Interaction of native and partially folded conformations of α-lactalbumin with lipid bilayers: characterization of two membrane-bound states

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Abstract α-Lactalbumin (αLA) can adopt two different membrane-bound states depending on the physical properties of the lipid bilayer, namely adsorbed and inserted. The latter, but not the adsorbed state, is able to disrupt the permeability barrier of the bilayer. The structure of both states is strongly affected by the conformational properties of the αLA conformer considered: as protein flexibility increases the helical content of the membrane-bound conformation decreases, especially in the adsorbed form. Moreover, the adsorbed and the inserted states of those conformers containing 3 or 4 disulfides can interconvert in response to changes in the physical properties of the host membrane.

Key words: Infrared; α-Lactalbumin; Lipid-protein interaction

1. Introduction

Association of water-soluble proteins with membranes is a complex process that has been postulated to occur through distinguishable steps which include: (i) adoption of a competent conformational state in solution by the protein; (ii) adsorption of this active state to the membrane surface; and (iii) partial or complete insertion into the membrane. There is growing experimental evidence for the first step, which suggests that under appropriate environmental conditions the previously water-soluble protein undergoes a conformational change which enables it to associate with membranes [1-4]. The second step leads to an adsorbed state that precedes insertion. Although the presence of this surface-bound insertion-competent state has been inferred from kinetic data [5,6], direct structural evidence for its existence is scarce. In the last step, the protein inserts into the lipid bilayer, adopting a conformation that closely resembles a 'molten globule'. The objective of the present study is to provide evidence for the existence of a membrane-bound adsorbed state different from the partially inserted form and to characterize its conformational properties. As a model system we have used α-lactalbumin (αLA), a secretory protein which is known to interact with lipid bilayers [7,8]. αLA contains four disulfide bonds and therefore it offers the additional advantage of trapping disulfide intermediates with characteristic membrane association properties [9]. We have used infrared and fluorescence spectroscopy to examine the structure of the membrane-bound conformers and their effect on membrane permeability. We have characterized two different membrane-associated states of the protein that show a similar loose tertiary structure but distinct secondary structure, depending on the physical state of the lipid vesicles and the conformational flexibility of the protein. Only the inserted, but not the adsorbed state is able to efficiently disrupt the membrane permeability barrier.

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Abbreviations: ANTS, 8-aminonaphthalene-1,2,3-trisulfonic acid; αLA, α-lactalbumin; $3SS_{cam}$, α-lactalbumin with the Cys6–120 disulfide bond reduced and blocked with iodoacetamide; R_{cam}, fully reduced and carboxyamidomethylated α-lactalbumin; DPPC, dipalmitoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; DOPG, dioleoylphosphatidylglycerol; DPX, *p*-xylenebispyridinium bromide; LUV, large unilamellar vesicles; IR, infrared

2. Materials and methods

2.1. Materials

Egg yolk lecithin (EYL, grade 1) was purchased from Lipid Products (South Nutfield, UK). 1,2-Dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG) and dipalmitoylphosphatidylcholine (DPPC) were from Avanti Polar Lipids (Birmingham, AL, USA). 8-Aminonaphthalene-1,3,6-trisulfonic acid sodium salt (ANTS) and *p*-xylenebispyridinium bromide (DPX) were obtained from Molecular Probes (Junction City, OR, USA). α-Lactalbumin (type I, calcium containing and type III, calcium free) and deuterium oxide (99.8% purity, D_2O) were obtained from Sigma (St. Louis, MO, USA).

2.2. Sample preparation

Reduction and carboxyamidomethylation of the disulfide bond between cysteine 6 and 120 ($3SS_{cam}$) or all disulfides (R_{cam}) of αLA was achieved as described by Shechter et al. [10], using monoiodoacetamide as the blocking agent. Protein concentration was determined spectrophotometrically as described previously [9]. Large unilamellar vesicles (LUV) of PC:PG (1:1 molar ratio) were prepared according to the extrusion method of Hope et al. [11]. The temperature of the samples containing saturated lipids was maintained at 48°C during the extrusion procedure. Lipid concentration was determined by phosphate analysis [12].

2.3. Fluorescence spectroscopy

Fluorimetric assays for vesicle leakage were performed in thermostatically controlled cuvettes using a Perkin-Elmer LS50-B spectrofluorimeter. The medium was continuously stirred to allow rapid mixing of protein and vesicles. Release of vesicular contents to the medium was monitored by the ANTS/DPX assay [13]. An aliquot (15 μl) of a protein solution at pH 6.0 (5 mM HEPES) was added to 1 ml LUV (100 μM), suspended in 20 mM Na_2HPO_4 or acetate, 100 mM NaCl, 1 mM EDTA at the desired pH to give a lipid:protein molar ratio of 350. Fluorescence measurements were carried out by setting the ANTS emission at 520 nm and the excitation at 355 nm. A cutoff filter (470 nm) was placed between the sample and the emission monochromator. 0% leakage corresponded to the fluorescence of the vesicles at time zero and 100% leakage was the fluorescence value obtained after the addition of Triton X-100 (0.5% v/v).

Intrinsic fluorescence measurements were performed on a Perkin-Elmer MPF-66 spectrofluorimeter using 1 cm path length quartz cuvettes. LUV suspensions and protein solution (1.1 μM) were mixed in the above buffer at pH 7.0, 20°C and a lipid:protein molar ratio of 350. After adjusting the pH, the samples were incubated at 20°C for 30 min. The emission spectra were recorded at selected temperatures

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between 300 and 400 nm using 3 and 5 nm bandwidths in the excitation and emission pathways, respectively, and excitation wavelength of 295 nm. Backgrounds of light scattering were determined with vesicles of identical composition and concentration, but in the absence of protein.

2.4. Infrared (IR) spectroscopy

The lipid-protein complexes prepared as described above were collected by centrifugation (120 000×*g*, 2 h, 14°C), and exchange of water by deuterium oxide was carried out by three cycles of centrifugation-resuspension in D_2O buffer of identical composition as the original H_2O medium. Direct binding measurements were performed by determining spectrophotometrically the concentration of the free protein after centrifugation. Infrared spectra were recorded in a Nicolet 520 spectrometer equipped with a MCT detector. Samples, at a protein concentration of ~10 mg/ml, were placed between two CaF_2 windows separated by a 50 μm thick teflon spacer. A total of 200 sample scans and 200 reference scans were taken for each spectrum, using a shuttle device. Thermal studies were carried out by a step-heating method with ~4°C steps, leaving the sample to stabilize for 5 min before recording the spectra. Spectra were analyzed in a personal computer where solvent subtraction, deconvolution and band-position determination were performed as previously described [14].

3. Results

In order to investigate the role of the lipid physical state in the membrane association of several αLA conformers, experiments were performed with vesicles composed of equimolar amounts of saturated lipids (DPPC:DPPG), which show a gel-to-liquid-crystalline phase transition at ~44°C (see below), and unsaturated lipids (EYL:DOPG), which are in the liquid-crystalline state within the temperature range used in this study. The effect of apo-αLA and two disulfide derivatives, namely apo-$3SS_{cam}$ and R_{cam}, on the stability of both types of lipid vesicles is illustrated in Fig. 1 (panels A–C). At 22°C, all conformers induce extensive leakage of ANTS from lipid vesicles in the liquid-crystalline state whereas content release from LUV in the gel phase is considerably reduced, indicating that the physical state of the bilayer drastically affects their membrane perturbing activity. In order to consider the possible effect of the bilayer physical state on the vesicle-binding ability of these conformers, we determined by centrifugation the amount of protein bound to LUV of both compositions at 22°C. Binding data demonstrate that under our experimental conditions (i.e. pH 4.5), more than 85% of the $3SS_{cam}$ and R_{cam} conformers are membrane bound regardless of the physical state of the bilayer. However, about 3 times less apo-protein binds to DPPC:DPPG (~35%) than to EYL:DOPG vesicles (>90%). To account for this difference, we measured the protein-induced content leakage from LUV made of unsaturated lipids at a lipid to protein molar ratio of 1000:1 (Fig. 1A, trace 1). Even at this high molar ratio the permeabilizing activity of apo-αLA on fluid bilayers is similar to that observed previously. Therefore, the differences detected in these experiments might reflect that these conformers bind to the lipid vesicles adopting different conformations depending on the physical state of the constituent phospholipids.

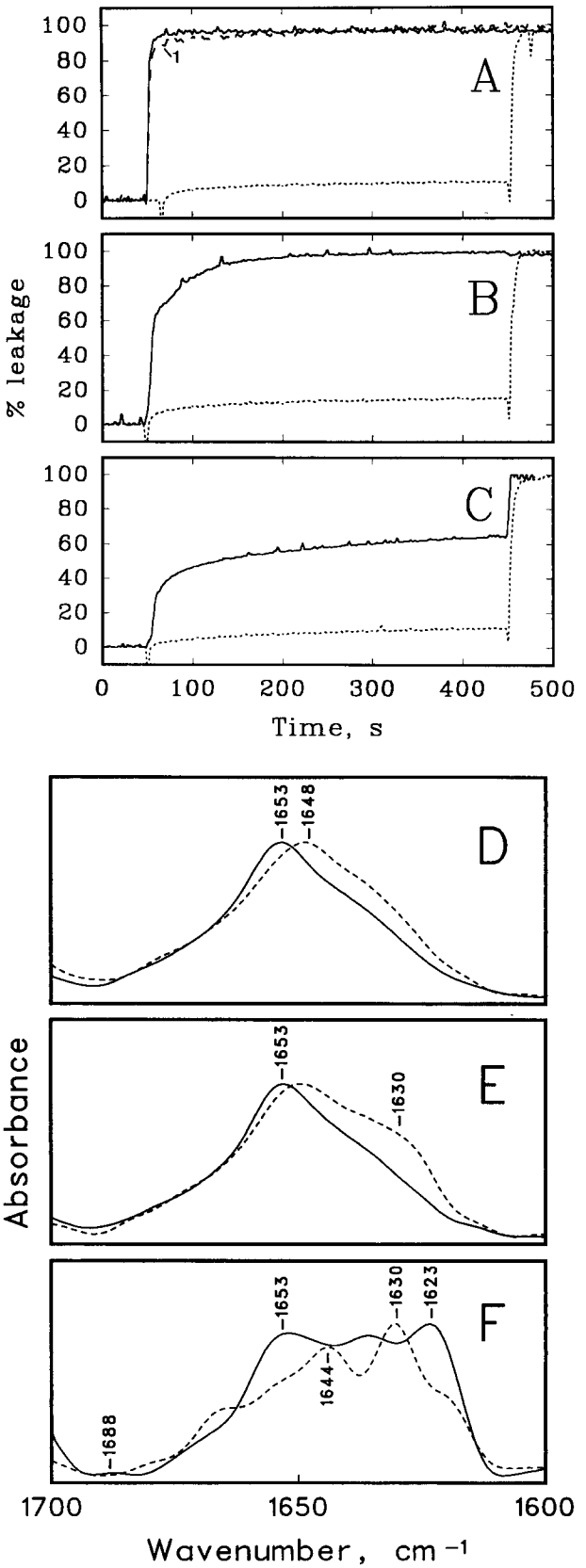

Fig. 1. Effect of the physical state of the bilayer and protein conformation on LUV destabilization by αLA. Kinetics of vesicle leakage induced by apo-native (A), apo-$3SS_{cam}$ (B) and R_{cam} (C). Deconvolved infrared spectra of membrane-bound apo-native (D), apo-$3SS_{cam}$ (E) and R_{cam} (F) αLA. Deconvolution parameters were h_w=18 and K=1.75. LUV were composed of equimolar amounts of EYL:DOPG (solid traces) or DPPC:DPPG (broken traces). All the experiments were performed at 22°C. Trace 1 in panel A shows the kinetics of vesicle leakage at a lipid to protein molar ratio of 1000:1.

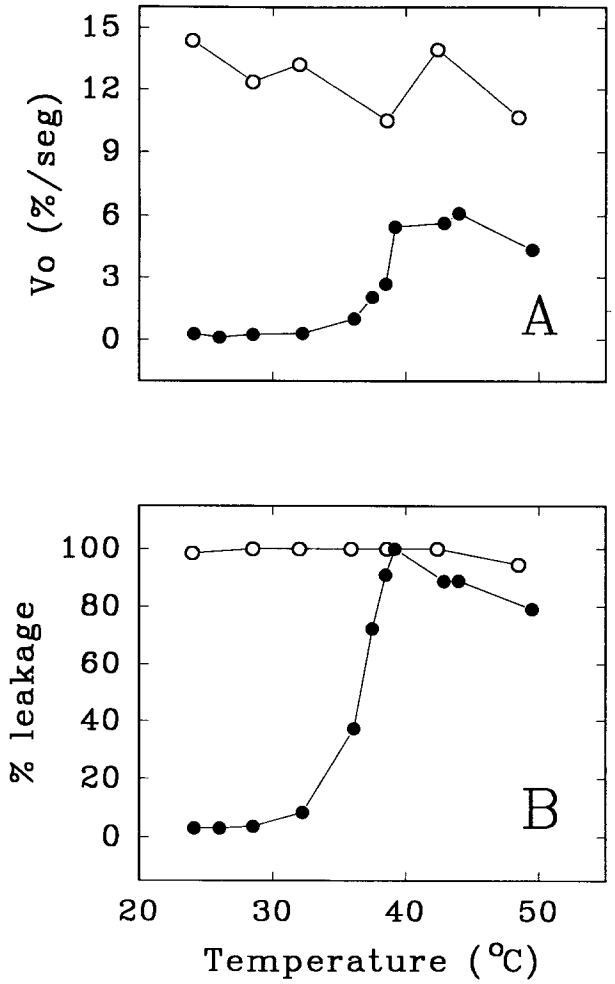

Fig. 2. Effect of temperature on the initial rate (A) and extent of vesicle leakage (B) induced by apo-$3SS_{cam}$ αLA. The lipid to protein molar ratio was 350:1. LUV (100 μM) were composed of equimolar amounts of DPPC: DPPG (filled circles) or EYL:DOPG (empty circles).

To test this postulate, we carried out an infrared analysis of the lipid-protein complexes (Fig. 1, panels D–F). The conformation of the native and partially reduced apo-proteins associated with fluid bilayers is very similar in spite of showing different structural properties in solution [15]. After deconvolution their conformation-sensitive amide I band exhibits several components that are assigned as previously described (Fig. 1, panels D and E) [15,16]: a major band located at 1653 cm^{-1} representing helical segments of the protein; a less intense and broad component at 1630 cm^{-1} characteristic of β-structure; and minor bands at around 1666 and 1675 cm^{-1} indicating the presence of turns, although the latter contains contributions from the high-frequency component of the antiparallel β-structure. Binding of these conformers to lipid bilayers in the gel phase results in a different protein conformation (Fig. 1, panels D and E). The absorption maximum of the amide I band is shifted to lower wave numbers, appearing at 1648 cm^{-1}, and the intensity of the component band at 1630 cm^{-1} is significantly higher for $3SS_{cam}$, suggesting that the extent of the spectral change depends on the conformer considered. Inspection of the amide I band of R_{cam} associated with lipid vesicles further corroborates this suggestion (Fig. 1, panel F). When the host lipid bilayer is in the liquid-crystalline state, the amide I mode of R_{cam} shows, together with bands at 1653 and 1636 cm^{-1}, a well-defined and strong component at 1623 cm^{-1}. Bands at similar positions, accompanied by high wave number counterparts around 1688 cm^{-1}, are indicative of intermolecular hydrogen bonding formed upon aggregation of the lipid-associated protein [17,18]. The lower permeabilizing activity of R_{cam} indicates that aggregation at the membrane surface leads to a protein conformation which is not competent to insert into lipid bilayers (Fig. 1, panels C and F). However, when bound to lipid bilayers in the gel state, the secondary structure of R_{cam} is mainly composed of non-structured (1644 cm^{-1}) and β (1630 cm^{-1}) conformations. The low intensity of the band at 1653 cm^{-1} indicates that ordered lipid bilayers prevent formation of a significant amount of helical structure in the membrane-associated protein.

To test if the physical state of the bilayer is able to modulate the conformation of the membrane-bound protein and to further characterize the transition between the adsorbed and inserted protein states, we analyzed the effect of the bilayer gel-to-liquid-crystalline phase transition on the protein-induced content leakage and protein conformation. Since DPPC:DPPG LUV are unstable, becoming spontaneously leaky, at pH 4.5 above 34°C, the following experiments were performed at pH 5.0. Binding measurements reveal that apo-$3SS_{cam}$ and R_{cam}, but not the apo-native conformer, bind almost equally to the gel (~80%) and to the fluid lipid phase (~90%). Therefore, we have used the reduced conformers to verify this postulate. Both the initial rate (Fig. 2A) and the extent (Fig. 2B) of content release produced after addition of apo-$3SS_{cam}$ to DPPC:DPPG LUV increase steeply between 34 and 41°C. In contrast, they do not show this temperature dependence when the protein interacts with fluid bilayers (Fig. 2).

The conformation of the membrane-bound protein also senses the physical state of the bilayer. At 22°C, the deconvolved amide I band of the lipid-associated $3SS_{cam}$ looks like that described previously at pH 4.5 (Fig. 3A; see Fig. 1, panel E), whereas at 50°C the component band at 1630 cm^{-1} is hardly detectable. Interestingly, above the transition temperature of the host lipid mixture (see below) the absorption maximum of the amide I band appears at similar wave numbers (1649 cm^{-1}) as that of the protein associated with unsaturated lipid bilayers (1650 cm^{-1}; data not shown). The relative fluorescence intensity and emission maxima (335 nm) of $3SS_{cam}$ bound to membranes are similar regardless of the physical state or the chemical composition of the bilayer (Fig. 3B). The intensity, about 2-fold higher than in solution, suggests the disappearance of the tertiary interactions that quench the protein fluorescence [19]. The blue shift of the emission maxima (≥5 nm) observed on membrane association reflects a change of the Trp residues of the protein to a more apolar environment which would also enhance its relative fluorescence intensity. The conformational transition detected by IR can be followed in greater detail by monitoring the thermal dependence of different bands of the membrane-bound protein spectrum. The intensity ratio of the bands at 1630 and 1650 cm^{-1} undergoes a thermal effect between 34 and 44°C (Fig. 4A), as previously observed in the content leakage experiments. A similar thermal behavior is observed for the ratio I_{1576}/I_{1650}, which accounts for the variation of the rela-

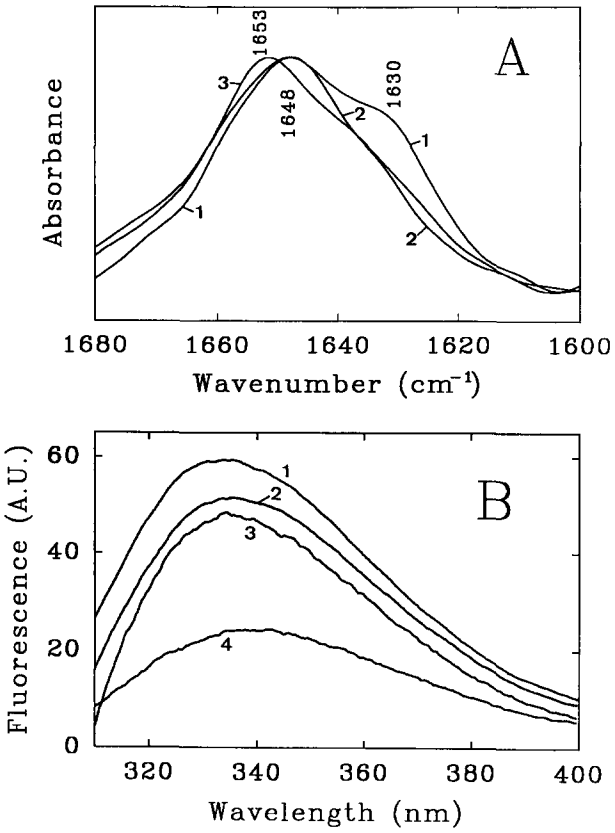

Fig. 3. Effect of temperature on the infrared (A) and fluorescence (B) spectra of apo-$3SS_{cam}$ αLA bound to LUV of DPPC:DPPG (1:1 molar ratio) at pH 5. The temperature was 22°C (1) and 50°C (2). Traces 3 correspond to the spectra of the same protein conformer bound to EYL:DOPG LUV at 22°C. Trace 4 in panel B represents the fluorescence spectrum of the protein in solution. The infrared spectra were deconvolved as in Fig. 1.

tive intensity of the band at 1576 cm^{-1}, originated from the COO^- stretching mode of the aspartic residues of the protein (Fig. 4B) [20]. At 50°C both parameters reach similar values for $3SS_{cam}$ associated with bilayers containing either saturated or unsaturated lipids. Similar temperature-induced changes are obtained for membrane-bound apo-native and apo-$3SS_{cam}$ αLA at pH 4.5, the absolute variation being less marked for apo-αLA (data not shown). In contrast, the IR spectrum of membrane-bound R_{cam} does not show an increase in the relative intensity of the 1653 cm^{-1} component with increasing temperatures (data not shown).

To correlate these thermal effects with the physical state of the bilayer, we studied the temperature dependence of the CH_2 symmetric stretching band position. The frequency of this band is conformation sensitive, the increase in wave number indicating an increase in the gauche/trans conformer ratio, characteristic of the gel-to-liquid-crystalline phase transition [21]. The phase transition temperature of the lipid mixture (~44°C) is slightly reduced (~42°C) in the presence of apo-$3SS_{cam}$ (Fig. 4C).

4. Discussion

α-Lactalbumin is an example of a water-soluble protein that, under specific environmental conditions, can associate with lipid bilayers. The membrane-bound protein can adopt several different conformations, depending on the physical state of the bilayer and the structural properties of the protein.

Below 34°C, αLA adsorbs onto the membrane surface of saturated lipid vesicles, adopting a conformation which depends on the protein conformational flexibility. When αLA is not conformationally restricted by disulfide bonds, as in the case of R_{cam}, the structure of the membrane-bound protein is largely a mixture of extended and unordered structures, devoid of α-helices. If conformational constraints, as three or four disulfides are introduced in the protein, the adsorbed state looks more structured. Therefore, as protein flexibility decreases the helical content of its membrane-adsorbed conformation increases. Its location at the membrane surface is supported by the following experimental findings: (i) the infrared band representing the α-helical structure appears at 1648 cm^{-1}, a position somewhat below the range typical for most helical proteins, in contrast to what was found for the partially inserted protein (1653 cm^{-1}); and (ii) none of the conformers is able to significantly disrupt the membrane permeability barrier. The downward shift of the helical component may be due to either of two reasons, or to a combination of them, namely: (i) some of the protein helical segments are protected against solvent exchange in the partially inserted but not in the adsorbed state [22,23], or (ii) other factors, imposed by the lipid bilayer, influence the folding pattern of the helical structure. The presence in the IR spectrum of the inserted form, at temperatures below 34°C, of a weak absorption band at ~1545 cm^{-1} originated by unexchanged N-H groups of the protein, and the fact that the IR spectra of both states become similar at ~55°C, supports that differences in solvent accessibility may account for the observed position shift.

Above 44°C, the gel-to-liquid-crystalline phase transition temperature, the structural differences detected between the adsorbed states of apo- and $3SS_{cam}$ αLA disappear, their conformation resembling that of the inserted conformers. One interesting finding in this study is that the conformation and permeabilizing activity of the membrane associated protein can be altered in response to changes in the physical properties of the bilayer. The temperature-induced conformational changes start at ~34°C, approx. 7°C below the main gel-to-liquid phase transition temperature (~42°C). The existence of a gel-to-ripple pretransition at ~34°C, already described for the pure lipid mixture [24], offers a rationale for the observed changes and emphasizes the importance of subtle defects in lipid packing for the insertion of proteins into lipid vesicles [25,26]. Our data clearly suggest that apo-native and -$3SS_{cam}$ αLA undergo a reversible and significant conformational change, more pronounced for the latter, between 34 and 44°C. This structural transition, which confers on the protein its membrane perturbing activity, is characterized by (i) an increase in the helical content of αLA at the expense of extended structures, and (ii) a further neutralization of the protein acidic side chains. Since both membrane-associated states, the adsorbed and inserted one, have few, if any, fixed tertiary interactions, the conformational transition would involve mainly changes in protein secondary structure. R_{cam}, however, does not experience the above temperature-induced conformational change. The reason for this different behavior is at present not clearly understood and could be related to the high stability of its partially aggregated, adsorbed state which

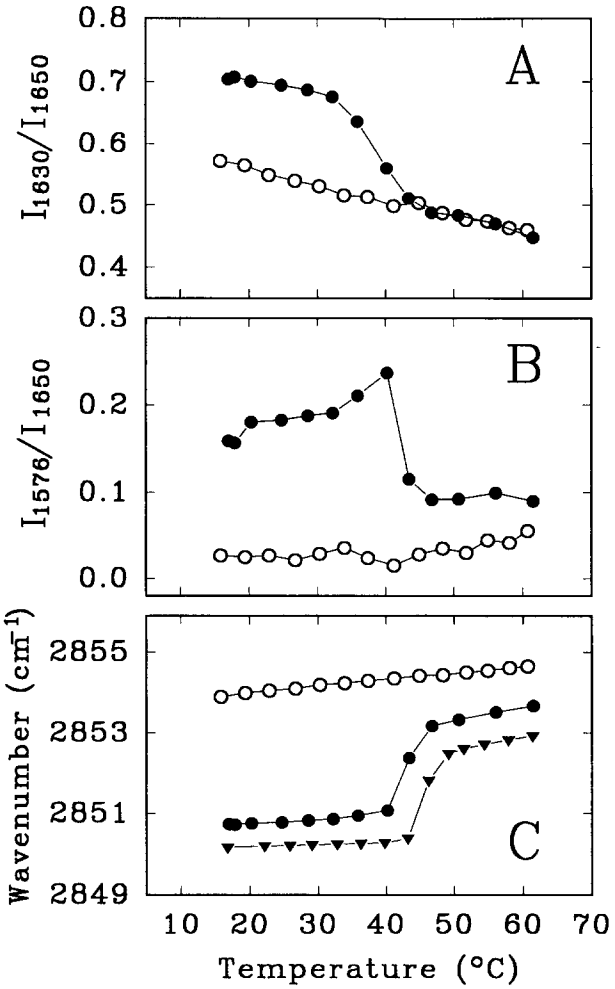

Fig. 4. Temperature dependence of the conformation of apo-$3SS_{cam}$ αLA and phospholipids in the lipid-protein complexes. Relative intensities of the amide I component corresponding to extended structures (A) and of the band originated from glutamate residues of the protein (B), as a function of temperature. Thermotropic behavior of the symmetric CH_2 stretching band of the phospholipids (C). Apo-$3SS_{cam}$ αLA bound to DPPC:DPPG (filled circles) or to EYL:-DOPG (1:1 molar ratio) LUV (empty circles). Filled triangles in panel C correspond to DPPC:DPPG LUV in the absence of protein.

would prevent the conformational transition to a helical structure.

In conclusion, the membrane-bound conformation and permeabilizing activity of αLA is strongly affected by its conformational flexibility and the physical state of the host lipid bilayer. The combination of both factors leads to more or less pronounced structural differences between the adsorbed and inserted states. Particularly significant is our evidence that the structure of the membrane-bound protein can be altered by changes in the physical properties of the lipid bilayer, since it offers a possible mechanism by which information about the physical properties of a lipid bilayer could be transmitted to the membrane-associated proteins and likely affect their biological activity.

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