Structural Requirements for the Association of Native and Partially Folded Conformations of α-Lactalbumin with Model Membranes†

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ABSTRACT: The effect of the structure and stability of several conformers of α-lactalbumin in aqueous solution on their association to negatively charged large unilamellar vesicles has been studied by circular dichroism, infrared spectroscopy, differential scanning calorimetry, and by content leakage experiments. Our results indicate that the affinity of αLA for negatively charged vesicles strongly depends on its conformational properties in solution. Analysis of the pH dependence of the interaction for the different conformers reveals that native-like, calcium-bound, ordered conformations become bilayer-associated through electrostatic forces. However, partially folded conformers are able to interact with negatively charged membranes at pHs higher than the protein isoelectric point, suggesting that hydrophobic interactions brought about by the exposure of hydrophobic residues at the protein surface are able to overcome the unfavorable electrostatic repulsion. Calorimetric and spectroscopic data in solution also indicate that substantial protein destabilization facilitates its subsequent membrane binding, and that the association process is favored for a set of conformers having significant secondary structure, but lacking native-like, stable tertiary structure. Aggregation of the unfolded α-lactalbumin molecules and burial of hydrophobic surfaces upon formation of ordered tertiary structure significantly reduce their membrane perturbing activity. These observations suggest that formation of a flexible structural intermediate of α-lactalbumin in solution is a prerequisite for its association with membranes.

The structural basis of the processes by which water-soluble proteins bind and insert into biological membranes is at present poorly understood. A conformational change of the previously water-soluble protein is believed to expose hydrophobic patches at the protein surface which will trigger protein partition into the membrane (Laine & Esser, 1989; Lakey *et al*., 1992; London, 1992; Helenius, 1992; Muga *et al*., 1993a; Meers & Mealy, 1993). The mechanism of this interaction is complex and involves both electrostatic and hydrophobic interactions. The role of each of these forces in driving the association process is unclear since the experimental approach often used to follow the association reaction requires acidification of the medium. Unfortunately, the pH-dependent exposure of hydrophobic surfaces poses significant problems to differentiate between electrostatic and hydrophobic effects.

α-Lactalbumin (αLA)[1] provides an interesting model system to study the interaction of different conformations of the same protein with lipid bilayers, under the same experimental conditions (i.e., pH, ionic strength, etc.). The native conformation of bovine αLA binds a single Ca^{2+} ion, which strongly stabilizes its structure (Ikeguchi *et al*., 1986; Kuwajima, 1989). This small, globular protein contains four disulfide bonds, the one between cysteines 6 and 120 being reduced by DTT^{SH}_{SH} 100 times faster than any of the other three disulfides (Schechter *et al*., 1973). The resultant three-disulfide form of αLA (designated as 3SS) has a near-native conformation in the presence of Ca^{2+}, while in its absence the protein adopts a molten globule conformational state (Kuwajima, 1989; Ikeguchi *et al*., 1992; Ewbank & Creighton, 1991). Thus, by combining Ca^{2+} dissociation and selective reduction of αLA, several different conformational states of this protein can be easily obtained.

αLA, like many other water-soluble proteins and peptides, can interact with lipid bilayers, especially under acidic conditions (Herreman *et al*., 1981; Kim & Kim, 1989). This interaction could be biologically relevant since αLA is a secretory protein and exerts its function in a membrane environment (Berliner & Koga, 1987; Ruoppolo & Freedman, 1994). To gain further insight into the structural requirements for the association of water-soluble proteins with membranes, we have characterized the conformation of αLA's different states in solution, under conditions where interaction with membranes is either favored or hindered. By using biophysical techniques that give complementary information, a relationship between protein structure and protein−membrane association can be established.

MATERIALS AND METHODS

Materials

Egg yolk lecithin (EYL, grade 1) was purchased from Lipid Products (South Nutfield, England) and 1,2-dio-

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[1] Abbreviations: αLA, α-lactalbumin; $3SS_{cam}$, α-lactalbumin with the Cys6−120 disulfide bond reduced and blocked with iodoacetamide; R_{cam}, fully reduced and carboxamidomethylated α-lactalbumin; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; CD, circular dichroism; DTT^{SH}_{SH}, reduced dithiothreitol; DOPG, L-α-dioleoylphosphatidylglycerol; DPX, *p*-xylylenebis(pyridinium bromide); DSC, differential scanning calorimetry; EDTA, ethylenediaminetetraacetic acid; EYL, egg yolk lecithin; LUV, large unilamellar vesicle(s); IR, infrared spectroscopy.

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leoylphosphatidylglycerol (DOPG) from Sigma (St. Louis, MO). 8-Aminonaphthalene-1,3,6-trisulfonic acid sodium salt (ANTS) and *p*-xylylenebis(pyridinium bromide) (DPX) were from Molecular Probes (Junction City, OR). Deuterium oxide (99.8% purity, D_2O) and bovine αLA (type I, calcium-containing; and type III, calcium-free) were obtained from Sigma and used without further purification.

Methods

Sample Preparation. Reduction and carboxamidomethylation of the disulfide bond between cysteines 6 and 120 ($3SS_{cam}$) or of all disulfides (R_{cam}) of αLA were achieved by the method previously described (Schechter *et al*., 1973), but instead of monoiodoacetic acid as the blocking agent we used monoiodoacetamide, which does not modify the net charge of the protein. Isoelectric focusing demonstrated that the isoelectric points of the different conformers were very similar, ranging from 5.0 for R_{cam} to 5.15 for holo-αLA. The apo- and holo-forms of the reduced proteins were obtained by extensive dialysis against neutral solutions containing 1 mM EDTA and 1 mM Ca^{2+}, respectively. The lyophilized proteins were redissolved in 20 mM Na_2HPO_4, 100 mM NaCl, pH 7.0, and the pH of the solution was adjusted to the desired value with citric acid. In addition to the above composition, the holo- and apo-buffers contained 1 mM Ca^{2+} and 1 mM EDTA, respectively. Protein concentration was determined spectrophotometrically as described previously (Ewbank & Creighton, 1993a).

Large unilamellar vesicles (LUV) of EYL/DOPG (1:1) were prepared according to the extrusion method of Hope *et al*. (1985). Briefly, dried lipid films were dispersed in 20 mM Na_2HPO_4/citric acid, 100 mM NaCl, 1 mM $CaCl_2$ (or 1 mM EDTA) at the desired pH and subjected to 10 freeze−thaw cycles prior to extrusion 10 times through two stacked polycarbonate membranes of pore size 0.1 μm (Nuclepore, Inc., Pleasanton, CA).

Leakage of Contents. Release of vesicular contents to the medium was monitored by the ANTS/DPX assay (Ellens *et al*., 1985). LUV containing 12.5 mM ANTS, 45 mM DPX, 20 mM NaCl, and 50 mM acetate buffer at the desired pH were prepared as described above and freed from the unencapsulated material by gel filtration in a Sephadex G-75 column eluted with 50 mM acetate buffer, 100 mM NaCl, and 1 mM $CaCl_2$ or EDTA. Lipid concentrations were determined by phosphate analysis (Bötcher *et al*., 1961). Fluorescence measurements were performed in a Perkin Elmer LS-50 spectrofluorimeter, by setting the excitation wavelength at 355 nm and the emission wavelength at 520 nm. A cutoff filter (470 nm) was placed between the sample and the emission monochromator. In all experiments, lipid concentration was 100 μM, and constant stirring was used. After recording the fluorescence of the liposome suspension to ensure a flat base line, an aliquot (15 μL) of a protein solution at pH 6.0 (5 mM Hepes) was added to 1 mL of vesicle suspension, and the fluorescence intensity changes were continuously monitored at 25 °C: 0% leakage corresponded to the fluorescence of the vesicles at time zero; 100% leakage was the fluorescence value obtained after addition of Triton X-100 (300 μM final concentration).

αLA Binding to Liposomes. Protein solutions (7 μM) and LUV were mixed in 2 mL of 20 mM Na_2HPO_4, 100 mM NaCl, pH 7.0, to give a final lipid:protein molar ratio of 300. After adjusting the pH to the desired value, samples were allowed to equilibrate for 30 min at room temperature and were centrifuged at 120000*g* during 4 h at 15 °C. Protein concentration in the supernatant was determined spectrophotometrically (Ewbank & Creighton, 1993a). To account for the sedimentation of the free protein, samples containing the same protein concentration in the absence of LUV were centrifuged under the same experimental conditions. Only R_{cam} was found to sediment, and therefore binding of this conformer to LUV was not estimated by this method.

Differential Scanning Calorimetry. The thermal stability of native and partially reduced αLA at different pH values was studied by high-sensitivity differential scanning calorimetry using a Microcal MC-2D instrument at a scanning rate of approximately 50 °C/h. Protein concentration varied between 2.0 and 3.5 mg/mL, depending on the sample. Calorimetric data were analyzed with software provided by Microcal, Inc.

Although the thermal unfolding of native αLA, at neutral pH, is reversible as demonstrated by repeated scans of the same sample (Xie *et al*., 1991) under certain experimental conditions, characteristic of each intermediate, the calorimetric denaturation becomes irreversible. This irreversibility, that is generally observed at low pH, comes from posttransitional protein aggregation and complicates the analysis of the calorimetric data (Sánchez Ruiz *et al*., 1988). Nevertheless, two parameters can be easily obtained from the symmetric excess heat capacity *versus* temperature curves of the protein: the midpoint transition temperature, T_m, and the calorimetric enthalpy of denaturation, ΔH_{cal}. These parameters provide a measure of the relative stability of each αLA conformation under conditions of different pH.

Circular Dichroism (CD) Spectroscopy. CD spectra were obtained at 25 °C on a Jasco J-600 or a Jasco J-720 spectropolarimeter. Spectra in the near-UV region were measured in a 0.5 cm quartz cylindrical cuvette at a protein concentration of 0.23 mg/mL. Those in the far-UV region were acquired using a protein concentration of 0.33 mg/mL and a 0.02 cm path length cell. Mean residue ellipticity values were calculated, after subtracting the corresponding value of the buffer, from the formula $\theta = \epsilon/10Cnl$, where ϵ is the ellipticity (mdeg), C is the protein concentration (mol/L), l is the path length of the cuvette (cm), and n is the number of amino acid residues in the protein (123 for αLA).

Infrared (IR) Spectroscopy. Samples for infrared measurements were prepared by disolving the freeze-dried proteins in 20 mM Na_2HPO_4, 100 mM NaCl, at the desired pH to achieve a protein concentration ranging from 2 to 12 mg/mL. The holo- and apo-buffer contained 1 mM Ca^{2+} and 1 mM EDTA, respectively. To complete the hydrogen−deuterium exchange of the accessible protein backbone N−H groups, samples were incubated in deuterated buffer for 48 h at 4 °C. Further changes in the spectra were not detected after this incubation time. Protein samples were placed between two CaF_2 windows separated by 50 μm (D_2O) or 6 μm (H_2O) thick Teflon spacers which were assembled in a demountable infrared cell (Harrick Scientific, Ossining, NY). Infrared spectra were recorded on a Nicolet 520 spectrometer equipped with an MCT detector. A total of 200 scans (sample) and 200 scans (background) were taken for each spectrum, using a shuttle device. Spectra were analyzed in a personal computer where solvent subtraction, Fourier self-

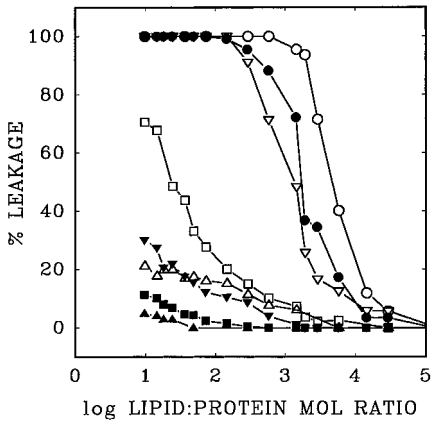

FIGURE 1: αLA-induced permeability of EPC/DOPG (1:1) LUV as a function of protein concentration and pH. Extent of leakage produced 7 min after the addition of holo-αLA (closed symbols) or apo-3SS$_{cam}$αLA (open symbols) at pH 6.0 (△), 5.5 (□), 5.0 (▽), and 4.5 (○).

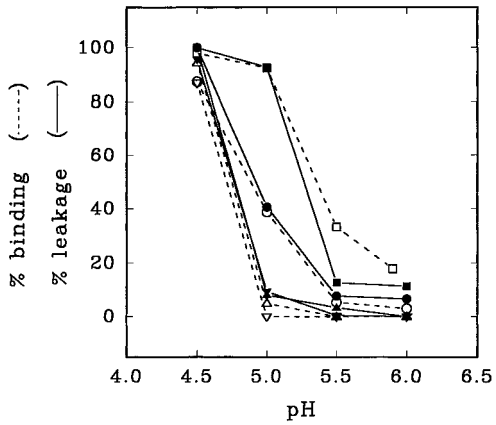

FIGURE 2: Effect of protein conformation on αLA binding to liposomal membranes (---) and protein-induced leakage from LUV (—) as a function of pH. Percentage of binding (open symbols) and leakage (closed symbols) for holo-αLA (▽), apo-αLA (○), holo-3SS$_{cam}$ (△), and apo-3SS$_{cam}$ (□). The lipid:protein molar ratio was 300:1. Each value was calculated from the average of three independent measurements of duplicates. Experimental error was estimated to be less than 8% and 7% for the binding and content release experiments, respectively.

deconvolution, and band position determination were performed as previously reported (Arrondo *et al*., 1989, 1993).

RESULTS

Interaction of Different αLA Conformers with Membranes. We have used the release of LUV content to monitor the time course and the extent of the interaction between different conformations of αLA and model membranes as a function of pH. Binding of αLA to LUV composed of equimolar amounts of EYL and DOPG promotes release of previously entrapped fluorescent dyes and therefore an increase in the total fluorescence of the sample until a stable value is obtained. The kinetics of the leakage process were studied by performing release experiments with increasing amounts of protein while maintaining constant lipid concentration. A comparison of the pH dependence of the extent of leakage induced by native, holo-αLA, and apo-3SS$_{cam}$ reveals that while the permeabilizing activity of the former becomes significant at pH 4.5, the latter is able to promote substantial leakage at pH 5.5 (Figure 1). In order to compare the effect of different conformations of αLA on content release, we have estimated, from experiments as shown in Figure 1, the lipid:protein molar ratios required for the onset of activity, 50% and 100% leakage at representative pHs (Table 1). These results show that holo-3SS$_{cam}$ interacts with negatively charged membranes in a similar way to that described for its native counterpart, i.e., promotes content release at pH values below the isoelectric point of the protein. Calcium removal from native and partially reduced αLA gradually changes the pH dependence of their membrane perturbing activity. In contrast to what was found for the holo-conformers, the apo-proteins are able to permeabilize lipid vesicles at pH values around (apo-native) and above (apo-3SS$_{cam}$) their isolectric points. The good correlation found between content leakage and protein binding, as measured by centrifugation, indicates that the former provides reliable information on the binding of each αLA conformation to model membranes (Figure 2).

Considering the reported pK_a values for PG in the absence and presence of Ca^{2+} (i.e., 2.3−3.1) (van Dijck *et al*., 1978; Schendel & Cramer, 1994), the surface charge of the membranes remains constant within the pH interval used in this study. Therefore, the possible effect of the ionic state of PG on the leakage measurements is negligible. It is important to note that neither αLA nor its partially reduced conformers induce content leakage from neutral liposomes, suggesting that protein-induced perturbation of the hydrophobic phospholipid barrier requires anionic phospholipids (data not shown).

Structural Properties of the Different Conformers in Solution. (1) Circular Dichroism and Differential Scanning Calorimetry. To provide a structural ground for the above-mentioned differences in affinity, the pH dependence of the ellipticity at 222 and 275 nm and the thermal stability of the different αLA conformers in solution have been measured as a function of pH. The data in Figure 3, corresponding to the holo-native protein, indicate: (1) release of aqueous contents is only significant below pH 5.0 (Figure 3A); (2) the onset of permeabilizing activity coincides with a modest decrease (20%) of the ellipticity value at 275 nm, while that at 222 nm remains virtually unchanged (Figure 3B); and (3) leakage is accompanied by an overall destabilization of the protein, as indicated by an abrupt decrease of both temper-

Table 1: Lipid:Protein Molar Ratios at Which Different αLA Conformers Produce the Onset, 50%, and 100% of Leakage from LUV (EYL:DOPG, 1:1 Molar Ratio)[a]

	pH 4.5			pH 5.0			pH 5.5		
	onset	50%	100%	onset	50%	100%	onset	50%	100%
holo-native	5.8×10^3	1.7×10^3	2.9×10^2	5.8×10^2	nd	nd	3.6×10^1	nd	nd
holo-3SS$_{cam}$	5.9×10^3	3.2×10^3	5.9×10^2	5.9×10^2	nd	nd	7.3×10^1	nd	nd
apo-native	1.4×10^4	4.9×10^3	1.4×10^3	5.8×10^3	1.8×10^2	2.4×10^1	5.8×10^2	nd	nd
apo-3SS$_{cam}$	3.0×10^4	4.9×10^3	1.9×10^3	5.9×10^3	1.5×10^3	2.9×10^2	1.5×10^3	2.4×10^1	nd

[a] Data derived from experiments as shown in Figure 1. See the corresponding figure legend for experimental details. nd: not detected.

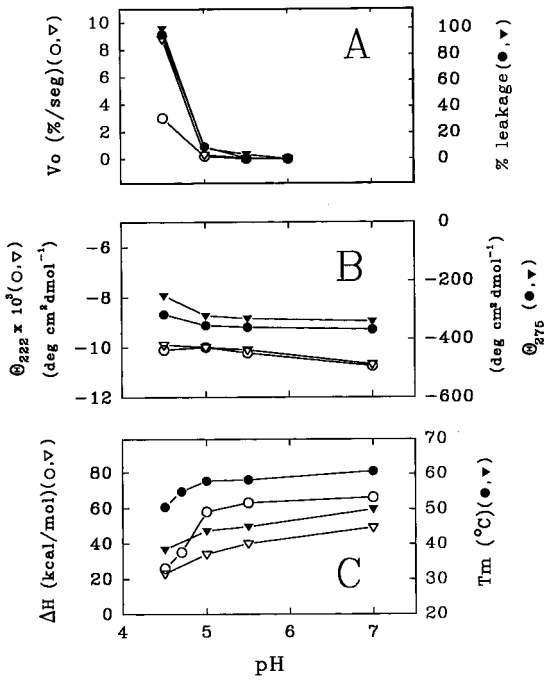

FIGURE 3: Comparison of the permeabilizing activity, the near- and far-UV CD amplitude, and the thermal stability of holo-αLA and holo-3SS$_{cam}$ as a function of pH. (A) Initial rate of ANTS/DPX leakage from LUV (EYL:DOPG, 1:1 molar ratio) (open symbols) and extent of content release (closed symbols) 7 min after the addition of holo-αLA (○) and holo-3SS$_{cam}$ (▽); other experimental details as in Figure 2. (B) Ellipticity values at 222 nm (open symbols) and 275 nm (closed symbols) for holo-αLA (○) and holo-3SS$_{cam}$ (▽) in solution. At least 10 measurements in 3 separate experiments were averaged for each data point. Estimated error was less than 8%. (C) pH dependence of the calorimetric enthalpy (open symbols) and temperature (closed symbols) of thermal denaturation of holo-αLA (○) and holo-3SS$_{cam}$ (▽). Each data point represents the average of three independent measurements. The experimental error was less than 10% for ΔH_{cal} and ±1 °C for T_m.

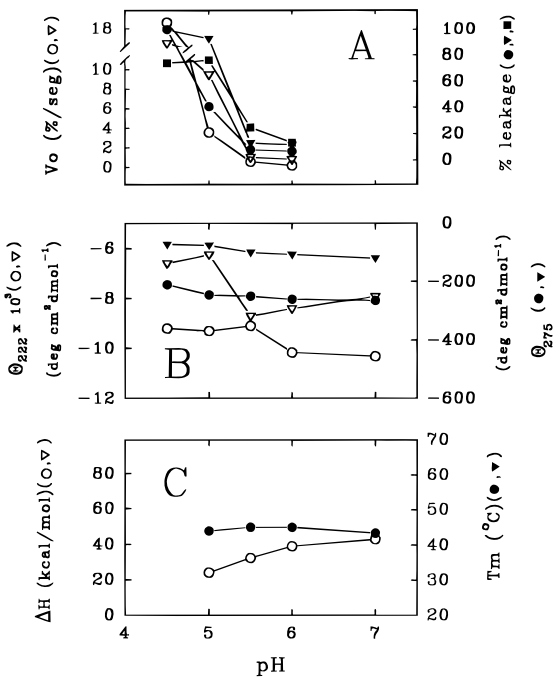

FIGURE 4: Comparison of the permeabilizing activity, the near- and far-UV CD amplitude, and the thermal stability of apo-αLA and apo-3SS$_{cam}$ as a function of pH. (A) Initial rate of leakage (open symbols) and extent of content release (closed symbols) induced by apo-αLA (○), apo-3SS$_{cam}$ (▽), and R$_{cam}$ (□). (B) Ellipticity values at 222 nm (open symbols) and 275 nm (closed symbols) for apo-αLA (○) and apo-3SS$_{cam}$ (▽) in solution. (C) Calorimetric enthalpy (open symbols) and temperature (closed symbols) of thermal denaturation of apo-αLA. Other experimental details as in Figure 3.

ature and enthalpy of denaturation below pH 5.0 (Figure 3C). A similar picture is observed for the partially reduced holoprotein (Figure 3). Differences between these conformers in the pH range studied include: (1) the initial rate of leakage at pH 4.5 is 2-fold higher for holo-3SS$_{cam}$ (Figure 3A), indicating that either this conformer undergoes the conformational change necessary for membrane association faster or it forms larger pores than its native counterpart; and (2) the values obtained for T_m and ΔH_{cal} along the pH interval 7.0−5.0 are significantly lower than those of the native holoprotein, suggesting that destabilization of the partially reduced protein was caused at least in part by enthalpic factors (Figure 3C).

As stated before, calcium removal from native αLA allows a significant interaction of the protein with lipid vesicles at pH 5.0 (Figure 4A). The ability to permeabilize lipid vesicles at this pH is accompanied by a decrease of the ellipticity values at 275 and 222 nm (Figure 4B) and of ΔH_{cal} (Figure 4C) in solution, as compared with the holo-conformers. Estimation of T_m and ΔH_{cal} at pH 4.5 was prevented by base line distortions, probably due to protein aggregation at posttransition temperatures. Inspection of the pH dependence of the permeabilizing activity of apo-3SS$_{cam}$ shows that this conformer is able to induce content leakage at pH 5.5, e.g., above the isoelectric point of the protein (Figures 1 and 4A). Under these conditions, the amplitude of the far- and near-UV CD spectra reveals that the amount of helical structure is considerably lower and the flexibility of the local environment of the aromatic side chains is greater than in the other conformers (Figure 4B). The calorimetric transition of apo-3SS$_{cam}$ was undetectable, indicating that the cumulative effect of cation removal and partial reduction destabilizes the protein structure to such an extent that the cooperative melting behavior is abolished. The drop of the ellipticity value at 222 nm observed for the apo-conformers below pH 5.5 might be due, at least in part, to protein aggregation (see IR data), and therefore should be analyzed only qualitatively. The pH-dependent stimulation of the membrane-permeabilizing activity detected for all the conformers shows a different profile for R$_{cam}$, which is partially inhibited at pH 4.5 (Figure 4A).

(2) Infrared Spectroscopy. Infrared spectra of proteins are dominated by the amide I band, which appears between 1700 and 1600 cm^{-1}. Specific secondary structures within proteins are associated with particular hydrogen-bonding patterns, which give rise to characteristic amide I bands (Surewicz & Mantsch, 1988). The deconvoluted spectra of the holo-conformers in the amide I region show several component bands at neutral pH, whose assignment has been carried out as previously reported (Figure 5; Prestrelski *et al*., 1991; Urbanova *et al*., 1991). The band at 1652 cm^{-1} is characteristic of helical structures, and that at 1640 cm^{-1} represents fully hydrated, extended chains connecting different types of secondary structure elements. Less intense components at around 1675 and 1630 cm^{-1} indicate the presence of turns and β-structure, respectively. Lowering the pH to values at which the interaction of holo-native αLA with membranes is favored induces the following changes in its amide I band

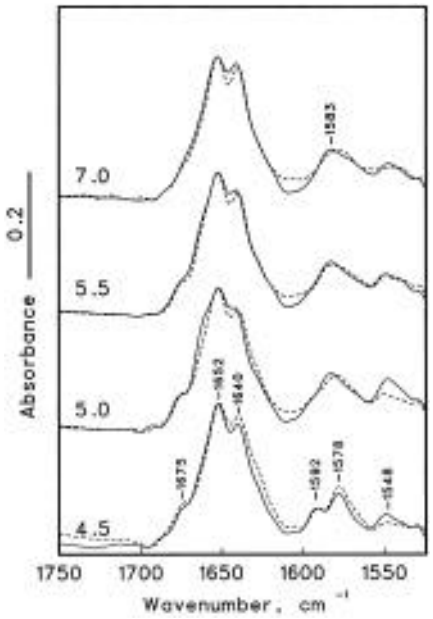

FIGURE 5: Deconvoluted infrared spectra of holo-native (solid traces) and holo-3SS$_{cam}$ αLA (broken traces) as a function of pH. Deconvolution was performed using a Lorentzian with half-bandwidth of 18 cm^{-1} and a band-narrowing factor of 2.

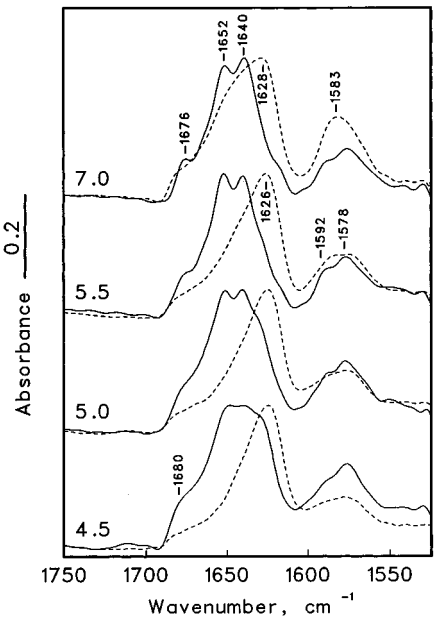

FIGURE 6: Deconvoluted infrared spectra corresponding to the apo-native (solid traces) and apo-3SS$_{cam}$ αLA (broken traces) at four representative pH values. Deconvolution was performed as described in Figure 5.

(Figure 5): (1) a decrease in the intensity of the 1640 cm^{-1} component relative to that at 1652 cm^{-1}; and (2) the appearance of a component band at around 1662 cm^{-1} at pH 5.0, which has been assigned to 3_{10} helices or turns (Kennedy *et al*., 1991; Byler & Susi, 1986). The amide I band of holo-3SS$_{cam}$ is similar, along the pH range studied, to that of the holo-native protein, except for the component band at 1662 cm^{-1} which is not detectable in the spectrum of the partially reduced protein at pH 5.0 (Figure 5). Therefore, the pH-induced conformational transition to a competent state, able to interact with lipid bilayers, involves local rearrangements of flexible connecting loops and helical segments of the protein rather than extensive alterations of its secondary structure. Another important pH-dependent difference observed in the infrared spectra of the holo-conformers concerns the bands located in the 1565−1595 cm^{-1} spectral region, coming from the antisymmetric COO$^-$ stretching mode of the aspartic and glutamic residues (Venyaminov & Kalnin, 1990). Above pH 5.0, the deconvoluted spectra display an asymmetric band with a maximum at around 1583 cm^{-1} which splits into two components at pH 4.5 (Figure 5). These components, located at around 1577 and 1590 cm^{-1}, are distinguishable in the spectra of apo-αLA at all pH values analyzed (Figure 6), indicating that cation removal modifies the coordination of at least part of the COO$^-$ groups of acidic amino acids, as expected from a protein whose calcium binding site is formed, among other chemical groups, by the side chain carboxylate of three, highly conserved, aspartate residues (Nara *et al.*, 1994).

The effect of calcium removal on the secondary structure of native and partially reduced αLA is illustrated in Figure 6. The main components of the amide I band at 1652 and 1640 cm^{-1} described above for the holo-native protein are preserved in the deconvoluted spectrum of apo-αLA at neutral pH. However, their relative intensities are altered, and the intensity of the residual amide II band at 1548 cm^{-1}, mainly due to unexchanged N−H groups, is strongly reduced (Figure 6). These findings are in agreement with previous results (Prestrelski *et al*., 1991) and are suggestive of a local unfolding of the protein helical segments and the overall loosening of its tertiary structure, which results in an increased access of solvent to the protein core. Calcium removal from 3SS$_{cam}$ induces gross protein conformational changes at neutral pH. First, the amount of α-helix is drastically reduced as reflected by the lower intensity of the band at 1652 cm^{-1}; and second, the protein self-associates as suggested by the presence of a strong band at 1628 cm^{-1} in the deconvoluted spectrum (Figure 6). Acidification of the medium promotes the progressive appearance of a band at 1628 cm^{-1} in the spectrum of apo-αLA below pH 5.5, indicating the gradual formation of intermolecular β structures as a consequence of protein aggregation (Figure 6; Muga *et al*., 1991, 1993b), and further decreases the helical content and increases the propensity to aggregate of the partially reduced apo-protein. It should be mentioned here that the relatively high protein concentration required for IR spectroscopy, as compared with CD, enhances the tendency of the apo-conformers to aggregate.

DISCUSSION

A general model for the membrane association of soluble proteins postulates that adoption of a mobile, highly dynamic conformation confers to the protein the ability to associate (Parker & Pattus, 1993). However, there are still some important questions that require a deeper understanding. The first one regards the nature of the driving force that governs the association reaction. Our results indicate that its nature is different for the various αLA conformations used in this study. The membrane-perturbing activity of the holo-conformers becomes significant only at pH 4.5 (i.e., below the isoelectric point of the protein), suggesting that electrostatics drive the initial interaction between the positively charged holo-proteins and the negative membrane surface. Moreover, the dependence of the kinetics of leakage on the amount of anionic lipid (data not shown) strengthens the idea that membrane association of these conformations is domi-

nated by electrostatic interactions. To explain the upward shift of the pH-dependent membrane destabilization across the isoelectric point of the protein, observed for the apo and R_{cam} conformers, one has to consider a driving force other than electrostatics, since the overall net charge of the different conformers is similar within the experimental pH interval. Exposure of apolar patches at the protein surface could facilitate hydrophobic interactions between these partially folded protein conformations and lipid vesicles, in order to overcome the unfavorable electrostatic repulsion. The fact that the ability of the apo-conformers to permeabilize lipid vesicles at pH values around and above the isoelectric point of the protein correlates well with their tendency to self-associate in solution supports this interpretation (see Figures 4, 5, and 6). An increased propensity to aggregate has also been described for partially folded states of many proteins, including αLA (Arakawa *et al*., 1987; Goto & Fink, 1989; Ewbank & Creighton, 1993b; Artigues *et al*., 1994). An efficient way for these relatively unstable conformations to hide hydrophobic residues and to avoid aggregation would be to associate with membranes. These observations indicate that intermediates that are believed to populate the folding pathway of αLA (Ewbank & Creighton, 1993b) exhibit different membrane association properties from the native protein as a consequence of the different driving forces involved in their association, which, in turn, might be determined by their conformational properties in solution.

The above finding leads to the second question which concerns the extent of protein unfolding necessary to become competent in membrane association. The possibility of a flexible intermediate, in which elements of secondary structure remain largely conserved in spite of tertiary structure changes, functioning in protein insertion and translocation was proposed by Bychova *et al*. (1988) and described for a number of systems (Cramer *et al*., 1990; van der Goot *et al*., 1991; London, 1992; Muga *et al*., 1993a). We can now define in greater detail the structural properties of this state. Our results demonstrate that a conformational transition to a somehow flexible structure in solution confers to the protein its membrane-destabilizing activity (see Figures 3 and 4). However, the fact that the amplitude of the near-UV CD spectra of the holo-native and holo-$3SS_{cam}$ conformers at pH 4.5 in solution is 80% and 70% of that at neutral pH, respectively, suggests that their native tertiary structure is largely retained. This is expected since the p*K* of the native to molten globule state transition is around 3.0 (Griko *et al*., 1994; Kuwajima, 1989), and challenges the hypothesis according to which the flexible molten globule state is an intermediate on the pathway to protein membrane insertion, as described for the colicin A channel polypeptide (van der Goot *et al*., 1991; Muga *et al*., 1993a; Schendel & Cramer, 1994). In this context, calorimetric data are essential as they demonstrate that substantial destabilization of the holo-conformers in solution occurs prior to their association with membranes. The electrostatic interactions, which drive the association reaction of these conformers with membranes, could cause further loosening of these partially destabilized structures as they encounter the surface of the membrane. This is confirmed by IR and CD studies which show that, at pH 4.5, the lipid-bound holo-conformers have a similar molten globule-like tertiary structure (Bañuelos & Muga, 1995). Formation of new electrostatic bonding, as a consequence of lipid−protein interaction, is generally an exothermic process that could provide the energy necessary for further protein unfolding which would trigger partial membrane insertion of the holo-conformers (Ramsay *et al*., 1986; Myers *et al*., 1987; Epand *et al*., 1990). Anionic phospholipid-induced conformational destabilization has been demonstrated for cytochrome *c* (Muga *et al*., 1991), human complement protein C9 (Lohner & Esser, 1991), and the pore-forming domain of colicin A (Muga *et al*., 1993a) and E1 (Zhang & Cramer, 1992). The fact that the secondary structure of the calcium-bound protein conformations is essentially maintained at pH 4.5 indicates that major rearrangements of this structural level are not essential for the association to occur.

The requirement of an optimum electrostatic interaction for membrane binding gradually disappears as the protein secondary structure and essentially tertiary structure progressively unfold in solution. This is obvious for the apo-$3SS_{cam}$ and R_{cam} conformers, and may reflect a less demanding energetic requirement for the initial unfolding. Apo-native αLA shows an intermediate behavior between the holo- and apo-$3SS_{cam}$ conformers regarding both conformation and pH-dependent membrane association. Interestingly, the calorimetric enthalpy of this conformer at pH 5.0 is similar to those of the holo-conformers at pH 4.5, suggesting that protein destabilization below a threshold value (≈28 kcal/mol), which occurs at different pHs depending on the conformational state, may facilitate the structural transition necessary for membrane association. It is worthwhile to mention that the structures of the membrane-bound native and partially reduced holo- and apo-conformers are remarkably similar, and thus adoption of a different conformation in the membrane can be ruled out as responsible for the observed differences in permeabilizing activity (Bañuelos & Muga, 1995). These differences rather reflect the specific pH-dependent structural properties of each conformer in solution.

Acidic conditions stimulate the membrane-permeabilizing activity of all conformers , except for R_{cam} which is partially inhibited at pH 4.5 (see Figure 4A). This particular behavior could be explained considering that at neutral pH R_{cam} possesses around 40% of the secondary structure of the native protein, the remaining parts of the molecule adopting an extended, unfolded conformation (Ewbank & Creighton, 1993a). The pH-dependent aggregation of the unfolded segments of R_{cam} in solution would compete with proper protein insertion into the bilayer. The presence of protein aggregates at the membrane surface, corroborated by IR spectroscopy (unpublished results), reveals a different way of protein binding to lipid bilayers which does not lead to membrane destabilization. Furthermore, leakage experiments show that preincubation of R_{cam} under conditions that promote protein aggregation (i.e., pH 4.5) considerably decrease the extent of content release (data not shown). In contrast, the effect of lowering the pH on the holo- and apo-native conformers is mainly to increase the proportion of protein molecules adopting the molten globular, flexible state (Ikeguchi *et al*., 1992; Griko *et al*., 1994; Figures 3 and 4).

Taken together, these observations suggest that the αLA conformations used in this study are especially efficient in destabilizing membranes under experimental conditions that favor the population of their flexible, molten globule-like conformation. Moreover, it is also shown that an ensemble of flexible conformations with a variable and considerable

amount of the secondary structure of the native state are able to interact with lipid bilayers (Figures 3, 4, 5 and 6). The similarity between the protein conformational requirements for αLA binding to the chaperonin GroEL (Hayer-Hartl *et al*., 1994) and to negatively charged membranes raises the intriguing possibility that these flexible conformations might play an important role in a variety of cell processes. Our findings may also be significant with respect to the membrane association of many water-soluble proteins and toxins, and suggest that some events required for the association could be explained, as pointed out by Fischer and Schmid (1990), by *in vitro* studies of the folding pathway, structure, and stability of these proteins.

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