1, this case, 5, appears less advantageous than case 4. This may be explained by the very different diffusion coefficients of ATP and glycerol, the different initial concentration values of these two substrates and the asymmetry of the system which makes this case, 5, not exactly the opposite of case 4.

Finally, what appears very clear, when looking at the P_i concentrations levels of cases 2 and 3 given in Table 1, is the key role played by the diffusion layer of thickness δ existing in compartment II.

More precisely, the P_i for case 2, for which the reaction can start only in compartment I, is far less pronounced than the P_i in case 3, for which the reaction can start only in the opposite compartment II.

In case 2, the diffusion layer seems to play the role of a passive additional resistance to that of the membrane, and this resulting global resistance does not prevent the leaving of both substrates from compartment I. In this case, 2, the driving force due to the diffusion of both substrates appears stronger than the capacity for the substrates to react with the enzymic side facing compartment II. Therefore no, or little, product can accumulate in the diffusion layer. Here diffusion has a negative regulatory effect on the membrane-bound enzyme activity.

In contrast, in case 3, the diffusion layer seems to play an active role. This can be explained because the reaction initially starts in this 'third compartment' and then the product can accumulate at the enzyme surface level facing compartment II, favouring in the first steps of the reaction time course the splitting of product toward compartment I and II. During the experiment, the substrates which diffuse to compartment I can also react concomitantly in this compartment, but the resulting product cannot diffuse toward compartment II because of the presence of the high product concentration existing in the diffusion layer, which then acts as a barrier. Here, diffusion has a positive regulatory effect on the membrane-bound enzyme activity.

CONCLUSIONS

Artificial enzyme membranes used as a catalytic barrier separating two solutions are an interesting tool for the understanding of metabolite compartmentation and of transport properties reported for biological membranes.

Previous studies dealing with artificial enzyme membranes in compartmentalized systems have demonstrated that a biochemical reaction can be made vectorial if it occurs in an anisotropic medium. Several factors may participate in the creation of this anisotropy, mainly (a) the existence of a diffusion layer in the vicinity of the enzymic surface (Engasser & Horvath, 1976), (b) the distribution of the enzyme on or within the membrane (Bunow & Caplan, 1984), (c) compartments of unequal volumes on both sides of the membrane (Maisterrena *et al.*, 1986) and (d) the substrate flow coming from a definite compartment (Bunow & Caplan, 1984).

However, most of these studies have been conducted in symmetrical compartmentalized systems with artificial membrane-bound enzymes and were mainly concerned with simpler Michaelis-Menten-type kinetics. Up to now no data have been reported concerning a two-substrate enzyme membrane behaviour versus the different possibilities in the spatial supply of its two substrates.

In our approach, a membrane-bound GK, chosen as a two-substrate enzyme membrane model, has been used to separate two compartments of widely different volumes. In this kind of topography, close to the situation reported to exist *in vivo* for many membrane-bound enzymes and specially for the liver GK (Fiek *et al.*, 1982; Östlund *et al.*, 1983; Benz *et al.*, 1988), the apparent enzyme activity and the product distribution in the system have been studied in respect of all the possibilities for ATP and glycerol supply.

Our results demonstrate that not only does the membrane-bound GK activity strongly depend on the different possibilities for ATP and glycerol issuing from the two compartments delimited by the enzymic membrane, but also that the different possibilities of substrate supply may play a prominent role in the distribution of the reaction product in an asymmetrical compartmentalized system. Even if our '*in vitro*' investigation involving artificial membranes and stirring conditions may appear approximate because it is far from a situation *in vivo*, this approach, however, did lead us to observe that the scenario proposed for the '*in vivo*' GK behaviour is also in our system the most advantageous with regard to the quantity of ATP involved. Such a result would merit more precise investigation with regard to various substrate concentrations.

This type of regulatory effect which has been observed in initial zero-order-kinetics conditions cannot be attributed to external diffusional limitations, but rather to a cumulative effect due to the asymmetry in volumes of the compartments, the presence of a diffusion layer, different possibilities of substrate supply and consequently by the resulting competition between reaction and diffusion occurring for substrates.

Then "We might be inclined to recognize that transport and metabolism as usually understood by biochemists may be considered advantageously as different aspects of one and the same process of vectorial metabolism". We very modestly quote this sentence of Peter Mitchell (1961), who sensed this important concept many years ago.

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