Binding of Molten Globule-like Conformations to Lipid Bilayers

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The effect of membrane binding on the structure and stability of several conformers of α-lactalbumin was studied by infrared spectroscopy, circular dichroism, and fluorescence spectroscopy. In solution, under experimental conditions where all conformers interact with negatively charged membranes, they show significant conformational differences. However, binding to negatively charged membranes, which causes considerable changes in the structure of these conformers, leads to a remarkably similar protein conformation. The membrane-associated conformations are characterized by 1) a high helical content, greater than any of those found in solution, 2) a lack of stable tertiary structure, and 3) the disappearance of their thermotropic transition. These observations indicate that association with negatively charged membranes induces a conformational change within α-lactalbumin to a flexible, molten globule-like state.

There is a large class of proteins (and peptides) which, although soluble in water, during the course of their action interact with plasma or intracellular membranes (1). The transition from the water-soluble to the membrane-associated state is believed to involve large structural changes (1–4). The nature of this conformational change is a central issue to understand the problem of protein folding in membrane environments. In this context, the important role of lipid-protein interactions in protein translocation across biological membranes and protein insertion into lipid vesicles has been recognized both in vivo and in vitro (5–7). The use of phospholipid biosynthetic mutants in Escherichia coli has revealed that anionic lipids are essential for efficient membrane insertion and translocation of newly synthesized proteins through both the "Sec"-dependent and -independent pathways (8). Investigations on simple defined model membranes have shown that anionic lipids are required for membrane insertion and translocation of proteins (9–11). Changes in protein structure, facilitated by interaction with negatively charged phospholipids, allows membrane insertion and subsequent translocation of proteins (8–12). The experimental difficulties encountered in conformational studies of membrane-associated proteins have led to the present lack of knowledge on the structural transition involved in such association processes.

To further examine the effect of membrane binding on protein structure we focus in this study on the interaction of native and several folding intermediates of α-lactalbumin (αLA) with model membranes. αLA is able to interact with lipid bilayers (13, 14) and provides a suitable model to characterize the structural transition involved in membrane association of water-soluble proteins, since its conformational properties have been extensively studied (15, 16). Moreover, the possibility of trapping stable reduction intermediates of this protein offers the opportunity to follow the conformational changes associated with binding of different folding intermediates to membranes (16, 17). As experimental techniques we use infrared (IR) spectroscopy, circular dichroism (CD), and fluorescence spectroscopy. The complementary information provided by these biophysical methods reveals that binding to negatively charged membranes induces a different structural change in the αLA conformers which leads to a very similar, membrane-bound flexible conformation.

EXPERIMENTAL PROCEDURES

Materials

Egg yolk lecithin (grade I) was purchased from Lipid Products (South Nutfield, United Kingdom). 1,2-Dioleoylphosphatidylglycerol, α-lactalbumin (type I, calcium-containing and type III, calcium-free) and deuterium oxide (99.8% purity, D_2O) from Sigma.

Methods

Selective reduction and carboxamidomethylation of the disulfide bond between cysteine 6 and 120 (3SS cam) or all disulfides (R cam) of αLA was achieved according to Shechter et al. (17). Protein concentration was determined spectrophotometrically as described previously (16). The freeze-dried proteins were dissolved in 20 mM NaHPO_4, 100 mM NaCl, pH 7.0, and the pH of the solution was adjusted to 4.5 with citric acid. In addition to the above composition, the holo- and apobuffers contained 1 mM Ca^{2+} and 1 mM EDTA, respectively. Large unilamellar vesicles (LUV) were prepared according to the extrusion method of Høst et al. (18), using polycarbonate membranes of a pore size of 0.1 μm (Nucleopore, Inc., Pleasanton, CA). For preparation of samples used to study the conformation of αLA and its derivatives bound to lipid vesicles, LUV suspensions and protein solutions were mixed at a starting lipid to protein molar ratio of 300 in the above buffer. After adjusting the pH, as described above, the samples were incubated at room temperature for 30 min.

IR Spectroscopy—Prior to infrared measurements, the lipid-protein complexes were collected by centrifugation (120,000 × g, 2 h). Exchange of water by D_2O was carried out by submitting the samples to three centrifugation-resuspension cycles in D_2O buffers of identical composition to that of the original H_2O media. Samples, at a protein concentration of ~0.7 mg/mL, were assembled between two calcium fluoride windows separated by a 50-μm-thick Teflon spacer. Infrared spectra were recorded at 25 °C in a Nicolet 520 spectrometer. A total of 200 scans (sample) and 200 scans (background) 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. During data acquisition, temperature was monitored with a thermocouple in contact with the

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The abbreviations used are: αLA, α-lactalbumin; 3SS cam, α-lactalbumin with the Cys6-Cys120 disulfide bond reduced and blocked with iodoacetamide; R cam, fully reduced and carboxamidomethylated α-lactalbumin; LUV, large unilamellar vesicles.
Fig. 1. Original (A) and deconvoluted (B) infrared spectra of αLA and its derivatives in solution (---) and in the presence of PC:PG (1:1) LUV at a lipid to protein molar ratio of 300:1 (-----). Trace 1, holo-αLA; trace 2, apo-αLA; trace 3, holo-3SS\textsubscript{amn}; trace 4, apo-3SS\textsubscript{amn}. The spectra were recorded in D\textsubscript{2}O media (20 mM Na\textsubscript{2}PO\textsubscript{4}, 100 mM NaCl (pD 4.5)) containing 1 mM Ca\textsuperscript{2+} (holobuffer) or 1 mM EDTA (apobuffer) at 25°C. Protein concentration was 0.7 mM. Deconvolution was performed using a Lorentzian with half-bandwidth of 18 cm\textsuperscript{-1} and a band-narrowing factor of 2.

\[ \text{degree cm}^2/(\text{mol cm}) \times 10^2 \]

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mal unfolding can be easily followed by plotting the amide I bandwidth versus temperature. The “melting curves” obtained for the apo- and holo-native proteins indicate that both undergo thermotropic transitions in solution (Fig. 2). In contrast, there is no evidence of a cooperative transition for the same membrane-associated conformers. Instead, the width of their amide I band follows a small, progressive temperature-induced increase similar to that obtained for their acid compact intermediate (Fig. 2). Identical results were obtained for the 35Scam conformers (data not shown).

To further explore the molten globule-like thermal stability of the membrane-bound conformers, we have compared their IR spectra with those of their corresponding acid compact intermediates (Fig. 3). Note that the terms molten globule and compact intermediate are used interchangeably. The deconvoluted IR spectra of the acid molten globule states with different conformational flexibility, as recently proposed by Redfield et al. (27). Remarkably, none of the above mentioned differences are seen between the membrane-bound conformers.

CD Spectroscopy—The far-UV CD spectrum of holo-αLA in solution is characteristic of a largely α-helical structure, in accordance with previous results (16, 28), displaying strong minima at 208 and 222 nm (Fig. 4, trace 1). The ellipticity value at 222 nm of holo-35Scam indicates that partial reduction of the Cys6-Cys120 disulfide bond slightly reduces the helical content of the protein (Fig. 4, trace 3). Removal of calcium from the native and selectively reduced protein results in a further and substantial reduction of the CD signal at 222 nm, respectively (Fig. 4, traces 2 and 4). In contrast, the far-UV spectra of the membrane-bound conformers look alike, showing ellipticity values higher than any of those measured in solution (Fig. 4, traces 5–8).

The CD spectrum in the near-UV region of native holo-αLA in solution shows a pronounced negative ellipticity at around 270 nm and a positive peak at 293 nm which have been assigned to Tyr and Trp residues, respectively (Fig. 5, trace 1) (29). This spectrum is characteristic of a native-like conformation with a fixed orientation of the αLA’s 12 aromatic residues, which are spread throughout the protein molecule (26). Inspection of the spectra corresponding to the other conformers indicates that the environment of the aromatic side chains becomes less rigid in the following order: holo-αLA > holo-35Scam > apo-αLA > apo-35Scam (Fig. 5, traces 1–4). Interestingly, the
spectra of the four conformers associated with negatively charged LUV are similar, lacking the fine structure characteristic of the native structure in solution (Fig. 5, traces 5–8). The general shape of these spectra resembles that of the "collapse" solution spectrum at acidic pH (15).

Fluorescence Spectroscopy—The intrinsic fluorescence of αLA reflects mainly the environments of its four tryptophan residues, which are spaced evenly throughout its sequence (30). We have determined the parameters \( \lambda_{\text{max}} \) and relative fluorescence emission for αLA and its derivatives in solution and associated with negatively charged LUV. In solution, the spectrum of the native, calcium-bound conformation is the most blue-shifted (Fig. 6A, trace 1), while the \( \lambda_{\text{max}} \) of apo-3SS_{cam} appears at the longest wavelength (Fig. 6B, trace 2). Calcium removal from native αLA (Fig. 6B, trace 1) and selective reduction of the holoprotein (Fig. 6A, trace 1) induces a red shift of \( \sim 2 \) nm. The fluorescence intensity of these conformers is comparable, except for apo-3SS_{cam} which is higher (Table I). A red shift of the fluorescence maximum reflects a change of the Trp residues to a more polar environment, while the increase in intensity indicates the disappearance of tertiary interactions that quench the fluorescence in the native state. Binding of these conformers to negatively charged liposomes results in protein conformations with similar fluorescence properties: emission maxima at around 335 nm and fluorescence intensities ranging from 45 to 55 (Fig. 6B, traces 5–8). Spectra were recorded at pH 4.5 in the absence (traces 1 and 2) and presence of PC:PG (1:1) LUV at a lipid to protein molar ratio of 300:1 (traces 3 and 4), and in solution at pH 2.5 (traces 5 and 6). Protein concentration was 1.1 μM.

![Fig. 4. Far-UV CD spectra of the different αLA conformers in solution (traces 1-4) and bound to PC:PG (1:1) LUV at a lipid to protein molar ratio of 300:1 (traces 5-8).](http://www.jbc.org/)

![Fig. 5. Near-UV CD spectra of native αLA (traces 1 and 2) and its 3SS_{cam} derivative (traces 3 and 4) in solution (traces 1-4) and bound to negatively charged vesicles (traces 5-8).](http://www.jbc.org/)

![Fig. 6. Effect of negatively charged membranes on the tryptophan fluorescence of αLA and its derivatives.](http://www.jbc.org/)

It should be mentioned that fluorescence intensity determinations in the presence of lipid vesicles are not as accurate as in solution, due to light scattering. After pH neutralization, the previously membrane-associated protein almost completely regains the fluorescence properties of the free conformer, indicating that the interaction is largely reversible (data not shown). A comparison between the fluorescence properties of the membrane-bound conformers and their acid compact intermediate states reveals that the \( \lambda_{\text{max}} \) of the later are red-shifted, while their relative fluorescence values are significantly lower (Fig. 6, traces 5 and 6; Table I).
Protein Conformation at Membrane Interfaces

Effect of membrane binding on the intrinsic fluorescence parameters of αLA and its trapped disulfide derivatives

The buffer was 20 mM Na₂PO₄, 100 mM NaCl (pH 4.5), and the excitation wavelength was 295 nm, a.u., arbitrary units.

| Conformation | Solution | Membrane-bound | Acid state
|--------------|----------|----------------|-------------
|               | h<sub>max</sub> | I<sub>r</sub> | h<sub>max</sub> | I<sub>r</sub> | h<sub>max</sub> | I<sub>r</sub> |
| Holo-αLA      | 326      | 22            | 335          | 45          | 340          | 24           |
| Apo-αLA       | 322      | 22            | 336          | 56          | 340          | 31           |
| Holo-3SS<sub>cam</sub> | 287      | 21            | 336          | 46          | 340          | 31           |
| Apo-3SS<sub>cam</sub> | 340      | 26            | 336          | 49          | 340          | 31           |
| R<sub>cam</sub> | 338      | 54            | 335          | 55          | 340          | 31           |

<sup>a</sup>The pH of the above buffer was adjusted to 2.5.

<sup>b</sup>Identical results were obtained at pH 5.0 for the membrane-bound form.

DISCUSSION

The results of this study demonstrate that the membrane-bound conformation of different αLA intermediates are remarkably similar, in spite of showing different structural properties in solution. The protein conformational changes induced on membrane binding may be summarized as follows.

Protonation of Aspartate and Glutamate Residues of the Protein—After an initial binding step, driven by electrostatic and/or hydrophobic interactions, the more acidic interfacial pH would mediate neutralization of acidic residues of αLA and thus facilitate subsequent membrane partial insertion of the protein. In particular, protonation of the sidechain carboxylates of three aspartates which participate in calcium binding could cause cation removal from the protein and the conformational transition to a state competent in membrane binding. The ability of the different αLA conformers to partially insert into negatively charged membranes is confirmed first by leakage experiments that show a pH-dependent release of encapsulated contents from LUV in the presence of the protein<sup>2</sup> and second by an increased protection of the membrane-bound proteins against solvent exchange (see “Results”). Our data provide a direct experimental observation of this process that has been postulated for several proteins, specially bacterial toxins, which in vitro penetrate into membranes upon exposure to acidic pH (14, 31).

Increase of the α-Helical Content of the Protein—The increased helicity of the membrane-associated conformers indicates that upon membrane insertion, relatively unstructured and/or flexible segments of the protein adopt a helical conformation (see Figs. 1 and 4). This is specially pronounced for the apoconformers, which have a less ordered structure and are prone to aggregation in solution. A similar folding behavior has been described for the interaction of apocytochrome c with negatively charged membranes (10, 32). Apocytochrome c latches ordered secondary structure in solution and becomes partly α-helical upon interacting with anionic lipids.

Loosening of the Protein Tertiary Structure—The lack of a stable tertiary structure in the membrane-bound states is evidenced by the collapse of the near-UV CD spectra (Fig. 5), the loss of the interactions responsible for the cooperative thermal unfolding (Fig. 2) and the increase of the relative fluorescence intensity (Fig. 6). This membrane-associated “unfoldase” activity becomes more efficient as the protein conformation in solution is more stable. Formation of less stable folding intermediates at the interface of anionic phospholipid vesicles has also been described for cytochrome c (22, 33), human complement protein C9 (34), and the thermolytic fragment of colicin A (12).

Taken together, these results indicate that negatively charged membranes bind a reduced number of similar highly helical and flexible protein conformations, which share structural properties with the acid molten globule state and the GroEL-bound protein (35). The fact that the conformations of the membrane-bound and acid compact intermediate states of αLA and its derivatives are not identical supports the view of the molten globule state not as a single conformation but rather as a family of more or less “ordered” conformers (27).

The membrane-bound, highly dynamic competent state, distinct from the native or aggregated state, could facilitate protein insertion and/or protein translocation across membranes, assembly of membrane protein complexes, or proper interaction of the protein with the transport machinery for its subsequent translocation. This would be consistent with the observation that protein translocation requires partial unfolding of its mature part (36). Negatively charged membranes could play a complementary role to that exerted by the variety of proteins which are directly or indirectly involved in protein translocation (37), as it has been proposed recently for the mitochondrial protein import (36).

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