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Membrane adsorption or penetration differentially modulates β-galactosidase activity against soluble substrates

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Abstract

We investigated if in complex environments, like those where β-galactosidase activity is usually assayed, the kinetics of hydrolysis against soluble substrates could be modulated through enzyme-surface interactions. Kinetic parameters were determined using *ortho*-nitrophenyl-β-D-galactopiranoside (ONPG) as substrate, in the absence or presence of multilamellar vesicles (MLVs) of pure phosphatidyl cholines (PCs) or PCs:cholesterol mixtures, by visible spectroscopy. Light scattering was carefully corrected by three different methods obtaining similar results. The spectroscopic behavior of the reaction product in the presence of liposomes was also taken into account in order to avoid overestimating the reaction rate calculated from absorbance data. At low [PC] (<0.0024 mM) K_M and V_{max} decreased compared with the control in the absence of lipids. At high [PC] (1.2 mM), enzyme interaction with highly packed bilayers of dpPC induced an increment in both kinetic parameters. Both kinetic parameters decreased upon the interaction with low packed bilayers (soybean PC) at very low concentration (24 μM) but at higher concentration (1.2 mM) only an increment in V_{max} was observed. The dpPC MLVs samples used were four times bigger than those of $PC_{soybean}$ (approximately 1 μm mean diameter) as measured by quasi elastic light scattering. The increments in V_{max} were due to a modulation of the kinetics of the enzymatic reaction and not to non-enzymatic hydrolysis of ONPG at the vesicle–water interface. Enzyme–membrane interaction was confirmed using monomolecular-layers at the air–water-interface. Interestingly, β-galactosidase showed a higher tendency to be localized at a lipid–water interface compared with the free air–water interface; membrane penetration was favored in lower packed membranes. Differences in surface curvature, and thus in surface molecular packing and hydration, might account for the effects observed as the main modulating factor. Our results suggest that β-galactosidase activity was differentially modulated according to the enzyme possibility to penetrate or just be adsorbed to a dimensionality restricted space.

Abbreviations: A, absorbance; ANOVA, analysis of variance; cmc, critical micellar concentration; dpPC, dipalmitoil-PC; ε, extinction coefficient; K_M, Michaelis constant; MLV, multilamellar vesicle; ONP, *ortho*-nitrophenol; ONPG, *ortho*-nitrophenyl β-D-galactopiranoside; ONPx, *ortho*-nitro phenoxide; PC, phosphatidylcholine; QELS, quasi-elastic light scattering; S.E.M., standard error of the mean; π, surface pressure; *T*c, gel to liquid crystalline phase transition temperature; V_o, initial velocity; V_{max}, maximal velocity; λ, wavelength.

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1. Introduction

β-galactosidase is an enzyme which has been widely studied. It has medical [1], nutritional [2], biotechnological [3,4] and therapeutic [5] importance. Particularly, the β-galactosidase from *Escherichia coli* was instrumental in the development of the operon model [6], and today is one of the most commonly used enzymes in molecular biology [7].

Not only in the cellular milieu but also in situations of technological interest like during encapsulation [8] the activity of this enzyme has to be evaluated in heterogeneous systems, plenty of interfaces. In these conditions, one or more of the reaction participants (substrate, product, enzyme) might be concentrated at the surfaces, steric restrictions may be imposed to their movements, concentrations of the chemical species in the liquid phase might change, protein conformation may be affected. As a consequence of those phenomena, changes in the reaction kinetics may be expected. Not only the presence of a surface is important but also the dynamics of its organization. Thus, the reductionist perspective which considers the enzymatic activity only dependent on enzyme–substrate interaction is not enough to describe the complexity of the system.

A number of examples appeared in the literature pointing to supramolecular organization as a modulator of the activities of enzymes against substrates that are components of biomembranes [9–12] but the effects of non-substrate surfaces in the kinetics of enzyme catalyzed reactions, except for a few examples [13], has not been widely studied. In the present paper we investigated if the activity of β-galactosidase in the presence of model membranes but against a soluble substrate could be modulated through enzyme–membrane interactions.

2. Materials and methods

2.1. Materials

The enzyme β-galactosidase from *E. coli* [EC 3.2.1.23] Grade VII (specific activity 650 UI/mg protein) as lyophilized powder and *ortho*-nitrophenol-β-D-galactopiranoside (ONPG) were obtained from Sigma Chemical Co (St Louis, 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.

2.2. Preparation of liposomes

Multilamellar vesicles (MLV) [14] were prepared by evaporating, under a stream of nitrogen, a chlorophormic solution of a pure PC (Egg-PC, Soybean-PC or dpPC) or PCs:cholesterol mixtures. The dry lipid was suspended in water at a final concentration ranging from 7 g/l to 11.8 g PC per l depending on the experiment, by repeating six consecutive cycles of heating for 2 min at 21 °C (Soybean PC and Egg-PC) or 60 °C (dpPC), which are values of temperature above the T_c of the corresponding phospholipid, and vortexing for 1 min. In these conditions, phospholipids self-aggregated into multilamellar vesicles [15].

2.3. Effect of environmental polarity on the spectroscopic behavior of ONP and ONPx

Solutions of 5.2 mM ONPx were prepared using water (dielectric constant $D_{water} = 78.36$) and an aqueous solutions of 70% V/V dioxane ($D_{70\%}$ dioxane = 17.69) and 20% V/V dioxane ($D_{20\%\ dioxane} = 60.29$) as solvents [16]). Absorbance spectra between 220 and 520 nm were recorded.

2.4. Correction of light scattering induced by lipidic vesicles

Three different procedures were applied:

- a double wavelength reading procedure where the absorbance values at 420 and 750 nm of 0.8–20 mg PC per ml aqueous dispersions were recorded. A_{420} was plotted against A_{750} and the regression equation of the straight line obtained was calculated then, the interference induced by vesicle's light scattering in the A_{420} of ONPx in samples containing lipids was corrected by a simultaneous determination of A_{420} and A_{750}; the latter was transformed in A_{420} by applying the regression equation and the resulted value, representing the contribution of light scattering to the total A_{420} values, was discounted from the experimentally determined A_{420} to obtain the A_{420} exclusively due to ONPx,
- centrifugation for 30 min at $10\,000 \times g$ before reading the absorbance of ONPx at 420 nm,
- addition of SDS at 10 mM final concentration in order to destabilized vesicles (up to 4 μm mean diameter) and turning them into micelles (10 nm diameter) which do not disperse light.

2.5. Determination of enzymatic activity

The method applied was essentially that of Wallenfels and Malhota [17]. The incubation system contained 0.7 ml of 0.1 M phosphate pH 6.8 buffer containing or not lipids dispersed as indicated above in order to reach the desired concentration, 0.6 ml of substrate (ONPG) at final concentrations ranging from 0 to 2 mM and 0.1 ml of β-galactosidase (specific activity 650 UI/mg prot; 1 UI = 1 μmol/min of ONP formed at 37 °C) at a final concentration of 0.033 mg/l (the rate of ONP production increased linearly with enzyme concentration up to a final β-galactosidase concentration of 0.05 mg/l, data not shown). Incubation at 37 °C lasted 15 min (the curve of ONP produced versus time was linear within the range 0–20 min, data not shown), the hydrolysis reaction was stopped by the addition of 0.2 ml of 1.4 M sodium carbonate. The absorbance of the ONPx formed was determined at 420 nm.

2.6. Determination of kinetic parameters of β-galactosidase

The values of K_M and V_{max} were determined by fitting the experimental data from the V_o versus substrate concentration plot to the equation of Michaelis–Menten by a computer aided nonlinear regression analysis by the least squares method.

2.7. Spontaneous interfacial hydrolysis of ONPG

About 1.3 mM ONPG was incubated, in a total volume of 1.4 ml, at 25 or 45 °C for 15 min in the presence of 0.84 mg/ml MLVs of Soybean-PC and the A_{420} was recorded after the addition of 0.2 ml of Na_2CO_3.

2.8. Monomolecular layers at the air–water interface

The equipment used was a Minitrough II (KSV, Finland) which measured the surface pressure with a precision of ± 0.004 mN/m by the Wilhelmy plate method. Data was automatically and continuously recorded as a function of time. In the present work we used a small Teflon circular trough with an internal diameter of 4.5 cm and 0.5 mm depth. Experiments were done at constant area in order to measure the changes of surface pressure ($\Delta\pi$) due to enzyme penetration into the monolayer, as a function of the initial surface pressure (π_i). Monolayers were prepared at 37 °C by spreading 5–13 nmol of Egg-PC, in less than 5–10 μl of chloroform; at least 5 min were allowed for solvent evaporation and monolayer stabilization until reaching a constant baseline at the desired π_i, then, the enzyme solution (at a final concentration of 0.4 μg protein/ml) was injected into the subphase (8 ml, 15.9 cm^2 of surface area) continuously stirred with a miniature Teflon coated rod spinning at 150–250 rpm.

2.9. Quasi-elastic light scattering

Vesicle size distribution were determined by using a Nicomp™ 380 particle sizer (Nicomp Particle Sizer Systems, Santa Barbara, CA) operating at 532 nm and at an average count rate

between 250 and 500 kHz; run time was around 8 min for most samples. This apparatus is optimized to determine particle sizes of less than about 1 μm, and where mean diameters of greater than 1 μm are given, the value should be considered to be an approximation. Aliquots of the lipid suspension were diluted appropriately (final concentration of 0.5 mg/ml), with water which had earlier been filtered through a polyvinilidene fluoride filters (GVWP, Millipore). Measurements were made at 22 °C. The time autocorrelation function of scattered light intensities was analyzed by an inverse Laplace transform (ILS) algorithm to obtain a volume weighted distribution of diameters. In a few samples, a Gaussian distribution analysis was applied.

2.10. Statistical calculations

The propagation error method was used to calculate the error associated to variables calculated from other several experimentally determined ones [18]. The effects on the kinetic parameters exerted by lipid concentration and the bilayer or micelle composition were analyzed by one or two-way ANOVA tests; the post-hoc test of LSD was used for individual comparisons [19].

3. Results

3.1. Effect of lipid on the spectroscopic behavior of ONP

The decrease in the polarity of the environment induced bathochromic and hyperchromic changes in the visible spectra of ONPx (λ_{max} of ONPx changed from 416 nm in water to 425 nm in 70% dioxane aqueous solution) and the value of A_{420} increased about 32.5% (Fig. 1a). In a 20% V/V dioxane solution ($D = 60.29$) the value of A_{420} increased only 9.3% respect to the control in water (not shown).

Fig. 1b shows that the values of A_{420} plotted against A_{750} of samples containing increasing amounts of PC (from 0.8 to 20 g/l) lay in a straight line. A similar behavior was observed with A_{560} and A_{660}. The regression equation describing this plot was used to correct possible artifactual absorbance values of ONPx at 420 nm due to light scattering in PC containing samples, using A_{750} data, according to:

$$A_{420} = -0.007 + 4.52\, A_{750}$$

3.2. Effect of enzyme-phosphatidylcholine interaction on the enzymatic kinetics

Fig. 2 shows that kinetics of ONPG hydrolysis has a Michaelian behavior even in the presence of PC. The same figure also shows that similar results were obtained from the hyperbolic plots of initial rate versus ONPG concentration in the presence of PC independently of the method applied to correct for light scattering. A detailed analysis of the kinetic parameters are shown in

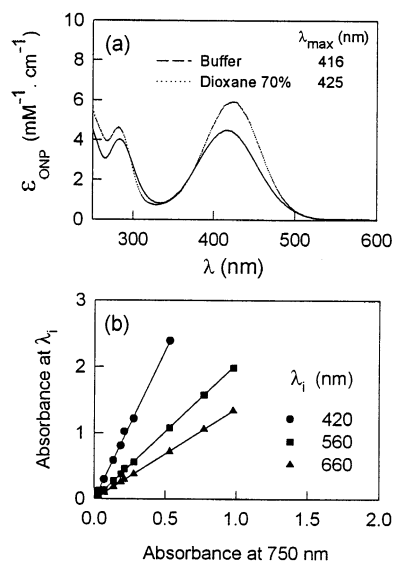


Fig. 1. Effect of lipids and detergents on the spectroscopic characteristics of ONP. (a) Absorbance spectra of ONPx in solution of different polarity; reference curves for correcting absorbance values of ONPx from the effects induced by the presence of phospholipid vesicles. The absorbance due to light scattering induced by PC dispersions with different PC concentration were recorded at the wavelengths indicated. (b) The absorbance of every sample at 420, 560 and 660 nm plotted against the absorbance of the corresponding solution at 750 nm gave the straight lines shown. The curve A_{420} versus A_{750} was used to interpolate A_{750} of samples containing ONPx + PC in order to obtained the effect of PC at $\lambda = 420$ nm and to calculate true A_{420} for ONPx.

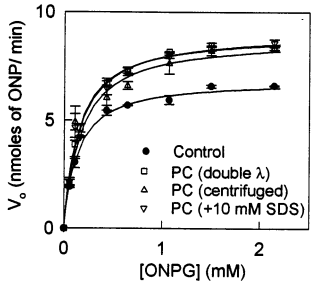


Fig. 2. Initial velocity as a function of substrate concentration in β-galactosidase-catalyzed ONPG hydrolysis. Effect of PC. Curves from typical experiments are shown. Experimental points could be adjusted to hyperbolic curves according to the Michaelis–Menten equation. Stimulatory effects of PC were similar independently of the correction method apply to discount the light scattering effect (see text and Table 1 for details). Reaction rate followed a pseudo first order regime up to 50 μg/l protein concentration in the incubation system and up to 20 min of incubation time (not shown).

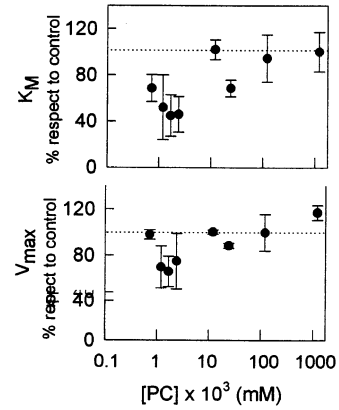


Fig. 3. Effect of the concentration of PC on the kinetic parameters of β-galactosidase. Values are the mean ± S.E.M. of at least two experiments performed in triplicates. (a) affinity, (b) maximal velocity. The dotted line represents the value of the control sample (100%). Kinetic parameters were obtained by adjusting the saturation curves to the Michaelis–Menten equation by a computer aided least squares method.

Table 1. This result strongly suggested that the effects observed in the presence of PC were really induced by the enzyme–membrane interaction affecting the reaction kinetics. This fact encouraged us to go further with the analysis of the ability of the surface to exert, in a surpramolecular organization-dependent manner, a modulation of the enzyme activity.

In Fig. 3 it is shown that the values of K_M and V_{max} decreased continuously as a function of PC concentration reaching a minimum at a PC concentration of 0.0024 mM; at higher concentrations, the kinetic parameters increased. At the highest PC concentration tested (1.2 mM) K_M reached (Fig. 3a) and V_{max} overpassed (Fig. 3b) the respective value for the control sample.

The ability of MLVs to affect β-galactosidase kinetic parameters depended on their lipidic composition (Table 2). dpPC alone or in mixtures with cholesterol induced a significant increment in K_M (one-way ANOVA $F_{(3,4)} = 14.09$, $P < 0.0136$). The post-hoc analysis showed a significant difference between the individual samples containing lipids and the control ($P = 0.025$) but not between samples containing dpPC plus 20 and 0% cholesterol ($P = 0.58$), and between those containing 5 and 0% cholesterol ($P = 0.15$); samples containing dpPC plus 5 and 20% of cholesterol showed a

Table 1
Different methods for correcting absorbance increments induced by phospholipid vesicles

Sample	PC	Blank	Other treatment	K_M (mM)	V_{max} (nmoles/min)
Control	No	Water	No	0.1316 ± 0.0137	6.9 ± 0.17
Control	No	Water	SDS	0.171 ± 0.034	7.18 ± 0.37
PC_{DWL}	Yes	PC	No	0.1398 ± 0.0125	9 ± 0.17[a]
PC_{centr}	Yes	Water	Centrifugation	0.165 ± 0.022	8.8 ± 0.26[a]
PC_{SDS}	Yes	Water	SDS	0.151 ± 0.018	9.1 ± 0.265[a]

DWL: double wavelength correction method. Values are the mean ± S.E.M. Determinations were performed in triplicates. All the 'other treatment' conditions were applied after the reaction was stopped. K_M values were not statistically different (one-way ANOVA: $F_{(4,10)} = 1.78$, $P = 0.21$); ANOVA on V_{max} values was significant ($F_{(4,10)} = 16.58$, $P < 0.0002$).

[a] Statistically different from control with or without SDS treatment (LSD post-hoc test).

Table 2
Modulation of β-galactosidase by its interaction with mixed bilayer of cholesterol and PC with different fatty acid composition

Sample	K_M (mM) mean ± S.D.	V_{max} (nmoles ONP/min)	MLVs mean diameter (nm)	
			Peak # 1	Peak # 2
1) Control	0.145 ± 0.006	6.9 ± 1	-	-
2) dpPC	0.272 ± 0.008[a]	8.8 ± 2.2	-	4000
3) dpPC: 5% Cho	0.230 ± 0.045[a]	9.0 ± 0.8	1026 (15)	3700
4) dpPC: 20% Cho	0.287 ± 0.012[a]	9.7 ± 1.2	-	3800
5) $PC_{soybean}$	0.146 ± 0.12	8.63 ± 0.65[b]	270 (8)	1077
6) $PC_{soybean}$: 5% Cho	0.156 ± 0.038	9.56 ± 0.46[b]	151(4)	926
7) $PC_{soybean}$: 20% Cho	0.120 ± 0.027	7.76 ± 0.65[b]	270 (8)	1102

Total lipid final concentration was 0.84 mg/ml. Other experimental conditions were those indicated in Section 2. The results shown are the mean ± S.E.M. of at least two experiments performed in triplicates. The values of K_M and V_{max} were calculated from the non-linear regression analysis of the initial velocity versus substrate concentration.

[a] Significantly different from control ($P<0.05$).

[b] Tend to be different from control ($P<0.055$). One-way ANOVA and LSD post-hoc tests (see text for details). Vesicle diameters shown are the results from a representative experiment. Numbers between brackets in column 4 (Peak # 1) are the percentage contribution of the corresponding particle population (see Fig. 6).

tendency to be different between one another ($P = 0.0765$). V_{max} values increased in the presence of dpPC and dpPC–cholesterol mixtures, however, the differences respect to the control were not statistically significant ($F_{(3,4)} = 1.17$, $P < 0.4263$). $PC_{soybean}$ alone or in mixtures with cholesterol did not induce any significant effect in K_M values respect to the control without PC ($F_{(3,4)} = 1.74$, $P = 0.296$). V_{max} values showed an evident and consistent increment ($F_{(3,4)} = 4.92$, $P < 0.079$) in the presence of $PC_{soybean}$ respect to the control. The post-hoc test on V_{max} values showed that the control was different from all the other samples containing $PC_{soybean}$ with a probability higher than 94.5% (Table 2). Similar results were obtained independently of the method applied to correct light scattering (Table 1).

3.3. Investigation of possible hydrolysis of ONPG at the lipid–water interface

Fig. 4 shows that A_{420} was not affected by the presence of ONPG but decreased with the increment of temperature.

3.4. Interaction of β-galactosidase with monomolecular layers at the air–water interface

Fig. 5 shows that the tendency of β-galactosidase to be localized at the air–water interface was significantly incremented in the presence of a phospholipid monomolecular layer ($\Delta\pi = 7$ mN/m at an initial surface pressure $\pi_i = 9$ mN/m) respect to the free interface ($\Delta\pi < 0.15$ mN/m;

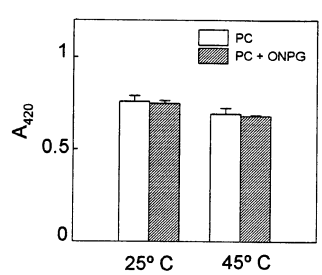


Fig. 4. Non-enzymatic hydrolysis of ONPG at the lipid-water interface. ONPG was incubation alone or in the presence of $PC_{soybean}$ without the addition of enzyme, at two different temperatures. After 15 min the absorbance at 420 nm indicative of ONP production was measured.

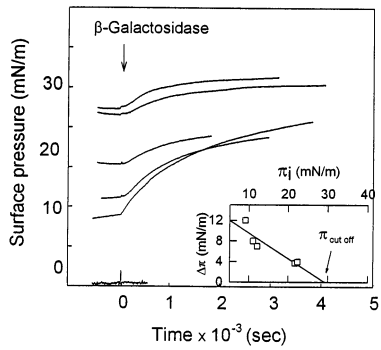


Fig. 5. Interaction of β-galactosidase with the lipid–water interface. Penetration of β-galactosidase in monolayers at different initial lateral surface pressures (π_i); insert: maximal $\Delta\pi$ decreased as a function of π_i; at a $\pi > 30$ mN/m ($\pi_{cut\text{-}off}$) penetration was not allowed.

$\pi_i = 0$ mN/m). Enzyme penetration into Egg-PC monolayer decreased as the molecular packing increased (at higher π_i) with a cut-off initial pressure $\pi_{i,\ cut\text{-}off} \cong 30$ mN/m.

3.5. Characterization of the lipidic dispersions used

It is known that MLVs formed by the hydration of dry lipid are under osmotic stress due to nonequilibrium solute distribution [20] nevertheless, they are stable structures. QELS experiments (Fig. 6) revealed the presence of a particle population representing between 85 and 100% of the total with a significant size difference between dpPC MLVs (near 4 μm diameter) and those of $PC_{soybean}$ (about 1 μm). The presence of cholesterol did not introduce qualitative significant differences in the mean size of those particles. A second particle population, with a mean size about 1000 nm, was observed in dpPC: 5%Cho samples. In all $PC_{soybean}$ containing samples, a second peak was also present but in this case the mean diameter was bellow 300 nm, being smaller in the samples containing 5% cholesterol ($\cong 150$ nm) than in those with 0 or 20% cholesterol ($\cong 270$ nm) (Table 2).

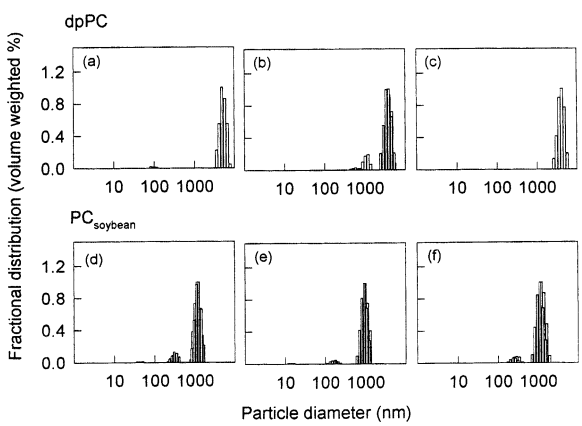


Fig. 6. Relative frequency distribution of diameters (volume weighted distribution) determined by quasi-elastic light scattering. MLVs of dpPC (a, b, c) and $PC_{soybean}$ (d, e, f) with different proportions of cholesterol (from left to right: 0, 5 and 20% of cholesterol in each row).

4. Discussion

4.1. Analysis of the experimental conditions applied

Early experiments were directed to assure a correct determination of the reaction product concentration. In media containing amphiphilic molecules, the formation of self-assembled structures has to be taken into account as they will lead to:

- physically perturbing phenomena like light scattering; and
- the appearance of surfaces and hydrophobic phases where substrates and products might be adsorbed and/or partitioned, changing their effective concentration in the reaction media and/or changing their spectral behavior.

PC is an amphipathic molecule which, according to the relative volumes of the hydrophilic and hydrophobic portions of the molecule, self-aggregate into bilayers [15] which in the present experimental conditions took the form of multilamellar vesicles with diameters up to approximately 4 μm (Fig. 6). So, the system consisted of a turbid suspension that caused a significant light scattering evidenced by high absorbance values in the whole UV-visible spectra, which had to be corrected. Light scattering is a wavelength dependent phenomenon and in long-wavelength spectrophotometry such as visible usually exerts a background absorbance that lies in a straight line [21]. In our assay system, light scattering induced by

PC vesicles caused a turbidity becoming significant over 0.1 g/l of PC and was corrected for in the experiments that followed (Fig. 2; Table 1).

Note that when the addition of SDS was the method applied to avoid light scattering, the absorbance was determined in an heterogeneous medium consisting of PC–SDS mixed micelles. So, it was necessary to evaluate not only if PC induced significant effects in absorbance of ONPx but also if the addition of SDS to avoid turbidity did cause additional artifacts.

According to the effect of the polarity of the media on the spectroscopic behavior of ONPx (Fig. 1a), the partitioning of ONPx towards an environment less polar than water would induce an increase in its extinction coefficient. We took this fact into account in order to avoid a misinterpretation of an eventual artifactual increase in the values of V_{max} (calculated from absorbance data). Taking together:

- the effect of environmental polarity on the value of ε_{420} (32% increment in an environment of $D = 17.69$ as shown in Fig. 1a and 9.3% increment when $D = 60$, like at the lipid–water interface),
- the value of 25 determined for the partition coefficient of ONP in PC MLVs (Ciclik, Sanchez and Perillo, unpublished),
- a value of 10^{-3} [22] for the fraction of the volume of the whole system corresponding to the particulate fraction (MLVs or micelles when the SDS is used to correct for light scattering)

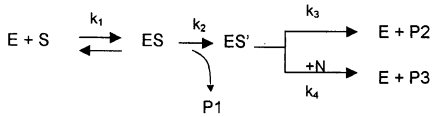
the absorbance experimentally measured would be: $A = \varepsilon_{420} b c_T$ in homogeneous media and, $A = \varepsilon_{420} b c_T [1 + P f_m + XP f_m]$ in the presence of lipids and/or detergents (X is the fractional increase in the value of ε_{420} due to a lower polarity of the medium, f_m the fraction of the total volume corresponding to the particulate phase, c_T is ONPx concentration in the whole system, P the lipid–water or detergent–water partition coefficient of ONPx and b the optical path (1 cm)) (see Appendix A for details).

Applying the numerical values for some particular variables and assuming a very low polarity of the region in the particulate phase where ONPx is localized, the absorbance results $A = 1.033 \cdot \varepsilon_{420} \cdot b \cdot c$. This value represent a 2.6% increase in the absorbance measured in the heterogeneous system compared with an aqueous solution. However, as ONPx tend to be localized at the polar head group region of the lipid-water interface where $D \cong 60$ (Ciclik, Sanchez and Perillo, unpublished) we should apply a lower value for X giving a lower correction factor (1.023). Moreover, in the presence of SDS the overestimation of A would be even lower due to the repulsive electrostatic interaction expected between ONPx and the negative charged surface of SDS micelles which will reduce P compared with PC. Therefore, the values of V_{max} calculated in the present paper might be overestimated by not more than 2.6%. Consequently, the approximate 30% increment observed in V_{max} in the presence of PC MLVs were of kinetical origin.

4.2. Modulation of enzyme kinetic parameters induced by lipidic vesicles

The reactions occurring at interfaces may change their mechanism respect to the ones taking place in the bulk of a solution, with a consequent modification of the values of activation energy [23]. Contrary to what would have been expected in the case that the hydrolysis of ONPG was non-enzymatically catalyzed by its partitioning within the environment of the lipid–water interface, the A_{420} did not increase in the presence of PC, indicating the absence of ONP production (Fig. 4). In this case the effect of light scattering exerted by the MLVs was not discounted. This let us observe that A_{420} was not affected by the presence of ONPG, suggesting that partitioning of ONPG towards the MLVs that might destabilize its structure and induce a decrement in their diameter, as shown earlier with other lipophilic drug [24], might have not occur. However, A_{420} decreased with temperature suggesting an effect of this parameter reducing the vesicular size.

The interaction of β-galactosidase with lipid–water interfaces did not change the hyperbolic shape of the saturation curve, indicative of a Michaelian behavior (Fig. 3) but, in some conditions affected the kinetic parameters of ONPG hydrolysis.



Scheme 1.

β-galactosidase (Fig. 5) as well as other proteins [9] and peptides [25] penetrated lipidic membranes more easily in lower packed membranes (lower lateral surface pressure) as demonstrated by experiments in monomolecular layers at the air–water interface. Different to what was observed with neuraminidase, β-galactosidase showed an increased tendency to be localized at the surface in the presence of a lipidic monomolecular layer compared to the free air–water interface (Fig. 5). This suggests that desolvation of the protein and membrane surfaces put in contact, in addition to changes in the structural dynamics of both the membrane and the protein might have lead to a thermodynamically favorable binding through a process possibly driven by the increase in the entropy of the water molecules originally bound to both surfaces. The inactivation of enzymes due to a high penetration in very low packed monolayers has already been described [9].

4.3. Penetration versus adsorption as modulators of enzymatic activity

Two mechanisms may participate in the modulatory effects of PC vesicles. The first one (Mechanism I) would induce an acompetitive inhibition through a sequestration of the enzyme-substrate complex by its penetration into the vesicular bilayer which, according to our results in monomolecular layers, would be favored when the bilayer is in the low packed state. The second mechanism (Mechanism II) might be a nucleophilic competition in the β-galactosidase-catalyzed reaction exerted by the polar head group of the phospholipid. The enzymatic hydrolysis of ONPG is accomplished in two steps [26] according to the Scheme 1: where E, S, ES and ES' stand for enzyme, substrate, Michaelis complex and second intermediate complex, P1 is the aglycone part of the substrate (ONPG), P2 the galactose and P3 the transfer product obtained by the action of the nucleophilic compound N on the ES' complex. The catalytic and the Michaelis constants for P1 are given by the expressions:

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

$$K_M = K_S \frac{(k_3 + k_4[N])}{k_1 + k_3 + k_4[N]}$$

In the presence of a nucleophile, K_M and k_{cat} increase if k_2 and k_3 are of the same order of magnitude as is the case with ONPG (K_S is the affinity binding constant for the substrate).

According to that rationale, mechanism II should prevail with dpPC bilayers (note that K_M and V_{max} increased over the control in the presence of these low curved, and consequently, high packed MLVs surfaces (Table 2)). Although, not on an exclusive basis, mechanism I would be expressed with low packed bilayers like that of $PC_{soybean}$, preferentially at low concentration (Table 2; Fig. 3). (Higher aggregation numbers, and as a consequence, higher vesicular size and concomitant lower surface curvature and molecular packing was described for the self-aggregation structures of amphiphilic compounds as a function of monomer concentration [27]). In this case, the effect observed would be the resultant of the expression of both mechanisms. So, increasing $PC_{soybean}$ concentration would change the prevalence of modulatory control from mechanism I (ES sequestration) to mechanism II (nucleophilic attack) (Fig. 3).

The addition of cholesterol is known to turn more rigid the fluid phases and to fluidify ordered phases [28]. However, although in bilayers there is a 'fluidity gradient' growing towards the methylene group end of the hydrocarbon chains, when comparing the structural order in bilayers above and below the transition temperature, a small difference was observed at C_2 compared with C_{10} [29]. Moreover, data from EPR experiments indicated that the fatty acid polymethylene chain in aqueous dispersions of dpPC and egg–PC mixtures with cholesterol (2:1 mole ratio) may be thought as relatively 'rigid rods' for a region of up to about eight carbon atoms from the fatty acid-glycerol ester linkage, with rapidly increasing probabilities for gauche states at large distances [29]. In our experimental system, the main difference between both dpPC and $PC_{soybean}$ samples was the presence of MLVs with

mean diameters predominantly of 4 μm in the former and 1 μm MLVs V plus less than 8% of particles bellow 300 nm diameter in the latter. This dissimilarity in size was interpreted as a difference in surface curvature and consequently in molecular packing. Cholesterol did not introduced a qualitatively significant difference in the particle size distribution neither of $PC_{soybean}$ samples nor in the biggest population of dpPC MLVs; for this reason the lack of effect of this lipid on the values of the kinetic parameters should not be surprising. So, penetrability of β-galactosidase (dependent on the surface molecular packing) and thus, possible modulatory effects though mechamism I, in PC bilayers might have been unaffected by cholesterol. Some details, like the presence of a small population of 1000 nm (15% of total weighted volume) mean diameter particles in dpPC: 5% Cho samples which disappeared when cholesterol content rose to 20%, might be associated to the significance in the difference between K_M values obtained in those experimental condition (Table 2). However, more experiments should be done in order to confirm this tendency. A possible interpretation of the lack of effect of cholesterol on the enzyme activity modulation through mechanims II upon its binding to MLVs surface, could be an eventual requirement of a nucleophile lattice where, in order to exert the modulatory effect, the phospholipid phosphate groups were separated by a specific distance which might be found even in the presence of mixed PC: Cho surfaces in spite of a decrease in the proposed nucleophile surface concentration by dilution of PC in the bilayer.

What is called 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 1). Then, we can change the viewpoint of the analysis from the molecular level towards the supramolecular organization of the hydrating sphere. It is known that hydration of a lipid–water interface will change according to the characteristics of the polar head group as well as molecular packing. Higher molecular spacing will increase the number of water molecules at the interface associated with a gradient of binding energies decreasing from the lipid polar head group towards the bulk of the solution. As 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 favored.

The amount of cholesterol also is known to affect the hydration level of PC-cholesterol mixed bilayers. In dimyristoyl PC–cholesterol mixtures it was demonstrated by neutron scattering [30] that several phase separation processes occur at molar fractions of cholesterol (x_{chol}) between 0.08 and 0.435, however, only at a x_{chol} of 0.5 the number of water molecules associated with the polar head group of PC levels off significantly [31]. Within the range of cholesterol content of the MLVs used in our experiments no changes in hydration should be expected. This may also contribute to explain the lack of difference between the effects of PC and PC-cholesterol mixtures on β-galactosidase activity.

5. Conclusions

β-galactosidase activity might be modulated differentially according to the enzyme possibility to penetrate or just be adsorbed to a dimensionality restricted space. This may affect the enzyme through changes in protein conformation or affecting the reaction mechanism. Surface topography is crucial for the modulatory effects. Partitioning of the reaction substrate and product towards the membrane may also play a role in the dynamics of the overall process and it is under study in our laboratory at present.

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Appendix A

The absorbance in an heterogeneous system will be:

$$A = A_w + A_m \quad (A.1)$$

where the subscripts w and m stand for water and membrane.

According to Lambert and Beer's law:

$$A_w = \varepsilon_w\ b\ c_w \text{ and } A_m = \varepsilon_m\ b\ c_m \quad (A.2)$$

ε, b and c being the extinction coefficient, the optical path and c molar concentration, respectively.

$$\varepsilon_m = \varepsilon_w + X \cdot \varepsilon_w \quad (A.3)$$

$$c_w = c_T - c_m \quad (A.4)$$

$$c_m = P\, c_T f_m \quad (A.5)$$

X is the fractional increase in the value of ε_{420} due to the effect of the polarity of the medium on the spectroscopic behavior of the absorbing chemical specie (ONPx in our case), c_T is ONPx concentration in the whole system,, f_m the fraction of the total volume corresponding to the particulate phase, P the lipid–water or detergent–water partition coefficient of ONPx (note that what is named c_m is the number of moles of ONPx in the membrane referred to the total volume of the system).

Replacing e_m, c_w and c_m for Eqs. (A.3), (A.4) and (A.5), using the resulting expressions in Eq. (A.2) and rearranging gives the solution for absorbance in a heterogeneous medium:

$$A = e_w\ b\ c_T[1 + P\, f_m\ (1 + X) \quad (A.6)$$

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