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Membrane topology modulates β-galactosidase activity against soluble substrates

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Abstract

The effect of bio-surfaces of contrasting curvature, on the kinetic parameters of ortho-nitrophenyl-β-D-galactopiranoside hydrolysis catalyzed by *E. coli* β-galactosidase, was investigated. The self-aggregating state and structure of the amphiphiles (Phosphatidylcholine, Lubrol-PX, Triton X-100, DocNa, SDS and CTAB) were inferred from their c.m.c. values and light-scattering measurements. Low curvature phosphatidylcholine or mixed phosphatidylcholine-detergent vesicles increased V_{max} without affecting K_M. High curvature micellar structures containing ionic detergents modulated negatively the enzyme activity (decreased or abolished V_{max} and increased K_M). Neither micelles containing non-ionic detergents nor the amphiphiles in a monomeric form, affected enzyme activity. CTAB at a concentration bellow its c.m.c but incorporated into a bilayer, became an activator (K_M decreased respect to the control). Non-enzymatic interfacial hydrolysis of the substrate was discarded. Enzyme–membrane interaction and membrane elasticity, were evaluated using monomolecular layers at the air–water interface. Beyond particular molecular structures, topology affected the direction of the modulatory effects exerted by these amphiphiles on β-galactosidase activity.

Keywords: β-galactosidase activity; Surface curvature; Enzyme–surface interaction; Vesicles; Micelles; Monomolecular layers

Abbreviations: ONPG: ortho-nitrophenyl β-D-galactopiranoside; ONP: ortho-nitrophenol; ONPx: ortho-nitro phenoxide; MLV: multilamellar vesicle; PC: phosphatidylcholine; SDS: sodium dodecyl sulfate; CTAB: cetyl-trimethyl-amonium-bromide; DocNa: sodium desoxicholate; *D*: dielectric constant; c.m.c.: critical micellar concentration; K_M: Michaelis constant; V_{max}: maximal velocity; V_o: initial velocity; S.E.M.: standard error of the mean; ANOVA: analysis of variance; T_c: gel to liquid crystalline phase transition temperature; *A*: absorbance; λ: wavelength; ε: extinction coefficient; π: surface pressure

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1. Introduction

Beta-galactosidase is an enzyme widely distributed, capable of catalyzing the hydrolysis of terminal β-glycosidic bonds present in carbohydrates, glycolipids, glycoproteins and glycosamineglicans [1].

This enzyme has medical [2], nutritional [3], biotechnological [4,5] and therapeutic [6] interests. Particularly, the β-galactosidase from *Escherichia coli* was instrumental in the development of the operon model [7], and today is one of the most commonly used enzymes in molecular biology [8].

The activity of β-galactosidase is usually determined in highly heterogeneous environments like cellular cytoplasm [8]. The use of detergents, sometimes in order to liberalize the enzyme from the interior of cells, others to measure the enzymatic activity in complex systems like blood, milk and fermented samples, is also frequent [9,10]. In these conditions the enzyme is working in a not only heterogeneous but also dynamic media where cooperative phenomena and new control levels, that are absent in one-phase systems, may appear. Thus, the reductionist perspective that considers the enzymatic activity only dependent on enzyme–substrate interaction is not enough to describe the complexity of the reaction kinetics taking place in that kind of system.

Enzymes have been studied in different environments, including normal and reverse micelles and lipid crystalline structures. It has been demonstrated that molecular crowding [11–13] and the coupling to the dynamics of microtubule polymerization–depolymerization [14] may have kinetic consequences in an enzymatic reaction by affecting the average degree of self-association of the enzyme in intracellular environments. Moreover, an enzymatic activity can be modulated by dimensionality restrictions like in a surface, a channel or dispersed clusters [15,16], by the topological organization of an amphiphilic substrate ([17–19] and references therein) and by the enzyme binding to a non-substrate surface [20–23].

Micellar enzymology have been used as media for enzymatic reactions [24–26]. These micellar systems are pseudo-homogeneous solvent/surfactant/water mixtures that spontaneously form different phases, the nature of which depends only on the relative ratio of the components. Beta-galactosidase has never been studied in a micellar system to the best of our knowledge. Even though, the effects of detergents on some β-galactosidases have already been investigated [27,28]. Those systems differed from the one used in the present paper because they dealt either with a membrane bound β-galactosidase [27] or with an amphipathic substrate self-organized as a membrane component [28].

In the present paper, we explored the possibility that the activity of a soluble β-galactosidase towards a soluble substrate could be modulated in heterogeneous media by enzyme–membrane interactions phenomena. Here, phospholipid vesicles and detergent micelles were used as model membranes of contrasting surface curvature. In addition to those conditions, the use of detergents in monomeric form as well as present in mixed vesicles, let us demonstrate that beyond its chemical structure, the effect of an enzyme modulator depends on the topology of the environment where it is located.

2. Experimentals

2.1. Materials

The enzyme β-galactosidase from *Escherichia coli* [EC 3.2.1.23] Grade VII (specific activity 650 UI/mg protein) as lyophilized powder and orthonitrophenol-β-D-galactopiranoside (ONPG) were obtained from Sigma Chem. Co (St. Louis, MO) and ONP from ICN Pharmaceuticals (Costa Mesa, CA). Lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Other reagents and solvents used were of analytical grade.

2.2. Methods

2.2.1. Preparation of liposomes

Multilamellar vesicles (MLV) [29] were prepared by evaporating, under a stream of nitrogen, a chlorophormic solution of pure Soybean-PC. The dry lipid was suspended in water at a final concentration of 11.8 g PC/l, by repeating six con-

secutive cycles of heating for 2 min at a temperature above the T_c of the phospholipid, and vortexing for 1 min. Detergents were dispersed in water at room temperature. In these conditions, phospholipids self aggregated into multilamellar vesicles [30]. Detergent self aggregated into spherical micelles when they were above their c.m.c. (the values of c.m.c. for SDS, Triton X-100, Lubrol PX, CTAB and DocNa are 1.33, 0.24, 0.8, 2.5 and 0.91 mM, respectively [31]).

2.2.2. Effect of detergents on the spectroscopic behavior of ONP

Aqueous solutions containing SDS, Triton X-100, Lubrol PX, DocNa and CTAB at concentrations below and above their respective c.m.c., were prepared in 0.1 M pH 6.8 phosphate buffer and in 0.177 M Na_2CO_3 and their UV-visible-spectra were recorded in a Beckman DU 7500 spectrophotometer equipped with a diode array detector and a sensitivity of 0.0001 AU. Moreover, solutions of ONP in 0.177 M Na_2CO_3 were prepared at ONP concentrations ranging from 0 to 10 mM in the presence of the same detergents at concentrations below and above their c.m.c. The molar extinction coefficient of ONPx (the dissociated form of ONP present in highly basic media) was determined from the slope of the A_{420} vs. ONP concentration plot. These plots were used not only as calibration curves to determine the amount of ONP produced, using absorbance data in the β-galactosidase catalyzed reaction, but also as a means to evaluate the possibility of ONPx partitioning towards the micellar–water interface, in the experiments that followed. Such a partitioning process would affect the interpretation of the results from β-galactosidase-catalyzed reaction kinetics in the presence of detergents.

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

Solutions of 0.25 mM ONPx were prepared using water (dielectric constant $D_{water} = 78.36$) and aqueous solution of 20% v/v dioxane ($D_{20\%\ dioxane} = 60.79$) and 70% v/v dioxane ($D_{70\%\ dioxane} = 17.69$) as solvents [32]). Absorbance spectra between 220 and 520 nm were recorded.

2.2.4. Correction of light scattering induced by lipidic vesicles

Three different procedures [22] were applied:

a. Double wavelength reading procedure where the absorbance values at 420 and 750 nm of 0.8–20 mg PC/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} due exclusively to ONPx;
b. Centrifugation for 30 min at $10\,000 \times g$ before reading the absorbance of ONPx at 420 nm;
c. Addition of SDS at 10 mM final concentration in order to destabilized vesicles (500-nm diameter) and turning them into micelles (10 nm diameter) that do not disperse light.

2.2.5. Determination of enzymatic activity

The method applied was essentially that of Wallenfels and Malhota [33]. The incubation system contained: 0.7 ml of 0.1 M phosphate pH 6.8 buffer with or without lipids and/or detergents dispersed as indicated above in order to reach the desired concentration; 0.6 ml of substrate (ONPG) at final concentrations ranging from 0 to 3 mM; and 0.1 ml of β-galactosidase (specific activity 650 UI/mg protein; 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 vs. 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 Na_2CO_3. The absorbance of the ONPx formed was determined at 420 nm.

2.2.6. 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 vs. substrate concentration plot to the equation of Michaelis–Menten by a computer aided non-linear regression analysis by the least squares method.

2.2.7. Spontaneous interfacial hydrolysis of ONPG

In a total volume of 1.4 ml, 1.3 mM ONPG was incubated at 37 °C for 15 min in the presence of 0.84 mg/ml MLVs of PC or detergent micelles (Triton X-100, SDS, or CTAB at 35 mM, a final concentration above their c.m.c. values). The A_{420} was recorded after the addition of 0.2 ml of 1.4 M Na_2CO_3 against a blank without either PC or the corresponding detergent.

2.2.8. Monomolecular layers at the air–water interface

The equipment used was a Minitrough II (KSV, Finland) that measured the surface pressure with a precision of ± 0.004 mN/m by the Wilhelmy plate method. Two kinds of experiments were performed:

2.2.8.1. (*a*) *Determination of the maximum value of lateral surface pressure* (π) *that allowed drug penetration in the monolayer* ($\pi_{cut\text{-}off}$). 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 in 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. The monolayers containing detergents were prepared similarly but using 1% methanol in chloroform as the solvent of the spreading solution, at a 100:1 molar ratio of the PC/detergent mixture. 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 in the subphase (8 ml, 15.9 cm^2 of surface area) of the reaction compartment (continuously stirred with a miniature Teflon coated rod spinning at 150–250 rev./min).

2.2.8.2. (*b*) *Surface pressure vs. molecular area isotherms*. For these experiments we used a rectangular trough fitted with two barriers that were moved synchronously by electronic switching. The signal corresponding to the surface area (automatically determined by the Minitrough according to the relative position of the two compression barriers) and the output from the surface pressure transducer (measured automatically by the Minitrough with a platinized Pt foil 5 mm wide × 20 mm long × 0.025 mm thick) were fed into a personal computer through a serial interface using a specific software. Before each experiment, the trough was rinsed and wiped with 70% ethanol and several times with bidistilled water. The absence of surface-active compounds in the pure solvents and in the subphase solution (bidistilled water) was checked before each run by reducing the available surface area to less than 10% of its original value after enough time was allowed for the adsorption of possible impurities that might have been present in trace amounts. The monolayer was compressed at a constant low rate of 20 mm^2/s at 21 ± 0.5 °C. A lower compression rate (12 mm^2/s) was tested, and identical results were obtained.

2.2.9. Effect of detergent concentration on the size of mixed PC-detergent self-aggregated structures

MLVs formed by mixtures of Egg-PC were prepared following the procedure described above, varying the concentration of detergent (Triton X-100, CTAB and SDS) from 10^{-4} to 35 mM. Turbidimetric measurements were performed at 650 nm, and started 0, 17 and 41 h after the mixing at room temperature.

2.2.10. Statistical calculations

The propagation error method was used to calculate the error associated to variables calculated from other several experimentally determined ones [34]. The effects on the kinetic parameters exerted by lipid concentration and the bilayer or

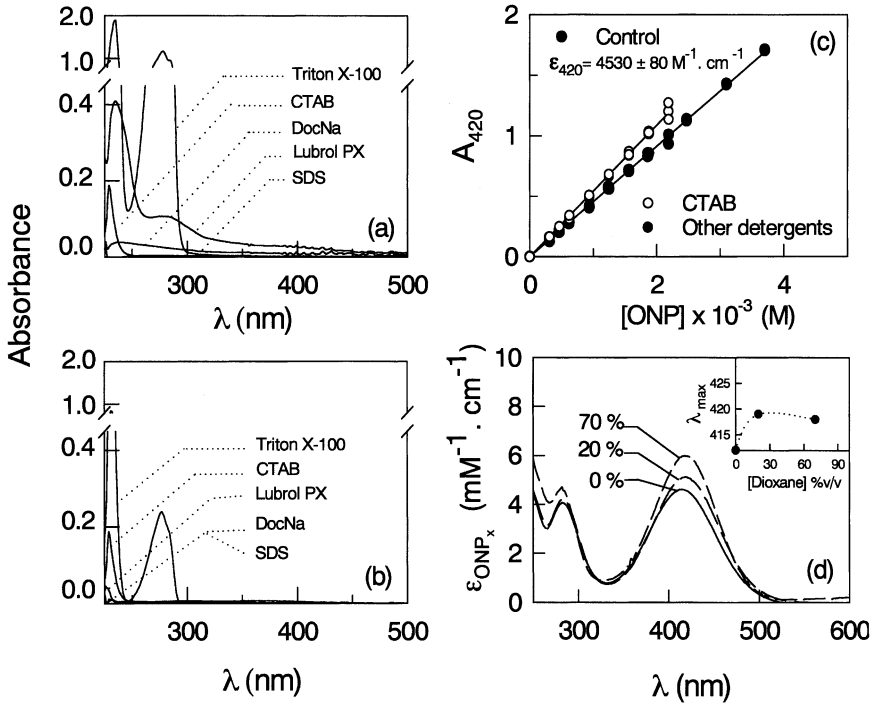

Fig. 1. Effect of lipids and detergents on the spectroscopic characteristics of ONP. Absorbance spectra of detergents (35 mM final concentration) in almost neutral (a) or highly basic (b) solutions. (c) Calibration curve of ONP in basic media in the absence or in the presence of detergents (note that only CTAB induced a significant change in ONPx absorbance at 420 nm). (d) Absorbance spectra of ONPx in solutions of different polarities.

micelle composition were analyzed by one or two-way ANOVA tests; the post hoc test of LSD was used for individual comparisons [35].

3. Results

3.1. Effect of lipid and detergents on the spectroscopic behavior of ONP

The aim of this experiment was to evaluate possible interference that might have been exerted by detergents on the values of ONPx concentration, and thus on enzyme's kinetic parameters, calculated from absorbance (A) data. At pH 6.8, the absorbance of Lubrol PX and DocNa were significant within the whole wavelength range analyzed (200 and 500 nm); Triton X-100, SDS and Lubrol did not show significant absorbance at any $\lambda >$ 300 nm (Fig. 1a). In highly basic medium (like in the presence of Na_2CO_3) none of the detergents absorbed electromagnetic radiation of $\lambda > 300$ nm (Fig. 1b). The plots of absorbance at 420 nm (A_{420}) vs. ONPx concentration in the absence and in the presence of detergents were adjusted to straight lines (Fig. 1c) and the values of extinction coefficient (ε) of ONPx was calculated from their slopes. A value of $\varepsilon = 4530 \pm 80$ AU M^{-1} cm^{-1} was obtained in the absence as well as in the presence of most of the detergents tested except CTAB which caused a 19% increment in ε when present at concentration of 25 mM (which was above its c.m.c.). The decrease in the polarity of the environment induced a bathochromic shift and a hyperchromic change in the visible spectra of ONPx; λ_{max} of ONPx changed from 412 nm in water to 419 and 418 nm in 20 and 70% dioxane aqueous solutions and the value of A_{420} increased approximately 9 and 31%, respectively (Fig. 1d).

3.2. Effect of phosphatidylcholine and detergents on the enzymatic kinetics

Fig. 2 shows V_o vs. substrate concentration plots of typical experiments performed in the absence or in the presence of detergents (Fig. 2a) or of PC (Fig. 2b).

At the PC concentration tested (1.2 mM) K_M was unaffected and V_{max} was a 30% higher than the control (this difference was statistically significant $P<0.05$) (Table 1). At this PC concentration the light dispersal effects of MLVs were important. For this reason, and in order to verify the true origin of the increments observed in V_{max}, three different methods were applied for correcting an eventual overestimation of A values that might be due to light scattering (see Sections 2.1 and 2.2) obtaining similar results with all of them (Fig. 2b). The values of A_{420} plotted against A_{750} of samples containing increasing amounts of PC (from 0.8 to 20 mg/ml) laid in a straight line [22]. The regression equation describing this plot was used to correct possible erroneous absorbance values of ONPx at 420 nm due to light scattering in PC containing samples, using A_{750} data, according to: $A_{420}=-0.007+4.52\times A_{750}$.

Another possible source of an apparent increment in V_{max} could be a reduced free volume of reaction due to a significant capture volume of the vesicles. This would cause the actual concentrations of all the chemical species participating in the reaction to be higher than those calculated by using the volume of the whole system. The theoretical capture volume in liters per gram of lipid has been calculated and published in a nomogram form relating the capture volume, the vesicle diameter and the lipid weight [36]. According to that nomogran, the capture volume of 1.2 mg of MLVs is less than 30 μl for a vesicle diameter of 500 nm like those used in the present work, as measured by quasi elastic light scattering [22]. This fact would cause an apparent increment in V_{max} in the vesicle containing system lower than 2% compared with the control. This value is 15 times lower than the 30% increment observed experimentally.

These results strongly suggested that, in the presence of PC the increments observed in V_{max} were actually 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 a modulation of the enzyme activity.

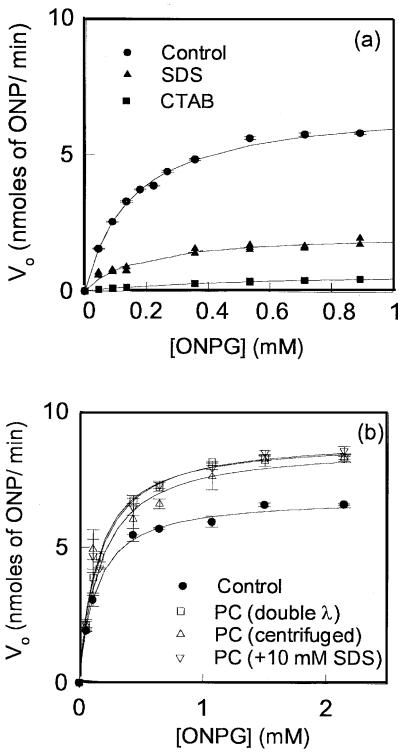

Fig. 2. Initial velocity as a function of substrate concentration in β-galactosidase-catalyzed ONPG hydrolysis. Effect of detergents and PC. Curves from typical experiments are shown. Experimental points could be adjusted to hyperbolic curves according to the Michaelis–Menten equation. (a) Inhibitory effects of ionic detergents. (b) Stimulatory effects observed in the presence of PC were similar independently of the correction method applied to discount the light scattering effect (see text 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. 2b was taken from Sanchez and Perillo [22]).

3.2.1. Non-ionic detergents

The values of K_M and V_{max} measured in the presence of the non-ionic detergents Triton X-100 (Fig. 3c Fig. 4c; Table 1), Lubrol-PX (Fig. 3dFig. 4d) and DocNa (Fig. 3e and Fig. 4e), at all the concentrations tested, were not statistically differ-

Table 1
PC–detergent mixtures: effects on the β-galactosidase kinetic parameters

Sample	Detergent (mM)	PC/detergent Ratio (mol/mol)	K_M (mM)	V_{max} (nmol/min)
Control	0	–	0.145±0.006	6.9±1
PC	0	–	0.158±0.013	9.16±0.5
		–		
Triton X-100	0.012	–	0.128±0.042	6.6±0.9
Triton X-100	35	–	0.143±0.03	7.2±0.8
PC/Triton X-100	0.012	100:1	0.15±0.1	9.67±0.38[b]
PC/Triton X-100	35	1:29	0.14±0.03	5.76±0.29[b]
		–		
SDS	0.013	–	0.16±0.01	7.1±0.1
SDS	35	–	0.7±0.096[a]	2.97±0.8[a]
PC/SDS	0.013	92:1	0.15±0.01	9.32±0.17[a]
PC/SDS	35	1:29	0.244±0.012[a,b]	3.35±0.05[a,b]
CTAB	0.012	–	0.13±0.03	5.9±1
CTAB	22	–	9817±nd[a]	<0.5[a]
PC/CTAB	0.012	100:1	0.041±0.024[a,b]	11.5±1[a]
PC/CTAB	22	1:18	8987±nd[a,b]	<0.5[a,b]

Soybean-PC was at 0.84 mg/ml final concentration in the incubation system. Other experimental conditions were those indicated in Sections 2.1 and 2.2. The values of K_M and V_{max} were calculated from the non-linear regression analysis of the initial velocity vs. substrate concentration. Values are the mean±S.E.M. of at least two experiments performed in triplicates. CTAB at a final concentration of 22 mM induced an almost total inhibition so the values shown for the kinetic parameters in this condition are only qualitative. Two-way ANOVAs performed both on K_M and on V_{max} values of samples containing SDS and CTAB as well as on V_{max} of Triton X-100 containing samples, were significant for the two factors analyzed [presence of PC (factor 1) and of detergent (factor 2)]; interaction 1×2 was also significant.

[a] Significantly different in respect to the control , with P«0.05 (LSD post hoc test).

[b] Significantly different in respect to PC samples, with P«0.05 (LSD post hoc test).

ent from the control values in the absence of detergents.

3.2.2. Ionic detergents

Ionic detergents SDS and CTAB, at concentrations equal or above their c.m.c., were able to affect the kinetic parameters of β-galactosidase in a statistically significant manner: SDS, at a concentration of 1.33 mM, incremented K_M in a 88% and decreased V_{max} in 25% with respect to the control values; in the presence of 35 mM SDS, K_M increased 4.4-fold and V_{max} decreased 2.4-fold (Fig. 3aFig. 4a, Table 1). CTAB at an intermediate concentration induced a 75% increment in K_M (Fig. 3b) and a 32% decrement in V_{max} (Fig. 4b), with respect to the control; at a concentration of 22 mM, CTAB inhibited β-galactosidase almost completely (Fig. 3bFig. 4b; Table 1).

3.2.3. PC–detergent mixtures

The effects of PC on the increase in V_{max} were unaffected when Triton X-100 was present in small amounts in addition to PC (molar ratio PC/Triton 100:1). However, when this detergent was in excess with respect to the lipid (molar ratio PC/Triton X100 1:29), the effects of PC on V_{max} completely disappeared (Table 1).

SDS, at a concentration below its c.m.c., in a 92:1 PC/SDS molar ratio, did not affect either K_M or V_{max} respect to the sample containing PC without any detergent.

Interestingly, CTAB at a concentration well below its c.m.c., in the presence of 0.84 mg/ml PC (PC/CTAB molar ratio was 100:1) significantly decreased K_M compared with the control; V_{max} remained incremented with respect to the control, as occurred in samples containing only

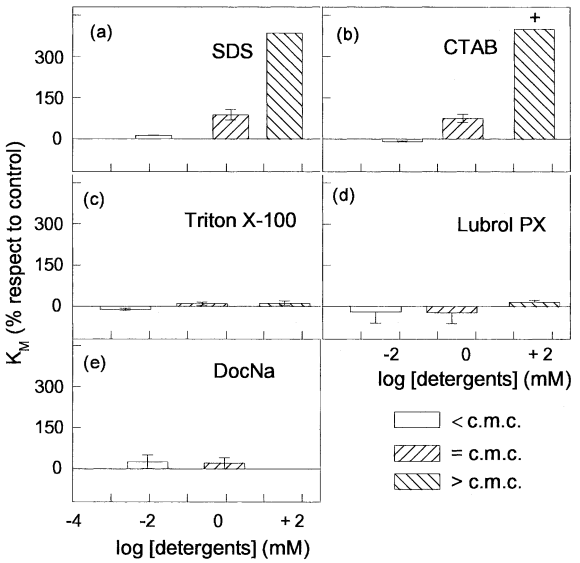

Fig. 3. Effect of detergents on the K_M values of β-galactosidase. K_M values for the β-galactosidase-catalyzed hydrolysis of ONPG in the presence of the detergents indicated which were tested at concentrations below, at and above their corresponding c.m.c. +: This value is qualitative just to indicate the almost complete inhibition of enzyme activity.

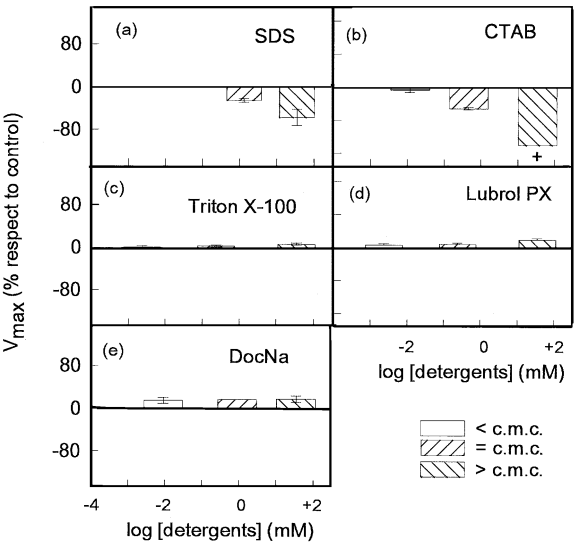

Fig. 4. Effect of detergents on the V_{max} values of β-galactosidase. See legend of Fig. 3 for details.

PC, without a significant difference compared to the latter. However, at the highest SDS and CTAB concentrations in molar ratios PC/SDS 1:29 and PC/CTAB 1:18, both detergents expressed the same effects they exerted when applied without PC, increasing K_M and decreasing V_{max} (Table 1).

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

Fig. 5 shows that A_{420} of a sample containing ONPG was not affected by the presence of self-aggregating structures of PC or detergents. This result indicated the absence of ONP becoming from the interfacial-catalyzed hydrolysis of ONPG [37].

3.4. Effect of detergent concentration on the size of mixed PC-detergent self-aggregated structures

The interaction of detergents with liposomes leads to the breakdown of lamellar structures and to the formation of lipid–surfactant mixed micelles; as a consequence, the turbidity of the dispersion decreases [38]. Accordingly, the turbidity of MLVs dispersion decreases dramatically at detergent concentrations above the corresponding c.m.c., becoming negligible at detergent concentrations above 10 mM (Fig. 6). At this point, the total solubilization of liposomes via mixed micelles formation was reached. These effects were

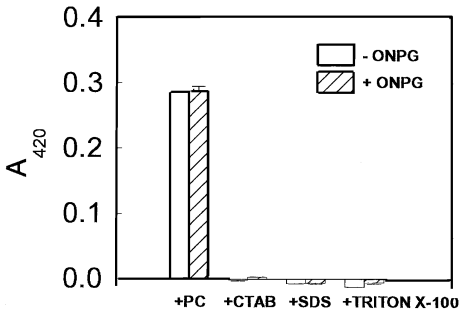

Fig. 5. Non-enzymatic hydrolysis of ONPG at the lipid–water interface. ONPG was incubated alone or in the presence of $PC_{soybean}$ without the addition of enzyme. After 15 min the absorbance at 420 nm indicative of ONP production was measured in the presence of 1.4 M Na_2CO_3. The high absorbance values observed in the presence of PC are due to light scattering.

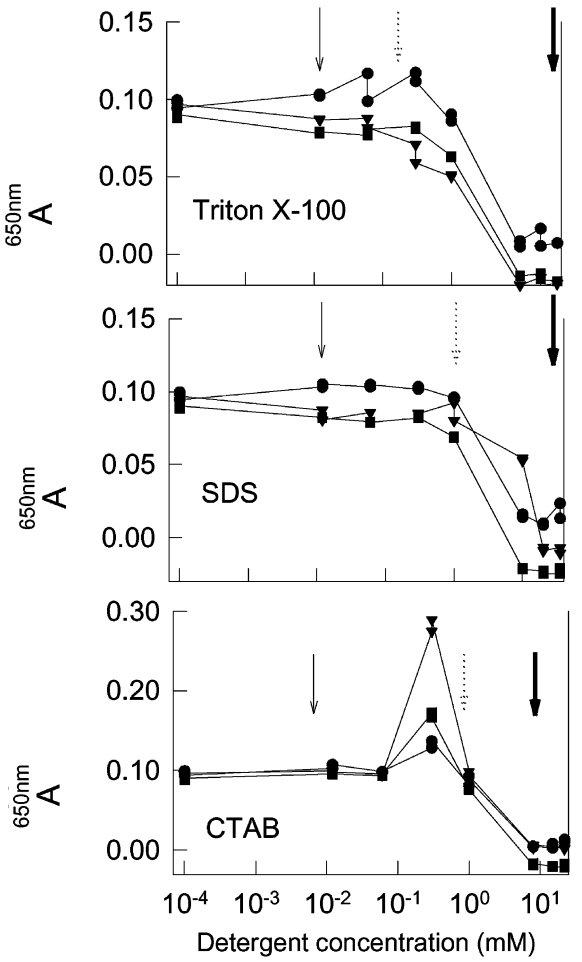

Fig. 6. Effect of detergent concentration on the size of mixed PC-detergent self-aggregated structures. Measurement were done at 0 h (circles), 17 h (squares) and 41 h (triangles) after the addition of the detergent. The arrows indicate the detergent concentration sufficient to reach the c.m.c. value of each detergent (dotted arrow) or the particular PC/detergent molar ratios 100:1 (thin solid arrow) or 1:29 (thick solid arrow), tested in the experiment shown in Table 1.

qualitatively similar at up to at least 41 h. In CTAB-PC mixtures, we observed a highly reproducible peak of turbidity at 0.3 mM CTAB (PC/CTAB molar ratio 4:1) that tended to disappear with time. This may be associated with a membrane expansion upon CTAB incorporation in the PC vesicles and a subsequent molecular reorganization.

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

Fig. 7a 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) with respect to the free interface ($\Delta\pi < 0.15$ mN/m at $\pi_i = 0$ mN/m). The penetration of β-galactosidase into Egg-PC monolayer decreased as the molecular packing increased (at higher π_i) and it showed a cut-off initial pressure $\pi_{i\ \text{cut-off}} \cong 30$ mN/m (Fig. 7e). Penetration of β-galactosidase in mixed-monomolecular layers of PC containing detergents in a 100:1 molar ratio was facilitated if it is compared to pure PC monolayers (Fig. 7b–d). The effect of all detergents tested was quantitatively similar ($\pi_{\text{cut-off}} = 35$ mN/m) (Fig. 7e).

In order to try to explain these results from the point of view of the type of viscoelastic changes induced by the detergents on Egg-PC monolayers, we ran π-area isotherms of the PC-detergent mixtures used in penetration studies (Fig. 8a). In addition, the values of compressibility modulus (K) as a function of molecular areas were calculated from π-area isotherms data, using the following equation [39]:

$$K = -(A_\pi)\cdot\left(\frac{\partial\pi}{\partial A}\right)_\pi$$

where A_π is the molecular area at the indicated surface pressure (Fig. 8b). This parameter reflected the physical state and the bi-dimensional phase transition of the monolayer. The higher the K values, the lower was the interfacial elasticity. CTAB and Triton X-100 increased the elasticity of PC monolayers, mainly at molecular areas corresponding to a surface pressure of 35 mN/m (the equilibrium surface pressure of bilayers). The effect of SDS was less marked. Contrary to what happened with CTAB and Triton X-100, in SDS the balance between the tendency towards membrane partitioning (stabilized through dispersion forces at the hydrocarbon chain level) and the water solubility (favored by the polar head group hydration) was displaced towards the latter [40].

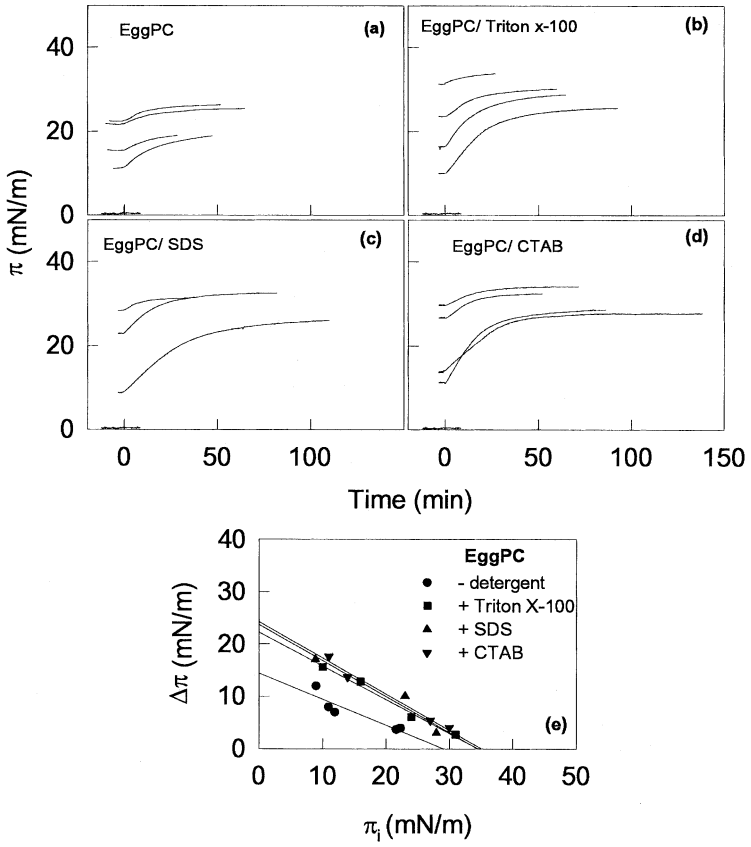

Fig. 7. Interaction of β-galactosidase with the lipid–water interface. (a–d) Penetration of β-galactosidase in monolayers of different composition. (e) Effect of molecular packing, represented by the initial lateral surface pressures (π_i), on enzyme penetration in monolayers; maximal $\Delta\pi$ decreased as a function of π_i; $\pi_{cut\text{-}off}$ represented the value of π_i over which penetration was not allowed. The difference between lateral pressure at time t (π_t) and initial surface pressure at time zero (π_i) was taken as a measure of penetration ($\Delta\pi = \pi_t - \pi_i$).

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 had to be taken into account as they would have lead to: (a) the appearance of surfaces and hydrophobic phases where substrates and products could be adsorbed and/or partitioned, changing their effective concentration in the reaction media and/or changing their spectral behavior; (b) physically perturbing phenomena like light scattering; (c) a decrement of the free aqueous volume of solubilization, and consequently, an increment in the actual concentration of the chemical species participating in the reaction, caused by a significant capture volume of the vesicles that would affect the apparent V_{max}; and (d) non-enzymatic catalysis of hydrolytic reactions at the lipid–water/detergent–water interfaces.

a. Although some of the detergents used exhibit measurable values of A_{420} in their absorbance spectra (Fig. 1a), in the present experimental conditions for ONP quantification (a highly alkaline medium containing the species ONPx),

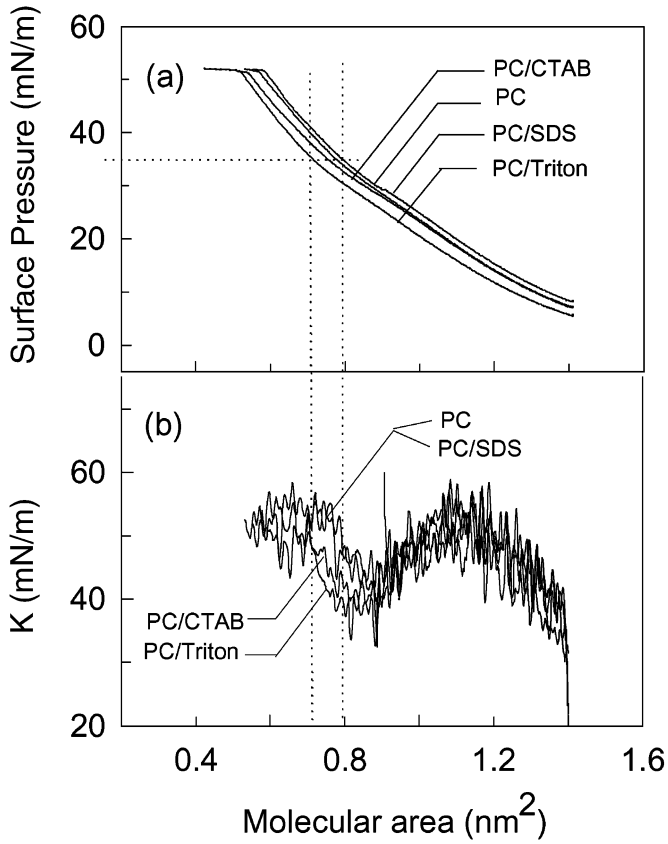

Fig. 8. Variation of surface pressure (a) and compressibility modulus (*K*) (b) as a function of the mean molecular area of EggPC–detergent mixtures. The compressibility modulus *K* was calculated as indicated in Section 3.5, using the values of the isotherms shown in panel a. The equilibrium lateral surface pressure of bilayers and the spanning range of the corresponding molecular areas are indicated by the horizontal dotted line in panel a and the vertical dotted lines in panel b, respectively.

A_{420} were negligible for all of the detergents tested, indicating that their presence did not introduce errors in A_{420} of ONPx (Fig. 1b). A similar conclusion could be achieved for eventual interactions ONPx-detergent except for CTAB that increased the extinction coefficient for ONPx (Fig. 1c). According to the effect of the polarity of the media on the spectroscopic behavior of ONPx (Fig. 1d), the partitioning of ONPx towards an environment less polar than water would induce an increase in its extinction coefficient. Moreover, as observed in Fig. 1c, a preferential partitioning is expected of the negatively charged ONPx towards the positively charged surface of CTAB micelles with respect to the other detergents, which offered either a neutral (Triton X-100, Lubrol-PX and DocNa) or negative (SDS) surfaces. The consequence on the enzymatic reaction kinetics that would be expected for this electrostatic interaction between CTAB and ONPx would be an apparent increase in the reaction rate due to an overestimation of ONPx concentration. This does not represent an artifact that invalidates the description of CTAB effects on the β-galactosidase kinetic parameters as inhibitory (see below) because both effects of CTAB, on the enzyme and on the $A_{420,\ ONPx,}$ are in opposite directions. At least what can be argued is that the enzymatic inhibition of CTAB may suffer of some degree

of underestimation.

b. Both PC and detergents, when used at final concentrations above their corresponding critical micellar concentrations (c.m.c.), according to the relative volumes of the hydrophilic and hydrophobic portions of their molecules, self-aggregate into bilayers and into spherical micelles, respectively [30,41]. Low amounts of detergents can be incorporated into PC bilayers but this fact will induce an increment in the surface curvature of the vesicles. If the molar ratio detergent/PC is very high, the bilayer structure may be disrupted and mixed micelles of detergents containing lipids may appear [38,41,42]. 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 [43]. In our assay system, light scattering induced by PC vesicles introduced a turbidity becoming significant over 0.1 g/l of PC and was corrected for in the experiments that followed (see Section 3 and Fig. 2b). Micellar dispersions of detergents did not induce any measurable light scattering effect that could distort the absorbance data.
c. The capture volume of the 500 nm-PC MLVs used, would induce a decrease in the effective reaction volume and a consequent apparent increase in V_{max} of less than 2% with respect to the values measured in vesicle free media [36]. This effect does not account for the 30% increments in V_{max} observed in the presence of MLVs, with respect to the control.
d. 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 [37]. With this concept in mind, possible non-enzymatically catalyzed hydrolysis of ONPG at either lipid–water or detergent–water interfaces was investigated, obtaining negative results in both cases ([22] and Fig. 5).

These facts supported the conclusion that the changes observed in the values of the kinetic parameters (Figs. 2–4, Table 1) were due to effects of lipids and detergents on the enzymatically catalyzed reaction.

4.2. Modulation of enzyme kinetic parameters induced by lipids and detergents. Is the presence of a surface a necessary requisite?

The interaction of β-galactosidase with lipid–water or detergent–water interfaces did not change the hyperbolic shape of the product vs. time plot (indicative of a Michaelian behavior) (Fig. 2) but, in some conditions affected the kinetic parameters of ONPG hydrolysis. The presence of a surface seemed crucial for the effects, if we take into account that none of the detergents tested affect K_M or V_{max} when present at concentrations below their c.m.c. (Figs. 3 and 4).

The surface curvature was also determinant: a neutral but highly curved surface like the one offered by Triton X-100 (micelles diameter was approx. 10 nm) did not affect the reaction kinetics (Figs. 3 and 4; Table 1) however, a less curved surface like zwitterionic PC vesicles (approximate diameter 500 nm) increased at least one of the kinetic parameters (Table 1). Moreover, the effect of PC on V_{max} disappeared when Triton X-100 was present in a great excess respect to the phospholipid (Table 1) due to either a great decrease in the diameter of the mixed PC-detergent vesicles or to its transformation into mixed PC-detergent micelles. This was indicated by the analysis of the effects induced by increasing proportions of detergents on the turbidity of PC-detergent suspensions (Fig. 6).

Charged surfaces, even those with high curvatures, independently of the sign of the electrical charge, were also able to modify the enzyme kinetic parameters (see the effects of SDS and CTAB at concentrations equal or higher than their c.m.c. in Figs. 3 and 4 and PC/SDS and PC/CTAB at high PC/detergent ratios in Table 1).

Muga et al. [44] demonstrated that 10 mg/ml (35 mM) SDS was able to affect the secondary structure and to inhibit the activity of β-galactosidase from *E. coli*. However, these authors neither indicated which of the kinetic parameters was affected nor associated the effects of SDS at high concentrations with the existence of a supramolec-

ular structure. The differences with respect to the control that we observed in β-galactosidase absorbance spectra in the presence of SDS, would reflect the conformational changes demonstrated by Muga et al. [44]. The effect of CTAB and of Lubrol PX on the absorbance spectra of β-galactosidase suggested that these detergents were also able to affect the enzyme conformation but through changes different from those exerted by SDS (not shown).

4.3. Enzyme–surface interaction

β-Galactosidase (Fig. 6) as well as other proteins [17] and peptides [45] 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 other enzymes, e.g. neuraminidase [17], 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. 7a). 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 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. In mixed PC-detergent monolayers, penetration of β-galactosidase was more efficient (Fig. 7b–d) and could be accomplished at higher surface pressures than in pure PC (Fig. 7e). This may be due to the higher elasticity (lower compressibility modulus) of the detergent containing monolayers, mainly at surface pressures equivalent to the equilibrium surface pressure of bilayers (Fig. 8b).

The enzymatic hydrolysis of ONPG is accomplished in two steps [46]. After the first kinetic step the free aglycone part of the substrate and a glicosyl-enzyme derivative (ES′) are obtained. In the second step the latter suffers the nucleophilic attack of water that breaks ES′ complex. This process can be enhanced by the presence of other nucleophiles like methanol, ethylenglycol and 2-mercaptoethanol. As suggested previously [22], the increment in V_{max} values observed in the presence of PC might be due to a nucleophilic behavior of the phosphate group from PC molecules or to a more favorable energetic configuration of the water molecules structured at the lipid surface, leading to a decrement in the activation energy of the second kinetic step. Perhaps a certain nucleophile lattice or a characteristic binding energy of the surface hydrating water molecules are required in order to observed the enhancing effect of PC on V_{max}. PC-Triton X-100 mixed self-aggregating structures are less packed and more curved than those composed of pure PC [42]. The decrease in V_{max} with respect to the value obtained in the presence of pure PC, may be interpreted on the basis of a disruption of the above-mentioned optimum arrangement of nucleophile lattice or changes in binding energies of water molecules induced by Triton X-100. The higher surface tension of the more curved structure may increase the adsorption of the enzyme. Moreover, the less packed surface may facilitate a deeper penetration of the enzyme in the bilayer that may also affect the overall process.

At a high PC/detergent molar ratio, and contrary to what happened with SDS, CTAB induced a decrement in the value of K_M with respect to the control (Table 1). Being itself an electrophile, CTAB might have induced an effect in the opposite direction respect to that exerted by nucleophiles. The latter result was very interesting because it pointed to the importance of topology and not just short-range interactions in determining the direction of the resulting modulatory effect. This conclusion was supported by the fact that the same compound (CTAB) as a monomer, in micelles or incorporated in a bilayer, induced different effects: not affecting, inhibiting, or stimulating, respectively, enzyme activity. At the lowest PC/detergent molar ratio both SDS and CTAB expressed the inhibitory effects they had shown when they were self-assembled in micellar structures.

5. Conclusions

The activity of β-galactosidase against a soluble substrate is modulated by enzyme–membrane interactions in a membrane-topology-dependent

manner. The enzyme confinement to that dimensionality-restricted space modifies the reaction kinetics; the enzyme conformation might also be affected. Experiments directed to clarify this point are being run in our laboratory at present.

A question that arises is how big the modulating effect should be in order to be considered of biological importance. In complex systems, nonlinearity is the rule [47]. So, even subtle changes in one variable may be amplified and the consequences may be observed at the same or at higher hierarchical levels of organization of the cellular dynamics. In this context, modulation of reaction kinetics through enzyme–membrane interactions, as proposed in the present work, may be coupled to subtle changes in the membrane organization triggered by physiological phenomena that may include signal transduction pathways.

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