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Keywords: β-Galactosidase, structure/activity relationship, thermal unfolding, DSC, CD, steady state fluorescence

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Abstract: Previously we demonstrated that E.coli beta-galactosidase (β-Gal) binds to zwitterionic lipid membranes improving its catalytic activity. To understand the activation mechanism from the protein perspective, here the thermal dependence of the catalytic activity was evaluated in conjunction with parameters derived from spectroscopy and calorimetry, in the presence and absence of egg-yolk phosphatidylcholine vesicles.

In solution, the native state of β-Gal exhibits a loose conformation according to the λmax of fluorescence emission which is in the upper end of the emission range for most proteins. A non-two state thermal unfolding mechanism was derived from DSC experiments and supported by the sequential unfolding temperatures exhibited by fluorescence (55ºC) and CD (60ºC) spectroscopies. Quenching of β-Gal´s intrinsic fluorescence, provided evidence for a novel and even looser folding for the lipid-bound protein. However, DSC data showed that the thermal unfolding in the presence of lipids occurred with a significant decrease in ΔH compared to what happened in solution, suggesting that only the population of non-bound protein molecules were involved in this process.

Concluding, upon binding to a lipid-water interface β-Gal becomes trapped in a partially unfolded state, more active than that of the native protein in solution.

Response to Reviewers: Answers to reviewers comments

Reviewer #1:

1- I would suggest to remove the paragraph of CD and made, as it is now, a quotation in the Discussion stating data not shown, or including it in the supplementary material.

Text about CD was removed from M&M and from Results sections and included in a Supplementary data section, as suggested.

2- Also, in the last paragraph of the Discussion the authors comment that contrary to what was expected... Probably, it should be expected because lipid bilayers generally thermally stabilize the proteins acting as an insulator, independent that changes in the conformation are produced, even if a more loose conformation in the extramembranal part is produced.

This statement was eliminated as suggested.

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5th of February, 2013

Prof. H. Ohshima
Editor
Colloids & Surfaces B: Biointerfaces
Tokyo University of Science,
Tokyo, Japan

Dear Prof. Oshima,

We are sending a revised version of the manuscript entitled: **β-Galactosidase at the membrane-water interface. A case of an active enzyme with non-native conformation.** Julieta M. Sánchez, Verónica Nolan and myself, for its publication in Colloids & Surfaces B: Biointerfaces.

Reviewer´s comments were addressed as described in the accompanying section. All changes are highlighted in the manuscript.

We thank the referee for his/her helpful comments and hope that the present version of our work could be suitable for publication.

Sincerely yours,

Prof. María Angélica Perillo, PhD

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Higlights

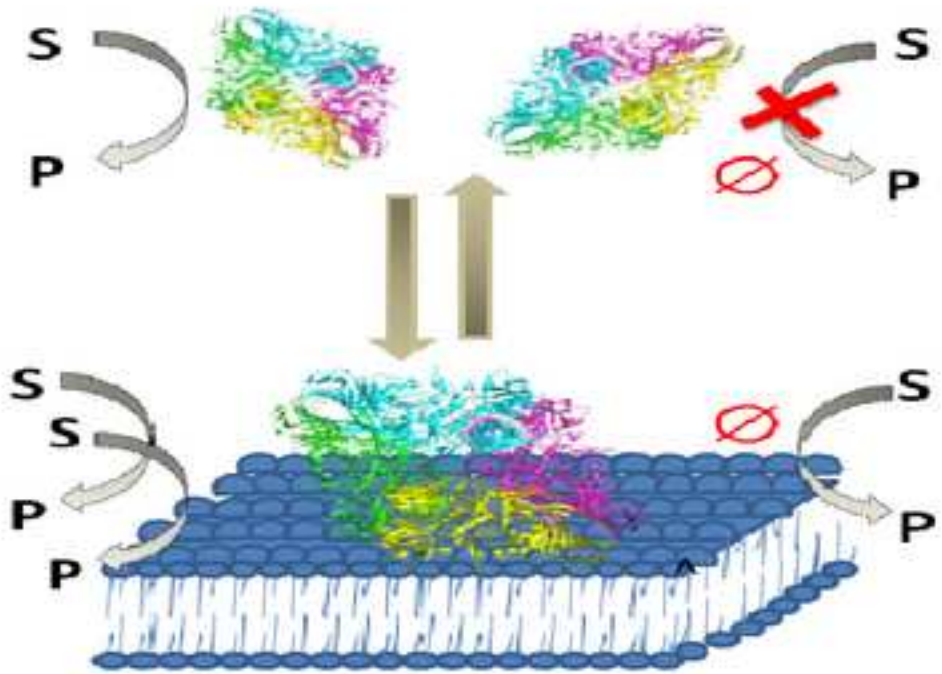
Up to 60ºC structural changes appeared almost exclusively in the tertiary structure

Changes in secondary structure appear mainly above 60 ºC.

In the presence of MLV_{EPC} only non-bound protein molecules were thermally unfolded

Lipid-bound β-Gal was trapped in a loosely packed active conformation

*Graphical Abstract (for review)



β-Galactosidase at the membrane-water interface.

A case of an active enzyme with non-native conformation.

Julieta M. Sánchez, Verónica Nolan and María A. Perillo*

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Abbreviations

β-Gal, *E. coli* beta-galactosidase; MLV, multilamellar vesicle; EPC, egg phosphatidylcholine; ONPG, *ortho*-nitro phenyl β-D-galactopyranoside; DSC, Differential Scanning Calorimetry; FI, fluorescence intensity; CD, circular dichroism; a.a: amino-acids; T_c, lipid phase transition temperature; Tm, temperature at the maximum of the transition peak; Trp, tryptophan; Tyr, tyrosine.

Abstract

Previously we demonstrated that *E.coli* beta-galactosidase (β-Gal) binds to zwitterionic lipid membranes improving its catalytic activity. To understand the activation mechanism from the protein perspective, here the thermal dependence of the catalytic activity was evaluated in conjunction with parameters derived from spectroscopy and calorimetry, in the presence and absence of egg-yolk phosphatidylcholine vesicles.

In solution, the native state of β-Gal exhibits a loose conformation according to the λ_{max} of fluorescence emission which is in the upper end of the emission range for most proteins. A non-two state thermal unfolding mechanism was derived from DSC experiments and supported by the sequential unfolding temperatures exhibited by fluorescence (55ºC) and CD (60ºC) spectroscopies. Quenching of β-Gal´s intrinsic fluorescence, provided evidence for a novel and even looser folding for the lipid-bound protein. However, DSC data showed that the thermal unfolding in the presence of lipids occurred with a significant decrease in ΔH compared to what happened in solution, suggesting that only the population of non-bound protein molecules were involved in this process.

Concluding, upon binding to a lipid-water interface β-Gal becomes trapped in a partially unfolded state, more active than that of the native protein in solution.

Keywords

β-Galactosidase, structure/activity relationship, thermal unfolding, DSC, CD, steady state fluorescence.

1. Introduction

Aqueos-soluble proteins can be defined as those capable to form a homogeneous system upon dispersing in water. In living organisms they may have extra- or intracellular locations. In spite of their water solubility, their binding at interfaces may be thermodynamically expectable whenever they are offered with appropriate high free energy surfaces (e.g. a lipid monolayer packed at a low lateral surface pressure, an air-water interface, etc.).

Binding of aqueous soluble proteins to a lipid-water interface is well documented and its nature can be electrostatic, hydrophobic, or both [1, 2]. In intracellular environments, such interactions regulate key biological functions such as apoptosis [3], signal transduction [4] and endocytosis [5]. They also play an essential role during membrane fusion [6], secretion [7] and the initial steps of membrane protein refolding [8]. Regulatory switches that control membrane-protein binding affinity include the membrane lipid composition and modifications of the protein itself by ligand binding, phosphorylation, or acylation [9].

The binding to a membrane surface can modulate the activity of aqueous soluble enzymes [4, 10-12] in a membrane organization- and topology- dependent manner [11-14]. It has been shown that the activity and stability of aqueous soluble enzymes, such as trypsin and α-chymotrypsin, increase drastically when they are encapsulated in reversed micelles of detergents according to the decrease in the total water content of the micellar lumen [15]. In addition, the interaction of the aqueos soluble proteins human α-amylase [11], *Escherichia coli* β-galactosidase (β-Gal) [12, 14] and aspartyl protease Rennet of *Mucor miehei* [16], with lipid membranes showed differential catalytic activities in the presence of different lipid environments.

In previous works we studied the modulation of the catalytic activity of β-Gal against a soluble substrate in heterogeneous media [12, 14]. This modulation was dependent on the

enzyme interaction with lipid/water interfaces and was exerted at different levels, i.e. the reaction mechanism and the type of β-Gal/bilayer interaction (adsorption or penetration). The former included an apparent effect associated with the partition to the membrane of two of the chemical species taking place in the reaction, the enzyme and one of the reaction products (*o*-nitrophenol, ONP) [17] as well whith the localization of these species within the polar head group region of the membrane [18]. Both modulatory mechanisms depended on the membrane composition, organization and topology. Furthermore, functional consequences of constraining β-Gal in a bi-dimensional space were studied at defined molecular packing densities and constant topology (Langmuir and Langmuir-Blodgett, LB, films) [19]. The β-Gal-monolayer binding equilibrium, mainly the adsorption rate and affinity, depended on the initial monolayer´s surface pressure (lower for higher π_i). Moreover, the β-Gal-catalyzed hydrolysis of *o*-nitrophenyl-galactopyranoside (ONPG) exhibited a qualitative change in its kinetics which resulted michaelian or fractal depending on the membrane molecular packing and the protein 2D distribution.

In all the experimental models tested, vesicles dispersion and LB films, the β-Gal in the membrane bound state, exhibited an enhanced activity compared with its behavior in solutions. As an attempt to understand the origin of the enzyme activation upon partitioning towards a membrane-water interface, in the present work we investigate the conformation-activity relationship of β-Gal in the presence and in the absence of lipid bilayer membranes.

2. Experimental Procedures

2.1 Materials

The enzyme β-Gal from *Escherichia coli* [EC 3.2.1.23] Grade VII (specific activity 650 UI/mg protein; 1UI= 1μmol/min of ONP formed at 37^{o}C) as lyophilized powder, *ortho*-nitrophenyl-β-D-galactopiranoside (ONPG) and acrylamide were obtained from Sigma Chemical Co (St Louis, MO). Egg-phosphatidylcholine (EPC) was purchased from Avanti Polar Lipids (Alabaster, Alabama). Other reagents and solvents used were of analytical grade.

2.2 Methods

2.2.1 Vesicles preparation

Multilamellar vesicles (MLVs) [20] were prepared by evaporating, under a stream of nitrogen, the chloroform from a solution of pure EPC. The dry lipid was suspended in water at a desired final concentration (from 0.3 to 15 g/L), by repeating six consecutive cycles of heating for 2 min at 21^{o}C (a temperature above the phospholipid T_c) and vortexing for 1 min. In these conditions, EPC self-aggregate into multilamellar vesicles MLV_{EPC} [21].

2.2.2 Thermal stability of enzymatic activity

The functional stability of β-Gal was studied by measuring the residual catalytic activity after a 30 min. pre-incubation period at different fixed temperatures (within 15 and 54ºC). For this, β-Gal solutions (0.23 mg/L) were prepared in 0.1 M pH 6,8 phosphate buffer with or without 6 g/L lipids as MLV_{EPC}. The method applied to evaluate the enzymatic activity was essentially that of Wallenfels and Malhota [22] modified as described previously [23]. Briefly, the incubation

system contained, in 0.7 mL final volume of 0.1 M phosphate pH 6.8 buffer, 3 mM ONPG and 0.033 mg/L β-Gal. Final lipid concentration of 0.84 g/L. After incubation for 15 min. at 37ºC, the hydrolysis reaction was stopped by the addition of 0.2 ml of 1.4 M Na_2CO_3. Then, SDS was added at 10 mM final concentration to destabilized vesicles (500 nm diameter) and turning them into micelles (10 nm diameter) avoiding light dispersion [12, 14]. The absorbance of the *ortho*-nitrophenoxide (ONPx) formed was determined at 420 nm with a sensibility of 0.0001 AUs, using a UV-vis Beckman DU® 7500, (Fullerton, CA., U.S.A.) spectrophometer equipped with a diode array detector and a thermostated cell.

2.2.3. Spectroscopic analysis of β-Gal structure in the presence or absence of a lipid-water interface.

2.2.3.1 Steady state fluorescence emission spectroscopy

The intrinsic fluorescence emission spectrum of β-Gal (0.2 g/L in 0.1 M sodium phosphate pH 6.8 buffer) was registered in the presence or in the absence of MLV_{EPC} (0-5.6 g/L) with a Fluoromax Spex-3 Join Yvon (Horiba, New Jersey, USA) spectrofluorimeter, equipped with a thermostated cell, a Xe arc lamp and a photomultiplier tube as signal detector, where light intensity was measured by a photon counting device. The excitation and emission slits were set at 2 nm-wide. Emission spectra were recorded between 300 and 550 nm (excitation wavelength, λ_{ex} = 295 nm). Samples were pre-incubated for 10 min. at a fixed temperature within the range 25ºC-70ºC, directly in the quartz cell hermetically closed with a Teflon cup to avoid solvent evaporation. Temperature was controlled and maintained with a water circulating bath. Data was corrected for light scattering induced by MLVs.

2.2.3.2. Acrylamide-induced quenching of β-Gal intrinsic fluorescence emission.

The emission spectra of β-Gal (0.1 g/L), mainly due to its Trp residues, were recorded in the presence or in the absence of MLV_{EPC} (5.6 g/L) and acrylamide between 0 and 5 M.

The FI (fluorescence intensity) dependence on the quencher concentration was evaluated according to Stern-Volmer theory (eq. 1):

$$\frac{F_0}{F} = 1 + K_{SV}.[Q] \qquad [1]$$

where, F_o and F correspond to FI in the absence or in the presence of [Q] quencher (acrylamide) concentration.

Modified Stern-Volmer plots [24] were obtained by plotting the variation of FI (ΔF) vs acrylamide concentration according to eq.2:

$$\frac{F_0}{\Delta F} = \left(\frac{1}{f_a.K} \right) \times \frac{1}{Q} + \frac{1}{f_a} \qquad [2]$$

where f_a is the initial fraction of fluorophores accessible to the quencher, K is the quenching constant of the fluorescence emitted by the fluorophores population accessible to the quencher. From the slope of eq.2 ($1/(f_a K)$), it was possible to determine K, which reciprocal value represents the quencher concentration necessary to reduce to a half the FI value obtained in the absence of quencher.

2.2.4 Differential scanning calorimetry

DSC experiments were conducted in a VP-DSC microcalorimeter (Microcal™, Inc, Nothampton, MA). Thermograms of β-Gal (0.7 μM) were recorded in the presence or in the absence of MLV_{EPC} (2.5 g/L EPC). Samples were degassed before the injection in the calorimeter

cell. In the reference cell, 0.1 M, pH 6.8 phosphate buffer, with or without EPC, was injected. Scans were performed over a temperature range 25-100ºC at a heating rate of 1ºC/min.

Data were analyzed with the specific software provided by Microcal™ (ORIGIN® 5.0). The temperature at the maximum of the transition peak [25] for a given transition was taken to be the point of maximum excess heat capacity. The molar enthalpy (ΔH) was calculated from the area under each calorimetric peak. Numerical integration of peak areas and resolution of overlapping peaks were performed using curve-fitting procedures after baseline subtraction. The ratio of the calorimetric to van't Hoff enthalpy ($\Delta H_{exp}/\Delta H_{vH}$) was calculated according to eq. 3:

$$\Delta H_{exp}/\Delta H_{vH} = \Delta H_{exp}^{2}/C_{p,max}.4RT_{m}^{2} \qquad [3]$$

where ΔH_{exp} is the experimental calorimetric enthalpy, $C_{p,max}$, is the maximum in the heat capacity function, R is the gas constant, and Tm, is the transition temperature. This ratio is 1 if the transition is from one state to another (two-state type), greater than 1 if more than two states are involved, and may be less than 1 in certain cases if the process is irreversible [26]. Our data exhibited a $\Delta H_{exp}/\Delta Hv_{vH} > 1$ so, heat capacity profiles were subjected to deconvolution analysis assuming a non two-state transition.

2.2.5 Protein bound at the membrane water interface.

The proportion of the total protein that remains bound to the lipid-water interface in each experimental condition (%β-Gal_L) was estimated according to eq.4, derived from the expression of the lipid-water partition coefficient of β-Gal ($P_{L/W}$ = 118) [17]:

$$\%\beta - Gal_L = \frac{118 \times V_L}{V_T + 117 \times V_L} \qquad [4]$$

where V_L and V_T are the volumes of the lipid phase (V_L) and the whole system (V_T), respectively. V_L was calculated from the lipid mass and density.

3. Results

3.1. The presence of lipid water interfaces affects the spectral behavior of β-Gal

3.1.1 Emission of β-Gal intrinsic fluorescence.

Fig. 1 shows β-Gal fluorescence emission spectra in the absence and in the presence of MLV_{EPC} at the indicated lipid concentrations. The spectrum of β-Gal obtained in buffer (λ_{max}=340 nm) is compatible with Trp residues localized in an environment quite polar if compared to what was reported for Trp localized in a buried or non-polar medium (λ_{max}~ 332 nm) [25]. In the presence of MLV_{EPC}, β-Gal spectrum was red shifted (Fig.1b) and fluorescence emission intensity decreased (Fig.1c) in a lipid concentration dependent manner. This indicated the accessibility of Trp residues to the solvent on average was higher when β-Gal was in the membrane-bound state than in solution. This suggested a conformational change in β-Gal upon binding to the lipid-water interface. Note that at the highest lipid concentration tested in this experiment, it can be estimated that a 40% of the total protein is bound to the lipid-water interface (right *"y"* axis in Figs. 1b and 1c).

3.1.2 Acrylamide-induced quenching of β-Gal intrinsic fluorescence in the presence or in the absence of liposomes.

The effect of acrylamide on β-Gal-Trp fluorescence emission spectra was evaluated in the absence and in the presence of MLV_{EPC}. Although acrylamide is a hydrophilic compound it has the ability to quench fluorophores present in both non-polar and polar environments [27]. It is important to note that acrylamide posseses a diffusion-controlled quenching mechanism so, the

number of collisional encounters of Trp with the quencher will decrease by the steric shielding of the fluorophores buried in the protein core. This should be considered for data interpretation.

Stern-Volmer plots obtained in the absence of lipids exhibited a linear behavior (Fig. 2a), and the value of K_{SV} (Table 1) was within the same order of magnitude as that reported in the literature [28]. In the presence of MLV_{EPC}, the Stern-Volmer plot showed a hyperbolic shape (Fig. 2a) suggesting a heterogeneity in the environment of Trp residues within the protein. In addition, in the presence of a lipid-water interface, the FI was more sensitive than in water to the acrylamide-induced quenching. This was reflected by the relationship $K_{SV,MLV} >> K_{SV,water}$ and by the lower acrylamide concentration required to reduce the FI to a half when the protein was interacting with liposomes compared with the protein in water solution ($[Q]_{0.5,MLV} < [Q]_{0.5,water}$) (Table 1).

The linearity of the Stern-Volmer plot obtained with the protein in water (Fig. 2a) suggested a uniform accessibility of acrylamide to Trps. This is also reflected by the value near unity obtained for f_a (Fig. 2b, Table 1). On the other hand, the non-linearity and the higher initial slope of the Stern-Volmer plot (Fig.2a), observed when the enzyme interacts with MLV_{EPC}, suggested that some of the buried Trp residues became more exposed to the solvent and more accessible to the quencher compared with average Trp residues. Moreover, upon increasing the acrylamide concentration it was observed not only a decrease in FI but also a blue shift of the whole spectrum, due to the fact that the residual fluorescence became from the emission of the non-quenched deeper Trp (not shown). The later result supported the idea that the small population of Trp residues (100% - 82%=18%, see Table 1) that remained unquenched in the presence of MLV_{EPC}, reside in a more hydrophobic environment (protein core or protein-lipid interface) not accessible to the quencher.

In particular, possible interactions established between the hydrophobic region of the bilayer and residues of the hydrophobic protein core could contribute to a conformational change of the enzyme. This phenomenon might be related to the enzyme superactivity observed in the presence of MLVs [12, 14] considering that some of the Trp residues of β-Gal (e.g. Trp 999 and Trp 568) are involved in the active site as reported by Huber et al. [29].

3.2 Thermal effect on the structure-activity relationship of β-Gal

3.2.1 Thermal effect on the enzyme's catalytic activity

In a homogeneous solution, the pre-incubation of β-Gal at or above 37°C for 30 min. inhibited the catalytic activity measured at 37°C (Fig. 3a). On the contrary, the presence of MLV_{EPC} in the pre-incubation medium prevented the lost of β-Gal's activity up to a pre-incubation temperature of 52°C.

The superactivity of β-Gal in the presence of MLV_{EPC} reported previously [14] was also evident in data shown in Fig.3a. Note that within the pre-incubation range 15-32 ºC, where the enzyme is active in both conditions tested, the reaction rate is significantly higher in the presence of MLV_{EPC} suspension compared with the aqueous solution.

3.2.2 Thermal effect on β-Gal molecular structure

3.2.2.1 Steady state fluorescence analysis

The thermally-induced unfolding of β-Gal in the absence as well as in the presence of lipid vesicles evidenced changes in the λ_{max} of Trp fluorescence (Fig.3b). This effect occurred in

a lipid concentration-dependent manner and started at a smaller temperature (50ºC) compared to what was registered through changes in $[\theta]_{216}^{MRW}$ in the CD experiments (60ºC) (Supplementary data), suggesting a biphasic-like unfolding.

The whole fluorescence spectrum at each temperature and lipid concentration can be interpreted as the linear combination of free and lipid-bound protein spectra, each one characterized by its own λ_{max} value, red shifted in the later (lipid-bound) compared with the former (free protein). Thus, in Fig.3b, as the lipid concentration increased, the whole thermal profile of λ_{max} value was displaced upwards. Moreover, the lift-off temperature point (where the λ_{max} value started to change) was displaced to lower values in samples containing MLVs. This result can be related to the oligomeric nature of β-Gal which might suffer a complex thermal response like that described by Burgos et al.[30]. This possibility is under investigation in our laboratory.

Taken together, all these observations suggest that bound and free protein molecules are in two different conformational states at room temperature, being the former more loosely packed and more prone to follow a thermal unfolding process than the latter.

3.2.2.2 Differential scanning calorimetry

Fig.4 and Table 2 synthesize results from calorimetric analysis of β-Gal thermal unfolding performed in the absence and in the presence of MLV_{EPC} (2.5 g/l). Fig.4 illustrates typical high resolution thermograms within a maximum range of scanning temperature spanning between 35 and 70°C before compensating for the continuous change in the specific heat through baseline subtraction. The thermogram obtained in the absence of lipids was characterized by one asymmetric calorimetric transition. This transition had $\Delta H_{exp}/\Delta H_{vH} > 1$, indicating that the thermal

event did not represent a single two-state transition [26]. Reversibility was found upon repetitive scanning after the first heating upto 57ºC. Therefore, data was subjected to thermodynamic deconvolution analysis (Table 2). The non two-state transition observed for β-Gal in aqueous solution was in accordance with data reported in the literature [31], resulting in two peaks. With respect to what was observed in the aqueous solution, a similar thermal profile was observed when the enzyme interacts with MLV_{EPC} but the calorimetric data analysis showed an enthalpy value slightly smaller than in water. Since enthalpy is related with the lost in the molecular interactions that maintain tertiary and secondary structure of β-Gal, this result suggest the possibility that in the presence of MLV_{EPC}, only the non-bound protein population was suffering the unfolding process (Table 2).

4. Discussion

In the present work we investigated if β-Gal suffered conformational changes upon binding to a lipid-water interface and also evaluated comparatively the structural stabilities of the conformations it acquired in free and lipid-bound states. These studies were aimed at contributing to understand the enhancement in the catalytic activity of β-Gal we had observed previously in heterogeneous media compared with the behavior of the enzyme in homogeneous solutions. For this, spectroscopic and calorimetric tools were applied.

The wild type β-Gal tetramer contains 39 Trp residues localized along the primary structure of each monomer that are the main contributors to the protein fluorescence after selective excitation at 295 nm. It is well known the sensitivity of Trp emission to the polarity of the environment, with residues in apolar microenvironments having the more ipsochromic shifted emission. So, a protein's spectrum should be rationalized as an average of the fluorescence emitted by its whole Trp population. For most native proteins the λ_{max} of fluorescence emission ranges within 308-350 nm and its unfolding almost always leads to a red shift in λ_{max} up to 345-355 nm [32]. In this context, the λ_{max}=340 nm for the intrinsic emission of native β-Gal in solution (Fig.1a) should be considered a relatively high value and suggested either a loosely packed native structure with high access to hydration water and/or an external location of its Trp residues. This may have a thermodynamic explanation if one considers that a high molecular mass proteins such as β-Gal (MW 465 kD), is expected to exhibit difficulties to energetically satisfy all its interactions in any given conformation [33]. Thus, such proteins would possess highly rough folding energy landscapes which anticipate that the probability of frustration of chains folding might be higher than with smaller proteins. This would lead to the acquisition of a

quite loosely packed native structure and would explain the relative high λ_{max} value of native β-Gal.

While the thermal unfolding up to 60ºC or the binding to a lipid-water interface induced an increase in λ_{max} of β-Gal fluorescence emission upto 348 and 349 nm, respectively, the unfolding in the presence of lipids raised the λ_{max} even more, reaching 357 nm (Fig.3b).

In addition to the information obtained from fluorescence spectra, the lower compactness of the lipid-bound β-Gal conformation is also supported by its significantly higher sensitivity for acrylamide-induced fluorescence quenching compared with the native protein in solution ($K_{SV,PC} >> K_{SV,W}$) (Fig.2, Table 1). This may indicate that in the lipid-bound state, β-Gal is in a conformation different not only from the native but also from the unfolded state in solution.

Far-UV-CD spectroscopy (Supplementary data) showed that the secondary structure of the enzyme is dominated by beta-sheet conformation which tends to get lost upon binding to the lipid-water interface. It has been reported that the secondary structure of β-Gal contains a low proportion of α-helix conformation [31]. More precisely, 40% of β-structures and 35% of α-structures content was demonstrated by FT-IR experiments [34]. Each monomer of the β-Gal homotetramer comprises 5 domains plus an α-peptide. This peptide has near 70% of disordered structure while the other domains exhibit around 45 to 60% α- or β- secondary structure. In accordance with statistical predictions of Malkov et al. [35], and against to what would be expected considering Trp physicochemical properties, 80% of Trp residues of the β-Gal are located in β-sheet structures [36]. However, in Domain 3, 50% of Trp residues appear within α-helices. Domain 3 forms a distorted TIM barrel and is the only domain where α-helix is the prevailing secondary structure and, interestingly, comprise much of the active site. This may explain why, upon membrane binding, β-Gal remains active (Figs.3a and refs. [12, 14]) in-spite

of loosing secondary structure (mainly β-sheet contents) through conformational changes (Supplementary data) that also affect significantly the polarity of Trps´ environment (Fig.1). Furthermore, β-Gal's catalytic activity is improved upon membrane binding. This might be due to concomitant favorable topological changes occurring within the active site which are associated with a new and more active conformation, different from the native structure of the protein in solution.

Thermal denaturation of the enzyme was analyzed by means of steady state fluorescence and DSC (also results from CD experiments shown in Fig.2S, Supplementary data). For DSC experiments a complex thermal transition was expected for a high molecular weight multimeric protein like β-Gal. After fitting a non two-state model, two peaks were resolved in the thermogram of the protein in solution, (Fig.4, Table 2). The first one appeared at T_m=56.7ºC (starting at 51ºC) and the other peak of higher enthalpy appeared at T_m=60.5ºC (starting at 55ºC) approximately. The thermal transitions observed with other techniques such as spectrofluorometry (Fig. 3b) on the one hand and CD (Fig.2S) on the other hand correlated well with the first and second calorimetric transitions, respectively (Fig. 4). In the presence of MLVs a decrease of ΔH was observed. This phenomenon might be related to the fact that the population of free enzyme molecules is the main participant in the unfolding process. Additionally, a decrease in the intramolecular interaction energy associated to a less packed conformation of the lipid bound β-Gal can also be considered.

It can be concluded that the first calorimetric transition (Fig. 4, Table 2) corresponds to a lost of structure at the tertiary/quaternary conformational level which was reflected by the thermally induced changes in FI (Fig. 3b) observed above 50ºC. Note that changes in $[\theta]_{216}^{MRW}$ began only

after 60ºC (Fig. 2S) suggesting that during most of the thermal unfolding process the secondary structure of the protein seemed to remain unaltered.

In homogeneous solution, if the heating proceeded beyond 37ºC and up to 50ºC, the activity of β-Gal was not recovered (Fig. 3a) although its structure did (calorimetric data). Above 60ºC, the secondary structure was completely lost (Fig. 2S). The fact that within the temperature range 50ºC- 60ºC the enzyme in solution was completely inactive reflect the requirement of a preserved tertiary structure for exhibiting full catalytic activity. However, in the presence of vesicles, the retention of the catalytic activity after preincubation between 37 and 55ºC would suggest that structural regions involved in the protein function (perhaps related with β-Gal lipid binding regions containing those Trp residues that were inaccessible to acrylamide quenching) could preserve their integrity during thermal inactivation.

Our non-two state thermal unfolding mechanism is in accordance with results obtained from experiments of refolding after chemical denaturation. Similarly to what happens with many proteins [37], the refolding of β-Gal takes place through a modular nucleation–condensation mechanism [38]. This mechanism, suggests that small protein modules fold through an extended nucleus involving the entire module in which elements of local structure are being formed concomitantly with and stabilized by tertiary interactions. So, going in the reverse direction, the lost of tertiary interactions at high temperature would trigger a subsequent destabilization of secondary structure unless an energy barrier gets in the path and traps the protein in a local minimum energy state. Thus, lipid binding provides β-Gal this trapping mechanism hindering the lost of structural elements relevant for the catalytic activity which allows to freeze the enzyme in an active state.

Concluding, the general scenario indicates that in the lipid bound state the enzyme acquired a more hydrated and flexible conformation that may explain the increase in the catalytic activity observed in the presence of bilayers vesicles. However, this flexible conformation is less susceptible to thermal inactivation probably due to an energetic difficulty in evolving towards complete protein unfolding which results favorable for the protein stability. The present results contribute to the understanding of the structure-activity modulation of proteins in complex environments and may be relevant for technological applications of the enzyme such as liposome encapsulation.

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FIGURE LEGENDS

Fig.1 Effect of MLVs on the spectrofluorimetric behavior of β-Gal. (a) β-Gal intrinsic fluorescence emission spectra in the absence or in the presence of MLV_{EPC} (increasing lipid concentrations from top to bottom) (λ_{ex} = 295 nm). (b) Maximal wavelength (●, λ_{max}) and (c) fluorescence intensity at 340 nm (●), taken from the emission spectra shown in (a). (○) in b and c represent the % of total β-Gal bound at the lipid-water interface.

Fig. 2 Fluorescence quenching of β-Gal Trp residues induced by acrylamide. (a) Stern-Volmer plot (Eq.1). (b) Modified Stern-Volmer plot (Eq. 2). Numbers inserted in panel (b) correspond to: *a*, the intercept, *b*, slope and *fa*, fraction of sites accessible to the quencher, calculated by fitting Eq.2 to the experimental data.

Fig. 3 Effect of temperature on β-Gal structure and activity.

Effect of temperature on β-Gal activity (a) and on β-Gal structure assessed by spectrofluorimetry (b), in the absence (●) and in the presence (○) of lipids.

In (a) β-Gal was pre-incubated during 30 min. at the indicated temperature and then the enzymatic activity was measured in optimal conditions (pH, 6.8 at 37° C) for 15 min. without (●) and with (○) 0.84 g/L MLV_{EPC} . In (b) numbers indicate EPC/concentrations. Small arrows point to the temperature at the discontinuous slope change.

Fig.4 Effect of lipids on the thermal unfolding of β-Gal assessed by differential scanning calorimetry.

Experiments performed at constant [β-Gal] = 0.7 μM, in the absence and in the presence of a dispersion of MLV_{EPC} (2.5 g/L). Results from the deconvolution analysis on these thermograms are shown in Table 2.

Table 1. Modified Stern-Volmer analysis of acrylamide-induced quenching of β–Gal intrinsic fluorescence in the presence or absence of MLV_{EPC}.

Treatment	K_{SV} (M^{-1})	$[Q]_{0.5}$ (M)	*f*a (%)
Water	6 ± 2	0.15 ± 0.01	100 ± 8
MLVs	46 ± 6	0.026 ± 0.003	82 ± 7

Parameters resulting from the modified Stern-Volmer analysis according to Eq. 2 fitted to the curves in Fig.2b through a linear regression by the least squares method. K_{SV}, Stern-Volmer constant calculated from the intercept/slope ratio; [Q], quencher concentration leading to half the fluorescence intensity; *f*a, fraction of fluorophores accessible to the quencher, determined from the reciprocal of the curve ordinate. Numbers are the mean ± S.E.M. of variables.

Table 2. Termodynamic data for β-Gal thermal unfolding in the presence and the absence of a lipid-water interface.

Sample	**Tm** (°C)	**ΔH_{exp}** (kJ/mol)	**$T_{1/2}$** (ºC)	**ΔH_{vH}** (kJ/mol)	**$\Delta H_{exp} / \Delta H_{vH}$**
Buffer	60	5284	5.7	543	9.73
MLV	59.6	4421.5	5.7	640.79	6.9
Peaks resulting from deconvolution analysis					
Buffer	56.7 ± 0.6	2345 ± 716	5		
	60.5 ± 0.5	3048 ± 708	4		
MLV	57± 1	1926 ± 963	6		
	60 ± 0.22	2608 ± 984	5		

Analysis of the thermograms obtained by DSC of β-Gal in solution or in the presence of a dispersion of MLV_{EPC}. Firstly, a two-state transition model was fitted. Due to $\Delta H_{exp} / \Delta H_{vH}$ >1data was submitted to a deconvolution analysis.

Figure 1

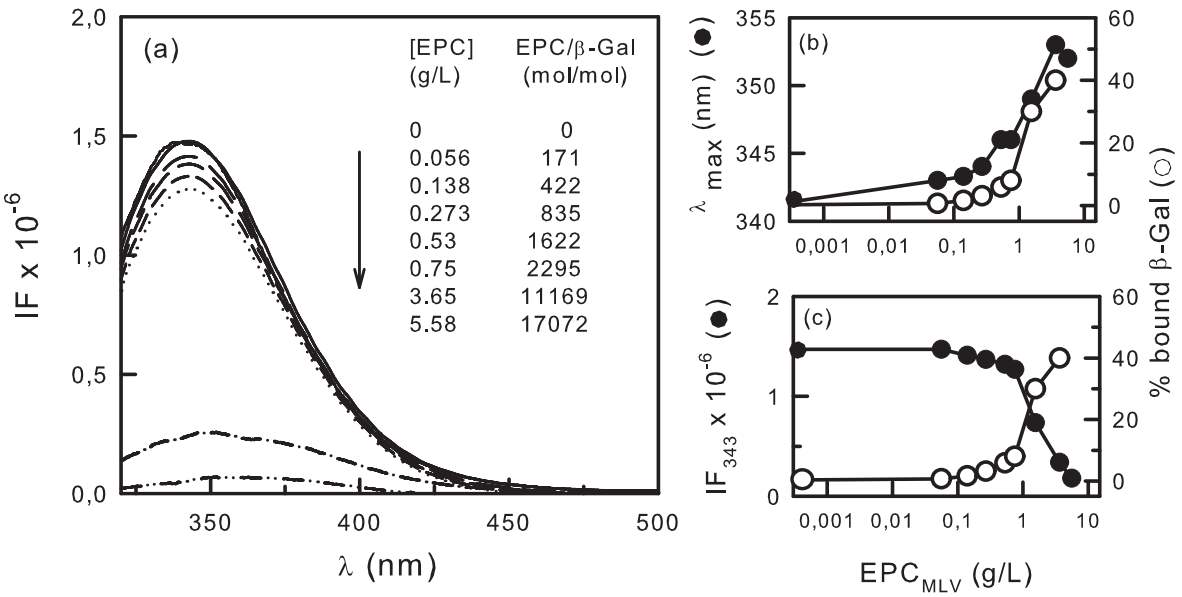
(a)
[EPC] (g/L)
EPC/β-Gal (mol/mol)
0 0
0.056 171
0.138 422
0.273 835
0.53 1622
0.75 2295
3.65 11169
5.58 17072
IF x 10-6
λ (nm)
(b)
λ max (nm) (●)
% bound β-Gal (○)
(c)
IF343 x 10-6 (●)
EPCMLV (g/L)

Figure 2

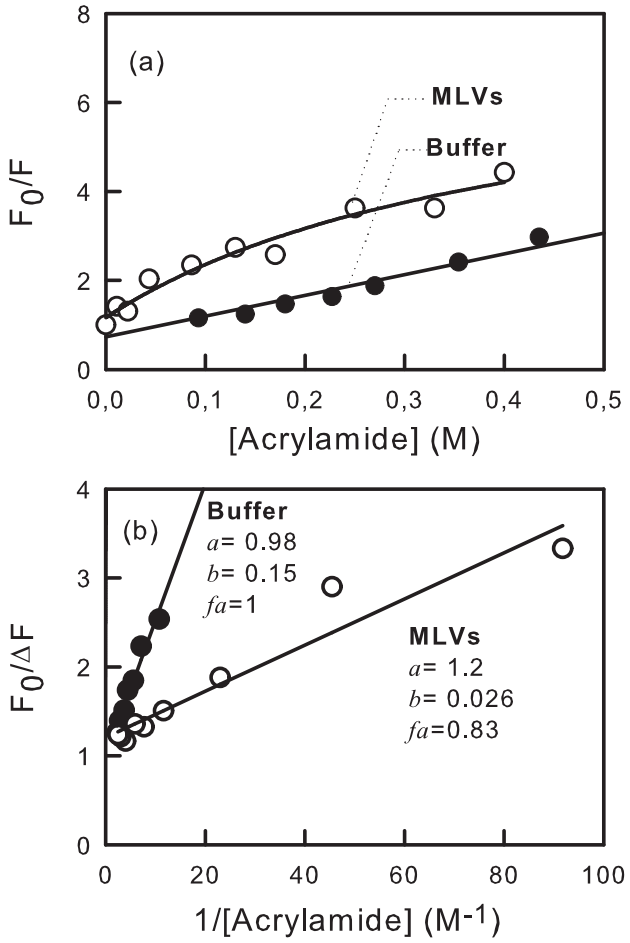
(a)
MLVs
Buffer
F_0/F
[Acrylamide] (M)
(b)
Buffer
a= 0.98
b= 0.15
fa=1
MLVs
a= 1.2
b= 0.026
fa=0.83
$F_0/\Delta F$
1/[Acrylamide] (M^{-1})

Figure 3

Click here to download high resolution image

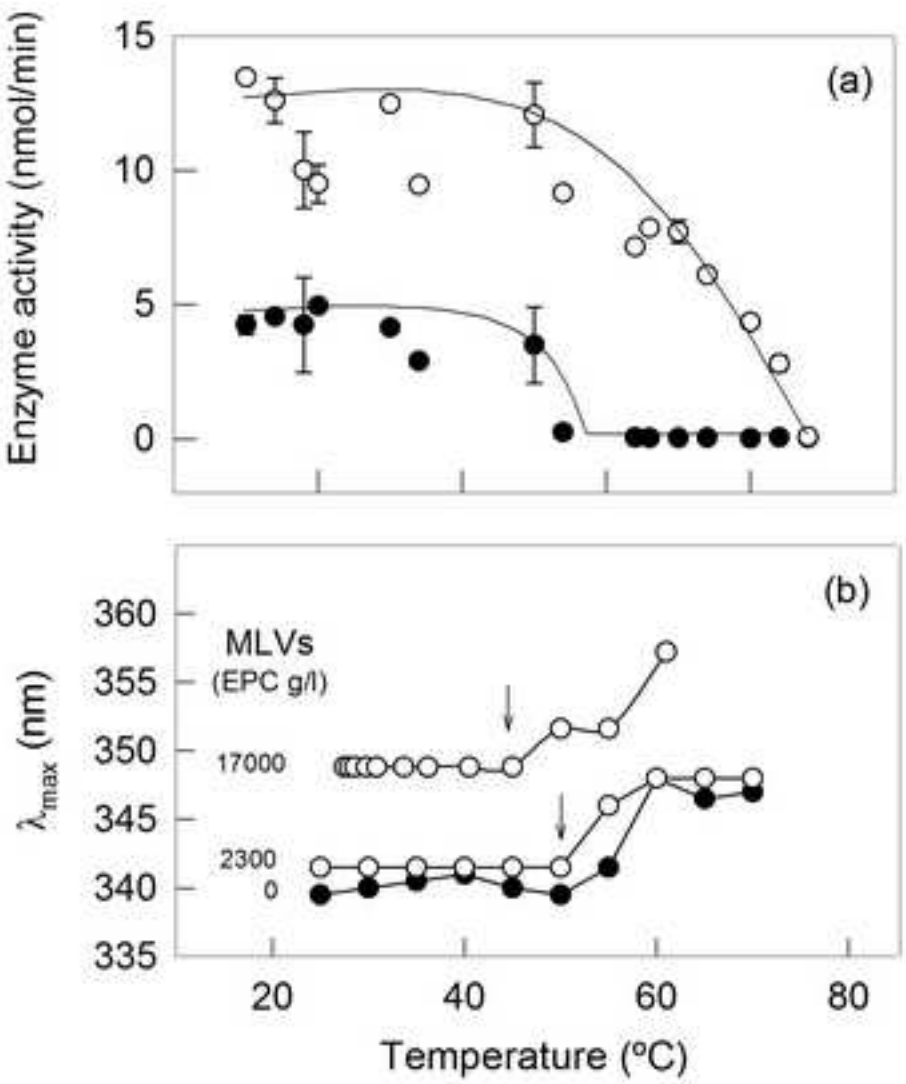

Figure 4

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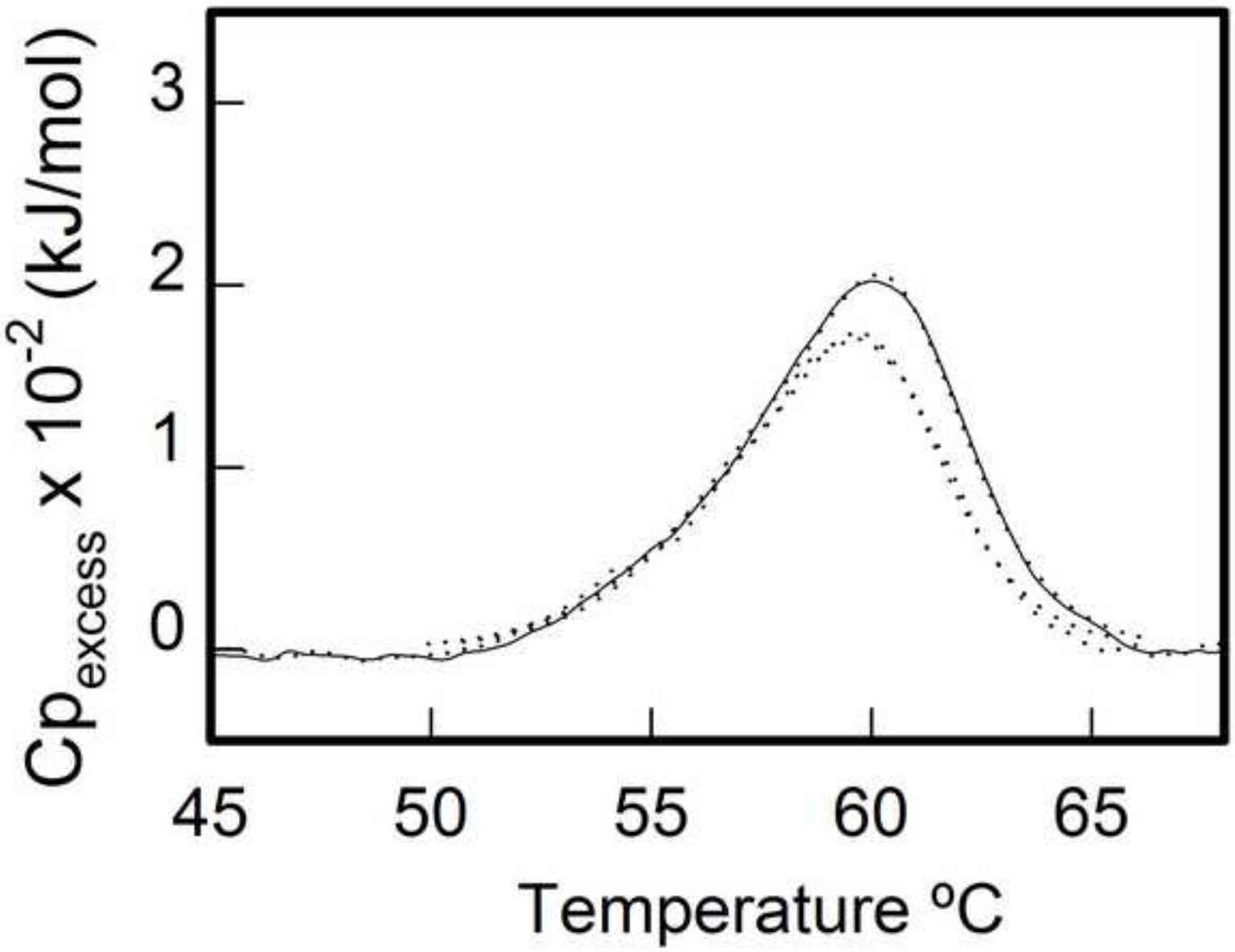

Supplementary Material

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