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Effect of partitioning equilibria on the activity of β-galactosidase in heterogeneous media

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Abstract

We had demonstrated that membrane adsorption or penetration differentially modulated β-Galactosidase (β-Gal) activity against soluble substrates (Coll. and Surf., 24, 21, 2002). In a heterogeneous media, not only the enzyme but also the rest of the chemical species taking part in a chemical reaction would eventually interact with the available surfaces. The aim of the present work was to investigate if, in addition to changes in the intrinsic mechanism of the reaction at the lipid–water interface, the kinetics of enzyme-catalyzed reactions could be significantly affected by the partitioning of the substrate (*ortho*-nitro-phenyl galactopyranoside (ONPG)), the product (*ortho*-nitro-phenol (ONP)) and the enzyme (*E. coli* β-Gal) towards the membrane. Multilamellar vesicles of sPC were used as model membranes. Membrane–water partition coefficients ($P_{m/w}$) were determined according to the theory and methodology developed previously (J. Neurosci. Meth. 36, 203, 1991). The values of $P_{m/w}$ obtained ($P_{ONPG}=0$, $P_{ONP}=50$ and $P_{\beta\text{-Gal}}=118$) were applied to a two-compartment model, which assumed a free access of the substrate to the enzyme and a nucleophile-like activatory effect exerted, within the membrane compartment, by the lipid–water interface. This model: (i) reproduced the lipid concentration-dependence we had observed previously in V_{max}, (ii) predicted the values of $k_4=3.54\times10^7\ s^{-1}$ and the extinction coefficient of the aglycone in the membrane phase, 4012 $M^{-1}\ cm^{-1}$, with $p<0.0001$ and $p<0.02$, respectively, as well as for $P_{\beta\text{-Gal}}=117$ (which was poor ($p=0.6716$) but gave a numerical value within the same order of magnitude that the experimental value) and (iii) emphasized the importance of the more efficient reaction mechanism in the membrane phase compared with that in the aqueous phase ($k_4 \gg k_3$).

Keywords: β-galactosidase; O-nitrophenyl-β-D-galactopyranoside; Partition coefficient; Phospholipids vesicles; Enzyme kinetics

1. Introduction

In previous works we demonstrated that beta-Galactosidase from *Escherichia coli* (β-Gal), which is a soluble protein, was able to interact with biomembranes. The type of interaction (adsorption or penetration) depended on the membrane composition, organization and topology, and differentially modulated the activity of the enzyme toward a soluble substrate [1,2]. However, in complex systems like membrane suspensions, not only the enzyme but also the other chemical species participating in the reaction could eventually partition or be adsorbed to the lipid–water interface. Many studies of enzyme kinetics in reverse micellar solutions addressed a similar problem ([3] and Refs. therein). However, in those cases the enzyme was totally associated to the micelles while the substrate was partitioned between the micelles and the external solvent. Our previous findings regarding the modulation of β-Gal [1,2] suggested that in the presence

Abbreviations: *A*, absorbance; *C*, concentration; *D*, dielectric constant; ε, extinction coefficient; f_m, volume fraction of membrane phase; f_w, volume fraction of water phase; MLV, multilamellar vesicle; ONPG, *ortho*-nitro-phenyl-β-D-galactopyranoside; ONP, *ortho*-nitro-phenol; ONPx, *ortho*-nitro-phenoxide; $P_{o/w}$, octanol–water partition coefficient; $P_{m/w}$, membrane–water partition coefficient; rpm, revolutions per minute; sPC, soybean phosphatidylcholine; V_{max}, maximal velocity; λ, wavelength; *X*, volume fraction of water phase trapped within the pellet.

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of lipid–water interfaces, in addition to partition equilibria and diffusional barriers, changes in the intrinsic mechanism of the reaction, would contribute to the modulation of the kinetics of substrate hydrolysis.

In order to get deeply in this problem, in the present work we determined the values of the membrane–water partition coefficients ($P_{m/w}$) of all the chemical species that participate in the reaction of hydrolysis of *o*-nitro-phenyl-β-D-galactopyranoside (ONPG) catalyzed by β-Gal.

The effect of nonzero $P_{m/w}$ values on the reaction kinetics was modeled and the results were compared with our previous experimental results of the hydrolysis of ONPG catalyzed by β-Gal in the presence of phospholipid bilayers [1].

2. Theory

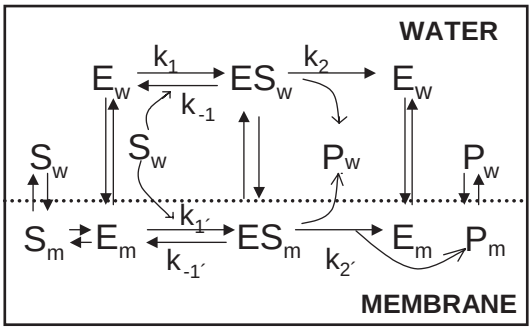
2.1. Modulation of β–gal activity in a heterogeneous system

A theoretical model was developed in order to describe the effects of PC multilamellar vesicles (MLVs) on the values of maximal velocity (V_{max}) of the hydrolysis of *ortho*-nitro phenyl β-D-galactopyranoside (ONPG) to *ortho*-nitro-phenol (ONP), catalyzed by the enzyme β-Gal.

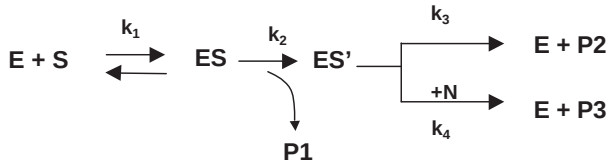
2.1.1. The model

The system consists of two compartments: the membrane phase and the water phase (see Scheme 1). The reaction can occur in both compartments with the following characteristics:

a) The reaction product and the enzyme are able to partition from the aqueous phase to the membrane and vice versa (eventual hysteresis between the adsorption and desorption processes, is not considered).
b) The reaction mechanism is different in each phase, resulting in differences in the rate constants. This can be due to the possible occurrence of a nucleophilic-like effect (see Scheme 2) [4] induced by the polar head group of the membrane phospholipids or by the water structured at the membrane surface proposed in previous works [1,2].



Scheme 1. *E*, ES, *S* and *P* represent the enzyme, enzyme–substrate complex, substrate and the product respectively. *k* = rate constants. Subindexes w and m refer to the water and the membrane phases, respectively.



Scheme 2. *P*1, ES′, *P*2, *P*3 and N, represent the aglycone, glycosylated enzyme, galactose, galactose derivative and the nucleophile, respectively. Other symbols were described in the text.

2.1.1.1. Mass balance. The total amount of each chemical species in the system is the sum of the amounts in each phase

$$C_T \cdot V_T = C_m \cdot V_m + C_w \cdot V_w \quad (1)$$

where C, V and the subindexes T, m and w refer to concentration, volume, total system, membranous phase and water phase, respectively.

The partition coefficient between the membrane and the aqueous phase is:

$$P = C_m / C_w \quad (2)$$

The sum of the fractions of the total volume corresponding to membrane ($f_m = V_m / V_T$) and water ($f_w = V_w / V_T$) phases correspond to

$$f_w + f_m = 1 \quad (3)$$

From the Eqs. (1), (2) and (3):

$$C_w = \frac{C_T}{f_w + P \cdot f_m} \quad \text{and} \quad C_m = \frac{C_T \cdot P}{P \cdot f_m + f_w} \quad (4)$$

From the mass of solute partitioned in each phase

$$m_w = C_w \cdot V_w \quad \text{and} \quad m_m = C_m \cdot V_m \quad (5)$$

the contribution of the amount of mass in the membranous and aqueous phases to the global concentration in the whole system can be calculated:

$$C'_w = \frac{m_w}{V_T} \quad \text{and} \quad C'_m = \frac{m_m}{V_T} \quad (6)$$

Then, combining Eqs. (3), (4) and (5), solving for C_T and C_m and replacing into Eq. (6) we got:

$$C'_w = \frac{C_T \cdot f_w}{P \cdot f_m + f_w} \quad \text{and} \quad C'_m = \frac{C_T \cdot P \cdot f_m}{P \cdot f_m + f_w}. \quad (7)$$

From the kinetics of releasing of the aglycone (ONP in this case) represented by *P*1 in the Scheme 2, k_{cat} in each phase results [4]:

$$k_{cat} = \frac{k_2 \cdot (k_3 + k_4 \cdot N)}{k_2 + k_3 + k_4 \cdot N}$$

where N represents the concentration of a nucleophile.

In the aqueous phase $k_4=0$ and in the membrane phase $k_3=0$, resulting:

$$k_{cat,w} = \frac{k_2 \cdot k_3}{k_2 + k_3} \quad \text{and} \quad k_{cat,m} = \frac{k_2 \cdot k_4 \cdot N}{k_2 + k_4 \cdot N}. \tag{8}$$

The maximal reaction rate of $P1$ production (V_{max}) is dependent on the maximal enzyme–substrate complex concentration ([ES]), which in turn is equivalent to the total enzyme concentration (E_T), then: $V_{max}=k_{cat} \cdot E_T$.

Taking in to account that:

a) The total rate comes from the sum of the individual contributions of the reaction rates in each phase:

$$V_{max,T} = V_{max,w} + V_{max,m} \tag{9}$$

b) The reaction rate in each phase depend on the reaction mechanism in each phase expressed by the corresponding catalytic rate constants ($k_{cat,w}$ or $k_{cat,m}$);

$$V_{max,w} = k_{cat,w} \cdot E_{T,w} \quad V_{max,m} = k_{cat,m} \cdot E_{T,m} \tag{10}$$

c) The enzyme concentration in each phase depends on E_T minus the amount of enzyme in the other phase, referred to the total volume (Eq. (7)), and depends on the partition coefficients of the enzyme ($P_{\beta\text{-Gal}}$) as well as on the volume fraction of each phase.

$$E_{T,w} = E_T \cdot \left(1 - \frac{P_{\beta-Gal} \cdot f_m}{P_{\beta-Gal} \cdot f_m + f_w}\right) \cdot \frac{1}{f_w} \quad \text{and}$$
$$E_{T,m} = E_T \cdot \left(1 - \frac{f_w}{P_{\beta-Gal} \cdot f_m + f_w}\right) \cdot \frac{1}{f_m} \tag{11}$$

d) For the calculation of the rate of $P1$ formation in each phase it is necessary to take into account the partition coefficient of $P1$ (P_{ONP}), in order to estimate the amount of product formed in one phase which is transferred to the other phase through a partitioning process, and that has to be added to $V_{max,w}$ and $V_{max,m}$.

$$m'_w = k_m \cdot E_{T,m} \cdot \frac{f_w}{f_m \cdot P_{ONP}} \quad \text{and}$$
$$m'_m = k_w \cdot E_{T,w} \cdot P_{ONP} \cdot \frac{f_m}{f_w}$$

$$V_{max,w} = k_w \cdot E_{T,w} \cdot \left(1 - P_{ONP} \cdot \frac{f_m}{f_w}\right) + k_m \cdot E_{T,m} \cdot \frac{f_w}{f_m \cdot P_{ONP}} \tag{12}$$

$$V_{max,m} = k_m \cdot E_{T,m} \cdot \left(1 - \frac{f_w}{f_m \cdot P_{ONP}}\right) + k_w \cdot E_{T,w} \cdot P_{ONP} \cdot \frac{f_m}{f_w} \tag{13}$$

e) The extinction coefficients of $P1$ in the aqueous and membrane phase (ε_w and ε_m) will depend on the polarity of both water and the membrane regions where it is localized.

Then, an expression that allowed to calculate, from spectroscopic measurements, the production rate of $P1$ as a function of the phospholipid amount present in the system, self-assembled in the form of MLVs and represented by a variable f_m value, can be proposed:

$$Abs = k_w \cdot E_{T,w} \cdot [\varepsilon_w + (\varepsilon_m - \varepsilon_w) \cdot c] + k_m \cdot E_{T,m} \cdot \left[\varepsilon_m + (\varepsilon_w - \varepsilon_m) \cdot \frac{1}{c}\right] \tag{14}$$

where

$$c = \frac{P_{ONP} \cdot f_m}{f_w}$$

Eq. (14) indicates that the transference of product from one phase to the other (due to partition), contributes to each component of the total absorbance with an intensity depending on:

i) the difference between the values of extinction coefficients of $P1$ in each phase,
ii) the value of the partition coefficient of $P1$,
iii) the volume ratio between both phases.

For the case of the chemical species measured as the product of the reaction studied in the present paper (*ortho*-nitro-phenoxide: ONPx), we considered the ε values determined from solutions of known dielectric constants (D) reported previously [2]. For our calculations we selected a $\varepsilon_w=4500\ M^{-1}\ cm^{-1}$ corresponding to $D=78$ of an aqueous media. In the case of the membrane phase, the value $\varepsilon_m=5900\ M^{-1}\ cm^{-1}$ should be expected if we consider that ONP is localized in a medium with $D=40$, characteristic of the polar head group region of the bilayer [5].

3. Materials and methods

3.1. Materials

The enzyme β-Gal from *E. coli* [EC 3.2.1.23] Grade VII (specific activity 650 UI/mg protein; 1 UI=1 μmol/min of ONP formed at 37 °C) as lyophilized powder and *ortho*-nitro-phenyl-β-D-galactopyranoside (ONPG) were obtained from Sigma Chem. Co (St. Luis, MO) and ONP from ICN Pharmaceuticals (Costa Mesa, CA). Lipids were purchased from Avanti Polar Lipids (Alabaster, Alabama). Other reagents and solvents used were of analytical grade.

3.2. Methods

3.2.1. Preparation of liposomes

Multilamellar vesicles (MLV) [6] were prepared by evaporating, under a stream of nitrogen, the chloroform from a solution of pure soybean phosphatidyl choline (sPC).

The dry lipid was suspended in water at a final concentration of 1 mg/ml, by repeating six consecutive cycles of heating for 2 min at 21 °C, (a temperature above the T_c of the phospholipid), and vortexing for 1 min. In these conditions, phospholipids self-aggregated into multilamellar vesicles [7].

3.2.2. Determination of the membrane–water partition coefficients of ONPG and ONP

The values of membrane–water partition coefficients ($P_{m/w}$) of ONPG (P_{ONPG}) and ONP (P_{ONP}) were determined as described elsewhere [8]. MLVs (1 mg/ml sPC), used as model membranes, were incubated at 21 °C during 15 min in the presence of the drug at variable concentrations. The mixture was centrifuged at 18 000 $\times g$ for 30 min to separate the free drug from that bound to the membrane. UV-Visible spectroscopy was applied to quantify ONPG and ONP in each phase. Changes in the extinction coefficients, due to differences between the polarity of the membrane and the aqueous phase, were taken into account [1,2].

The amount of drug that is "observed" in the membrane fraction arising from 1000 ml of incubation system ($Drug_{obs}$), was plotted as a function of the total drug concentration $[Drug]_T$. This plot could be described by the following equation (see Ref. [8] for details):

$$Drug_{obs} = \left(P_{m/w} \cdot f_m + X \cdot f_w\right) \cdot [Drug]_T. \quad (15)$$

From the slope of this plot, equivalent to $[P_{m/w} \cdot f_m] + [Xf_w]$, the value of $P_{m/w}$ was calculated. As defined previously, f_m and f_w were the volume fractions that corresponded to the membrane and the water phases, respectively. The parameter X was the volume fraction of the supernatant retained by the sediment during the centrifugation process done to separate the aqueous and the membrane phases. If $P_{m/w}=0$, the first term zeroed and the slope resulted equal to Xf_w. Hence, the latter was obtained in an experiment run in parallel, but using $K_2Cr_2O_7$, taking advantage of the fact that this is a highly hydrophilic substance the $P_{m/w}$ value of which could be assumed to be zero.

Octanol was also used to mimic the membrane environment and the partition coefficient of ONP between water and octanol ($P_{ONP,o/w}$) was also determined. This drug was dissolved in an octanol–water mixture. After 30 min at room temperature, the partition equilibrium was reached; then the mixture was centrifuged at 3000 $\times g$ for 30 min to allow a clear separation between the octanolic and aqueous phases. The drug present in aliquots of both phases was quantified by visible spectrophotometry.

3.2.3. Determination of the membrane–water partition coefficient of β-Gal

The enzyme was incubated for 30 min at room temperature in the presence of sPC MLVs (1 mg/ml). The free and bound enzyme fractions were separated by a procedure similar to that applied for ONP and ONPG. The amount of enzyme retained in the water trapped within the pellet was discounted from that present in the whole sediment, and the β-Gal partition coefficient was calculated as indicated above. The enzyme retained in the sediment was quantified as a protein by the spectrophotometric method of Lowry et al. [9].

3.2.4. Statistical calculations

The least squares method was applied to fit functions through a regression analysis. Student's *t*-test was applied to compare individual averages. The propagation error method was used to evaluate the error associated to variables calculated from other ones determined experimentally [10,11].

4. Results

4.1. The octanol–water partition coefficient of ONP

The upper and the lower phases of an octanol–water mixture were used as solvents to prepare ONP solutions of known concentration and their absorbance at 420 nm (A_{420}) were measured. The experimental A_{420} vs [ONP] using, as solvents, either the upper (Eq. (16)) or the lower phase (Eq. (17)), were fitted to straight lines by a linear regression analysis by the least squares method leading to:

$$A_{420} = 6.531.10^{-2}[ONP] + 1.522.10^{-2} \quad (16)$$

$$A_{420} = 0.1627[ONP] - 1.695. \quad (17)$$

The average values of A_{420} from the upper and the lower phases, obtained in the experiment of ONP partitioning in the octanol–water system (described in the Materials and methods section) applied to Eqs. (16) and (17), respectively, gave 8.75 and 0.35 mM as the values for ONP concentration in each phase, and a value of $P_{ONP,o/w}=25$ mM/mM for the octanol–water partition coefficient of ONP was calculated (Fig. 1).

4.2. Membrane–water partition coefficient of ONP and ONPG

The amount of $K_2Cr_2O_7$, ONPG or ONP present in the insoluble phase of a suspension of sPC became from measurements of the absorbance values obtained by UV-visible spectrophotometry. These data were transformed in mass values using appropriate calibration curves (data not shown) and, after plot them as a function of the total drug concentration in the incubation system, a straight lined relationship was observed (Fig. 2). Eq. (15) could be fitted to these data through a highly significant linear regression

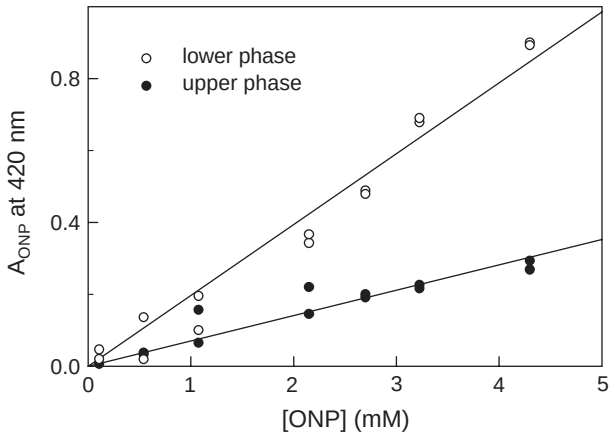


Fig. 1. Calibration curves and determination of partition coefficients for ONP in an octanol–water system. Blanks consisted of ONP solutions of known concentrations prepared with the theoretical upper or lower phases of an octanol–water mixture system. Lines represent the fit to linear equations by the least square method. $P_{o/w}$: octanol–water partition coefficient.

analysis ($p<0.0001$) with zero ordinate values (see Table 1 for details).

The results obtained with $K_2Cr_2O_7$ are shown in Fig. 2a. This is a hydrophilic drug with a partition coefficient assumed to be equal to zero, so the slope of this plot represents the value $[X \cdot f_a]$ (see Eq. (15)). The membrane–water partitioning of ONPG and ONP were calculated from the plots shown in Fig. 2b and c, the slopes of which are defined by the expression $[P_{m/w} \cdot f_m] + [Xf_w]$. So, the value obtained by subtracting from the latter the slope of the curve obtained with $K_2Cr_2PO_7$, (Xf_w) is equivalent to $P_{m/w} \cdot f_m$. The partition coefficient could be calculated once the volume fraction corresponding to the membrane (f_m) was known. The slope values determined were: Slope $_{K2Cr2PO7}=0.0347$; Slope $_{O\ NPG}=0.0280$; Slope $_{ONP}=0.106$.

The result of the difference [Slope $_{ONPG}$ − Slope $_{K2Cr2PO7}$] tended to zero so $P_{ONPG} \cong 0$. The difference [Slope $_{ONP}$ − Slope $_{K2Cr2O7}$] was 0.0717. The value of $f_m = 1.41 \times 10^{-3}$ was taken from our previous work [8] and a value of $P_{ONP}=51$, could be calculated (see Table 1 for a detailed analysis).

4.3. Membrane–water partition coefficient of β-Gal

The presence of the enzyme in each fraction (Total, sediment and supernatant) was checked measuring the protein concentration (Fig. 3), and the β-Gal membrane–water partition coefficient resulted was $P_{\beta\text{-Gal}}=118$.

4.4. Evaluation of the effect of the partitioning of the product and the enzyme on the kinetics of the reaction of hydrolysis of ONP catalyzed by β-Gal in the presence of lipid–water interfaces

For the description of the reaction system, we propose a two-compartment model (Scheme 1). According to this model, the reaction can take place simultaneously in the water phase as well as at the membrane–water interface. The enzyme (E) is partitioned from the aqueous phase towards the membrane. The soluble substrate (S) interacts with both, the free and the membrane-bound enzyme pools. The product (P) obtained, through the enzyme–substrate complex (ES) formed in each compartment, is redistributed within the system according to its $P_{m/w}$ value.

The mechanism for ONPG hydrolysis catalyzed by β-Gal (Scheme 2) was proposed by Viratelle and Yon [4]. In the first stage the enzyme–substrate complex ES is formed and then $P1$, the aglycone part of the substrate (ONP in this

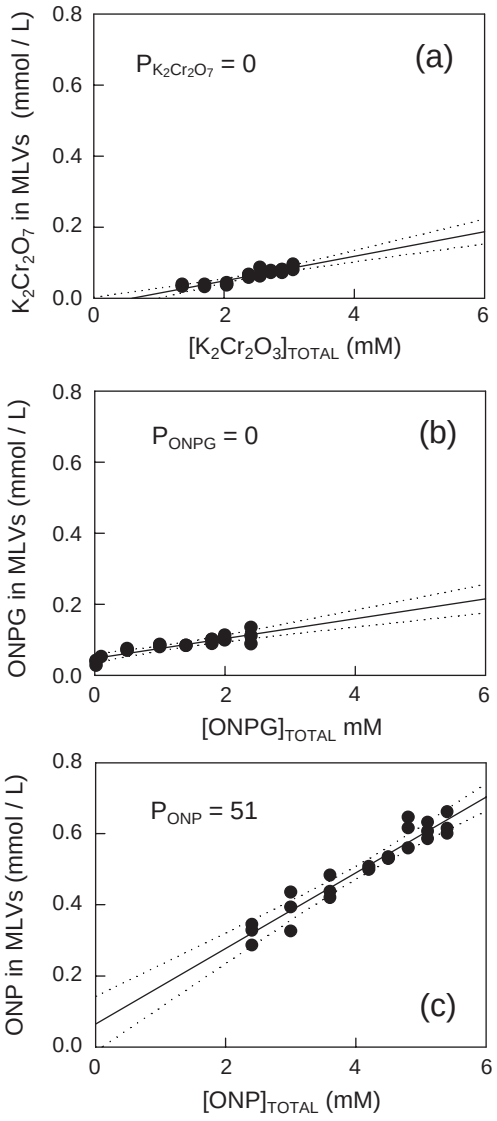


Fig. 2. Drug quantity partitioned in the membrane phase vs the total drug concentration in the incubation system. The amount of drug in the membrane phase present in 1000 ml of incubation system, as a function of total drug concentration are shown for $K_2Cr_2O_7$ (a), ONPG (b) and ONP (c). The 99% confidence intervals are indicated with dotted lines. $P_{m/w}$: membrane–water partition coefficients were calculated from the slopes as explained in the text.

Table 1
Determination of the membrane/water partition coefficients of ONPG and ONP

Drug	Ordinate[#] (mmol/l)	Slope (mmol/l/mM)	Regression coefficient (R)	Membrane–water partition coefficient ($P_{m/w}$)[d]
$K_2Cr_2O_7$	−0.02±0.02	0.035±0.003[a]	0.84479	$P_{K2Cr2O7}=0$
ONPG	0.048±0.004	0.028±0.003[b]	0.82172	$P_{ONPG}\cong 0$
ONP	0.06±0.03	0.106±0.007[b]	0.92513	$P_{ONP}=51$

The values of the slope, ordinate, and the regression coefficients were obtained from the fitness of Eq. (15) to the data shown in Fig. 2, by linear regression analysis by the least squares method (the three regressions resulted statistically significant with $p<0.0001$). Note that ordinates are expressed in mmol of drug trapped in the amount of pellet becoming from 1 l of incubation system.
[#]The values of ordinate were not statistically different from zero with $p<0.01$ (look at the confidence intervals in Fig. 2).
[a]($X\cdot f_w$); [b]($P\cdot f_m+X\cdot f_w$); [d]calculated from the difference between [b] and [a], and using the value $f_m=1.41\times10^{-3}$ [8].

case), is hydrolyzed. Then, in the second stage the hydrolysis of the second intermediate complex ES′ (galactosylated enzyme) gives a product $P2$ that correspond to galactose, when the reaction occurs in water. However, in the presence of a nucleophilic compound N, such as when the reaction occurs in the lipid–water interface compartment, a transfer product $P3$ is obtained.

According to this landscape, we tried to model the experimental values of V_{max} obtained in a previous work [1] by fitting Eq. (14). After 10000 iterations at a step-size of 1000, the function converged with a tolerance=0.0001 and a correlation coefficient $R=0.6568$ so, the solid line in Fig. 4 was predicted. This nonlinear regression analysis was performed using three constraints ($k_4>0$, $P_{\beta\text{-Gal}}>0$ and $\varepsilon_m>0$). The values of f_m were calculated from the lipid concentrations (C_T) $C_T=f_m\cdot\delta\cdot10^3$, being the lipid density $\delta=1$ g/ml. Nucleophile concentration was assumed to be the amount of lipid polar groups exposed to the water phase,

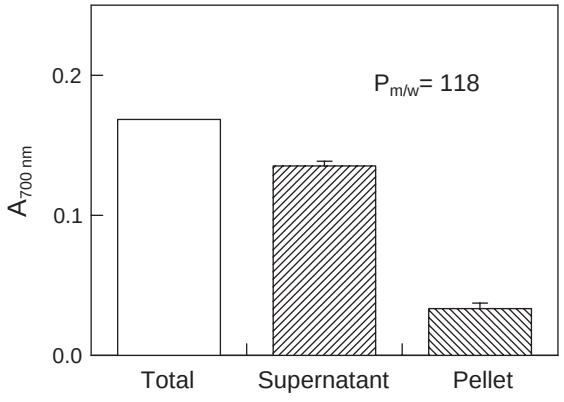


Fig. 3. Partition coefficient of β-Gal in a membrane–water system. MLVs of sPC were used as a model membrane. The quantity of enzyme partitioned in the sediment and the free one in the supernatant were quantified by the method of Lowry. Details of the experiments were as described in Materials and methods.

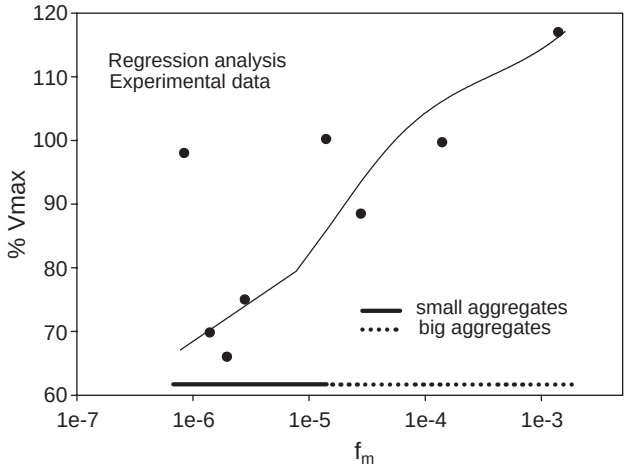


Fig. 4. Effect of the factional membrane volume on the maximal rate of ONP production through the hydrolysis of ONPG catalyzed by β-Gal. Thin solid line: fitting of Eq. (14) to the experimental data taken from Ref. [1] (black circles) by a nonlinear regression analysis, using $k_4>0$, $P_{\beta\text{-Gal}}$ and $\varepsilon_m>0$ as constraints. High lipid concentrations are coincident with the presence of vesicles of high diameter (thick dotted line); low lipid concentrations were associated to vesicles of low diameter (thick continuous line).

equivalent to $C_T/4$. The other constant parameters used were: $E_T=7.10^{-11}$ M (Ref. [1]), $P_{ONP}=51$ (present work), and $\varepsilon_a=4500$ M^{-1} cm^{-1} [1,2]. The reaction mechanism within the water phase, hence the values of rate constants applied ($k_2=2100$ $s^{-1}$and $k_3=1200$ s^{-1}), were assumed to be the same as those reported previously [4]. The fitting of Eq. (14) let calculate $k_4=(3.54\pm1.3)\times10^7$ s^{-1} ($p<0.0001$), $P_{\beta\text{-Gal}}=117\pm259$ ($p=0.6716$) and $\varepsilon_m=4012\pm1184$ M^{-1} cm^{-1} ($p<0.02$). The value of k_4, obtained was two orders of magnitude higher than that reported by Viratelle and Yon [4] in the presence of 2-mercaptoethanol (49000 s^{-1}), which was the most efficient nucleophile tested by this author. The value predicted for $P_{\beta\text{-Gal}}$ was not statistically different from the experimental value (Fig. 3). The extinction coefficient (ε_m) for ONP in the membrane environment was very close to that in the aqueous solution according to its localization near the polar head group–water interface [5].

5. Discussion

5.1. Partition coefficients

The membrane–water partition coefficients were determined for all the chemical species that participate in the hydrolysis of ONPG catalyzed by β-Gal.

5.1.1. ONPG

The membrane–water partition coefficients of ONPG was $P_{ONPG}=0$ so, this drug can be considered as hydrophilic. As a consequence the presence of a lipidic phase

would not induce changes in the kinetics of ONPG hydrolysis that could be associated to a partitioning phenomenon of this compound.

5.1.2. ONP

We determined a nonzero $P_{m/w}$ value for ONP. For this reason, in the case of this compound, not only the membrane–water but also the octanol–water partition coefficient were determined, in order to obtained more information about the characteristics of the partitioning process. The latter solvent system is frequently used to estimate the hydrophobicity of a drug as well as to predict its partition coefficient in a membrane–water system (note that octanol is a hydrophobic solvent immiscible with water and here it was used to mimic the membrane environment). The partition coefficient obtained in the octanol–water system was $P_{ONP,o/w}=25$. This value, which do not considered the acid–base equilibrium of *o*-nitro-phenol, was between those reported for the neutral ($P_{ONP,o/w}=78$) and the dissociated ($P_{ONPx,o/w}=0.017$) species [12]. The partition coefficient obtained in the membrane–water system was $P_{ONP}=51$. The difference between $P_{ONP,o/w}$ and P_{ONP} values may be due to the different kind of interaction forces between ONP and each of the molecular media. Moreover, it is important to consider that the octanol–water mixture consist of two isotropic phases in contrast with the membrane–water system where the membrane phase constitute an anisotropic environment [13]. Hence, the $P_{ONP,o/w}$ value underestimates the actual P_{ONP}. The latter reflects an average value, dependent on the different concentrations that ONP can reach in each of the structural domains that might be laterally separated in a membrane [14].

Our results suggest that ONP is a weak hydrophobic substance, that concentrate in the membrane with respect to the aqueous phase through stabilization forces, which are probably more closely related with dipolar interactions between ONP and the polar head groups of the membrane components than with the hydrophobic effect [5].

5.1.3. β-galactosidase

We had already reported that the interaction of the enzyme with lipid–water interfaces modulated its ability to catalyze the reaction of hydrolysis of a soluble substrate [1]. This effect could be explained, at least in part, by the physical insertion and/or adsorption of β-Gal to membranes as demonstrated by experiments of protein penetration in phospholipid monomolecular layers at the air–water interface [2]. In the present work we evaluated β-Gal distribution between the water and membrane phases. By determining its concentration through a protein quantification method, we showed that the enzyme is concentrated 118 times in the membrane with respect to the aqueous phase. The theoretical prediction for $P_{β\text{-Gal}}$, although statistically poor ($p=0.6716$), was within the same order of magnitude of the experimental value.

5.2. Modulation of the reaction kinetics by ONP and β-Gal interaction with the membrane

The effect exerted on the reaction rate by the partitioning of the enzyme and the product to the membrane was evaluated by applying the $P_{m/w}$ values determined for the enzyme ($P_{β\text{-Gal}}$) and for the reaction product (P_{ONP}), on the equations that defined $V_{max,w}$ and $V_{max,m}$ (see Eqs. (12) and (13)). At the same time, another intrinsic modulatory effect of the reaction was taken into account by the use of a different k_{cat} for the reaction that occurred at the interface compartment ($k_{cat,m}$) which was different from that of the aqueous compartment ($k_{cat,w}$). This expressed the presence of a nucleophile or a nucleophilic-like phenomenon occurring at the lipid–water interface, which would have caused the increment in the V_{max} value through a modification of the intrinsic reaction mechanism. This nucleophilic-like behavior of PC may be interpreted at the molecular level as a condition where the water molecules structured at the surface may have a more favorable energetic configuration compared with free water, leading to a decrease in the activating energy of the second kinetic step (Scheme 2). The number of water molecules at the interface is associated with a gradient of binding energies decreasing from the lipid polar head group towards the bulk of the solution. Moreover, higher aggregation numbers, and as a consequence, higher vesicular size and concomitant lower surface curvature and higher molecular packing was described for the self-aggregation structures of amphiphilic compounds as a function of monomer concentration [15]. Hence, the increase in molecular packing would increase the proportion of water molecules of high binding energy. The ability of PC to enhance the hydrolysis of ES′ complex will be more evident as the lipid concentration increase because of the growing contribution that the membrane compartment makes to the volume of the whole system. This rationale is based on our previous work where we correlated the reaction rate with the organization, composition, size and curvature of the vesicles used as model membranes [1].

At low lipid concentration the size, curvature and, in turn, the molecular packing of the membrane would be lower. In these conditions the enzyme would penetrate more deeply in the structure of the bilayer. This would cause either a change in the enzyme conformation and/or restrictions to its accessibility to the substrate, leading to a decrement in $V_{max.}$ This is supported by our findings on membrane penetration in monomolecular layers at the air–water interface, performed in controlled molecular packing conditions [2].

The effect of water structure on the enzyme activity is supported by the experiments in molecular crowded environments that are currently being developed in our laboratory [16,17].

From the numerical analysis of the model it follows that, as expected the enzyme concentration in the water phase decreases and in the membrane phase increases as a function

of membrane fractional volume (f_m) in the system (Fig. 5a and b). However, because $P_{\beta\text{-Gal}}>1$, the increments in the latter grows faster than the decrements in the former (compare Fig. 5a and b). The value of kcat1 (the catalytical rate constant in the presence of a nucleophile-like behavior within the membrane phase) also increases with f_m in a non-monotonous way (Fig. 5c). So, the nonzero $P_{\beta\text{-Gal}}$ as well as the increasing amount of nucleophile contribute together to the increment in the reaction rate within the membrane phase (Fig. 5d), which in turn will make a positive contribution to the whole value of the reaction rate in the system. In experimental terms, the reaction rate will be determined by absorbance values of the product formed. The latter magnitude will be affected by a nonzero $P_{m/w}$ for the product ($P_{ONP}>0$) as well as by the departure of the extinction coefficient of the product within the membrane phase from its value in water (Fig. 5e and f). As this difference becomes more positive, the presence of a minimum in the plot of absorbance vs f_m will be more evident (compare lines within Fig. 5e and f). Moreover, the increase in k_4 will cause not only a magnification in the changes in the absorbance values due to $(\varepsilon_m-\varepsilon_w)\neq 0$ but also a displacement of the minimum to the left within the abscissa (compare Fig. 5e and f).

The experimental data showed a growing up behavior which lead to a statistically significant fitting of Eq. (14) with a minimum located at any value of $f_m>0$ (Fig. 4). This would imply that the nonzero values for $P_{\beta\text{-Gal}}$, k_4, and P_{ONP} are necessary but insufficient to explain the experimental effect of enzyme–membrane interaction. As suggested previously, changes in enzyme conformation do to its incorporation within the membrane structure in low packed bilayers would contribute to displace the minimum of reaction rate to lower f_m values.

6. Conclusions

In a heterogeneous media, the reaction studied can be modulated at least at two levels. One of them corresponds to the modulatory nucleophile-like effect, which would modify the reaction intrinsic mechanism, possibly associated with the steric and dipolar organization of structured water. The other one would correspond to the apparent effect induced by the partition of the product and the enzyme between the membrane and the water phases at the lipid–water interface. The localization of the product within the polar head group region of the membrane might play a significant role in the modulation of the hydrolytic reaction mechanism. The model proposed in the present paper took into account both modulatory effects on V_{max} values and was able to reproduce the experimental results previously obtained in the presence of high concentrations of MLVs. Changes in enzyme conformation due to its incorporation to the membrane structure in low packed bilayers would contribute to displace the minimum of reaction rate to f_m values lower than those expected if only the nonzero values of $P_{\beta\text{-Gal}}$, k_4, P_{ONP} and $(\varepsilon_m-\varepsilon_a)$ were required.

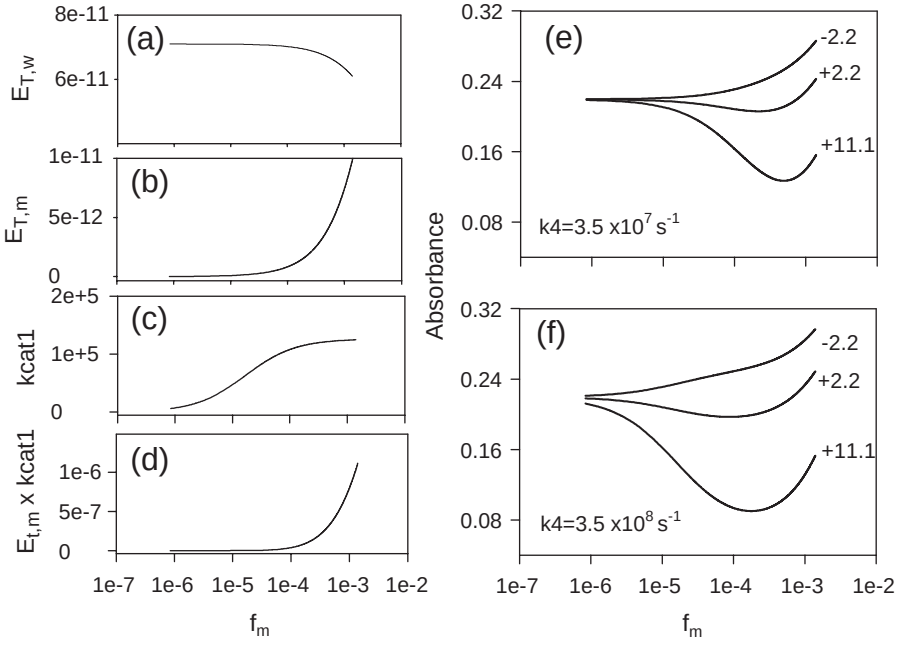


Fig. 5. Numerical analysis of the model. Effect of the fractional volume of membrane phase on: (a) and (b), the enzyme concentration in the water and membrane phases, respectively, referred to the total volume of the system (calculated from Eq. (11)); (c), the catalytical rate constant in the presence of a nucleophile-like behavior within the membrane phase (Eq. (8), right); (d), reaction rate within the membrane phase; (e) and (f) effect of the percent departure of the extinction coefficient of ONPx within the membrane phase from its value in water, at two different values of the rate constants for nucleophile derivative formation, indicated by the k_4 (Eq. (14)). Numbers next to the lines in panels (e) and (f) represent the percent departure of the extinction coefficient of ONPx in the membrane phase (ε_m) from the corresponding value in the water phase (ε_w) calculated as $[(\varepsilon_m-\varepsilon_w)\varepsilon_w/100)]$.

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