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Interactions of chicken liver basic fatty acid-binding protein with lipid membranes

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Abstract

The interactions of chicken liver basic fatty acid-binding protein (Lb-FABP) with large unilamellar vesicles (LUVs) of palmitoyloleoyl phosphatidylcholine (POPC) and palmitoyloleoyl phosphatidylglycerol (POPG) were studied by binding assays, Fourier transform infrared (FT-IR) spectroscopy, monolayers at air–water interface, and low-angle X-ray diffraction. Lb-FABP binds to POPG LUVs at low ionic strength but not at 0.1 M NaCl. The infrared (IR) spectra of the POPG membrane-bound protein showed a decrease of the band corresponding to β-structures as compared to the protein in solution. In addition, a cooperative decrease of the β-edge band above 70 °C in solution was also evident, while the transition was less cooperative and took place at lower temperature for the POPG membrane-bound protein. Low- and wide-angle X-ray diffraction experiments with lipid multilayers indicate that binding of the protein produces a rearrangement of the membrane structure, increasing the interlamellar spacing and decreasing the compactness of the lipids.

Keywords: Chicken liver basic fatty acid-binding protein; Lipid membrane; FT-IR; Monolayer; Low-angle X-ray diffraction

1. Introduction

The fatty acid-binding proteins (FABPs) are a large family of low molecular weight proteins (14–15 kDa) that share structural homology and the ability to bind fatty acids and other nonpolar ligands. The common structural feature is a β-barrel of 10 antiparallel β-strands arranged in two orthogonal β-sheets enclosing a large inner cavity that accommodates the nonpolar ligand. The proteins of this family have been isolated from a wide variety of eukaryotic tissues and are believed to be involved in the uptake, transport and targeting of fatty acids and/or other nonpolar compounds (for a review see Banaszak et al. [1] and references therein). Two mechanisms have been described for the transfer of fatty acids from the FABPs to the lipid membrane. The FABP from rat liver, for example, transfers the fatty acid to the membrane by a diffusion-controlled mechanism: the fatty acid is released into the aqueous medium and reaches the membrane by diffusion [2] whereas in the case of rat heart FABP, the ligand is released after direct collision of the protein with the membrane [3]. These mechanisms do not seem to be exclusive for a particular protein: If the affinity for the ligand is high, FABP from rat liver does not exchange the fatty acid by a diffusional mechanism but the collisional process is observed instead [4]. For both mechanisms, it is relevant to understand the interactions that the protein can establish with the lipid membrane.

In this work we describe the interactions of chicken liver basic FABP (Lb-FABP) with lipid membranes. The protein has a particularly high isoelectric point of 9.0 [5] and binds fatty acids with an equilibrium constant of $K_d = 10^{-6}$ M [6]. Its amino acid sequence is known [7], and the 2.7-Å crystal structure [8] showed that it presents the canonical 10 stranded β-barrel and the two α-helices resembling a lid over the internal cavity.

Abbreviations: Lb-FABP, chicken liver basic fatty acid-binding protein; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; FT-IR, Fourier transform infrared; IR, infrared; LUV, large unilamellar vesicle

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Using molecular exclusion chromatography, filtration assays, and penetration into lipid monolayers at liquid–air interface, we have found that Lb-FABP interacts preferentially with the anionic lipid phosphatidylglycerol as compared with the zwitterionic phosphatidylcholine. By Fourier transform infrared spectroscopy (FT-IR), we have observed changes in the conformation and thermal stability of the bound protein as compared with the protein in solution. Low- and wide-angle X-ray diffraction indicated changes in the membrane organisation upon protein binding.

2. Materials and methods

2.1. Materials

Basic Lb-FABP was purified according to Scapin et al. [5] and stored in aqueous solution at −20 °C. Palmitoyloleoyl phosphatidylcholine (POPC) and palmitoyloleoyl phosphatidylglycerol (POPG) were obtained from Avanti Polar Lipids (Alabaster, AL). D_2O 99+%, NaOD, DCl, NaCl, Tris (tris(hydroximethyl)aminomethane) and Sephacryl S-300-HR where from Sigma (St. Louis, MO). Centricon 100 concentrators were from Amicon (Beverly, MA).

2.2. Large unilamellar vesicle (LUV) preparation

LUVs were prepared by freeze–thaw and extrusion through polycarbonate filters (pore diameter 100 nm) [9] in an extrusion device from Avestin (Ottawa, Canada). The method produced vesicles of 93 nm in diameter according to dynamic light scattering measurements.

2.3. Gel-filtration binding assay

A Sephacryl S-300-HR column, 5-ml bed volume, was equilibrated with a buffer at the desired ionic strength. Once the column was equilibrated, 100 μl of a sample containing pure FABP, LUVs or a mixture of LUVs, and protein was loaded. The flow rate was set at 0.125 ml/min. The UV absorbance of the column output was continuously monitored. The protein distribution coefficient, K_d, was calculated according to $K_d=(V_e-V_o)/V_s$, where V_e is the protein elution volume, V_o is the void volume, and V_s is the volume of the stationary phase.

2.4. Centricon-filtration binding assay

Samples containing pure protein and lipid–protein mixtures were loaded in the upper chamber of Centricon 100 concentrators (Amicon) and spun down at 3000 rpm at room temperature until 60–70% of the initial volume was eluted. The protein concentration in the initial sample and in the eluted fraction was quantified by measuring the absorbance at 280 nm.

2.5. Penetration of Lb-FABP into lipid monolayers

Monolayers of POPC or POPG were prepared with a monofilmmeter (Mayer Feintechnik, Göttingen, Germany) in which the original circular trough was replaced by another that is specially designed, teflon-coated, and has several compartments of different surface areas and volumes, connected through narrow and shallow slits (5-mm wide × 5-mm long × 1-mm deep). The signal corresponding to the surface area and the surface pressure (platinized Pt foil 12.5-mm wide × 20-mm long × 0.025-mm thick) were fed into a double channel X-Y-Y recorder (Yokogawa Corp., Japan). Before each experiment, the trough was wiped clean and rinsed with 70% ethanol and twice with double distilled water. After spreading 10–20 μl of the lipid solution in chloroform/methanol 2:1, 5 min was allowed for solvent evaporation and the films were compressed to the desired initial surface pressure which was maintained constant. The desired amount of protein was injected into the subphase solution containing 10 mM Tris–HCl buffer or 50 mM Tris–HCl plus 0.1 M NaCl buffer under continuous magnetic stirring at 25 °C.

2.6. FT-IR spectroscopy and sample preparation

Spectra were recorded in a Nicolet Nexus spectrometer using a thermostatted demountable cell for liquid samples with CaF_2 windows and 100-μm teflon spacers. The spectrometer was flushed with dry nitrogen to reduce water vapour distortions of the spectra. Normally, 100 scans were collected both for the background and the sample at a nominal resolution of 2 cm^{-1}.

For sample preparation, about 1-mg lipid dissolved in chloroform/methanol solution was dried as a thin film in a glass tube. The film was hydrated and resuspended with 20 μl of a solution containing 200 μg of protein in D_2O. The final lipid to protein molar ratio was about 100:1. The protein was first lyophilized from an aqueous solution, dissolved in the appropriate buffer in D_2O, and incubated 24 h at room temperature to allow deuterium exchange of the amide protons.

2.7. X-ray scattering

Lipids were transferred as lyophilized powder into 1-mm glass capillaries. Tris–HCl buffer pH = 7.4 (either 10 mM Tris–HCl or 50 mM Tris–HCl plus 0.1 M NaCl) was added to reach about 40% lipid concentration (w/v). The capillaries were then flame-sealed and centrifuged five times, reversing the orientation each time to favour homogenization. The capillaries were exposed to X-rays at room temperature at a capillary to detector distance of 120 mm for 2 h. The Rigaku RU-200 rotating anode generator was run at 50 kV and 160 mA. The Cu Kα radiation was selected with a graphite crystal monochromator and a 0.5-mm-diameter collimator was used for the exposures. Still frames

were recorded using a Rigaku R-Axis II-C image plate area detector.

3. Results

3.1. Binding of Lb-FABP to LUVs

LUVs of POPC or POPG are excluded from a Sephacryl S-300-HR column whereas pure Lb-FABP is retained instead with a K_d of about 0.5. When the column was loaded with Lb-FABP mixed with POPC LUVs at high or low ionic strength or POPG at high ionic strength, the protein eluted with the same K_d as the pure protein and it was not detected in the lipid fraction. When the sample contained POPG LUVs in a low ionic strength buffer, no Lb-FABP was detected in the elution volume corresponding to the pure protein but it was found instead in the lipid fraction. These observations indicate that Lb-FABP was bound to the anionic lipid at low ionic strength and it was not bound to POPC or to POPG at high ionic strength although it can not be ruled out that some weak binding to these interfaces may occur.

As a further control in a sample in which the protein was bound to POPG, the ionic strength was increased to 0.1 M by adding a concentrated NaCl solution, and the sample was chromatographed through the size exclusion column. More than 95% of the protein eluted as free protein and only a small proportion was found in the lipid fraction, indicating that the binding was reversible and that the protein was released from the membrane by high salt concentration. This behaviour is indicative of peripheral binding, driven by electrostatic forces, without deep penetration of the protein into the hydrophobic core of the membrane. The importance of electrostatic interactions to drive the binding to the membrane has also been demonstrated for several other FABPs [4,10–12].

The binding to LUVs was also studied using a filtration assay. The upper chamber of Centricon 100 concentrators was loaded with samples containing Lb-FABP, pure or in the presence of LUVs. Lb-FABP can freely pass through the membranes of Centricon 100 concentrators, while the LUVs are retained. After centrifugation, the protein concentration measured in the eluant is equal to the concentration of free protein in equilibrium with the lipid-bound protein, irrespective of the amount of solution eluted. When the protein was incubated with POPC LUVs both at low and high ionic strength or POPG LUVs at high ionic strength, the protein concentration in the eluant was more than 80% of that obtained when the upper chamber was loaded with pure protein in the absence of lipids. In addition, a sample was tested in which the protein was incubated with POPG initially at low ionic strength and subsequently taken to high ionic strength and the same result was obtained. Only when the protein was incubated with POPG LUVs at low ionic strength no protein was detected in the eluant, indicating that it was bound to the anionic lipid. These results confirm those obtained by size exclusion chromatography.

3.2. FT-IR spectroscopy

The infrared (IR) absorbance spectra of Lb-FABP in the absence and in the presence of POPG is shown in Fig. 1. The amide I′ band, between 1600 and 1700 cm^{-1}, is mainly due to the C=O stretching of the peptide bonds and provides information on the secondary structure of the protein [13,14]. The spectral shape is similar to that observed for other FABPs like human liver and heart FABPs [15] and for adipocyte FABP [10], and it is typical of a

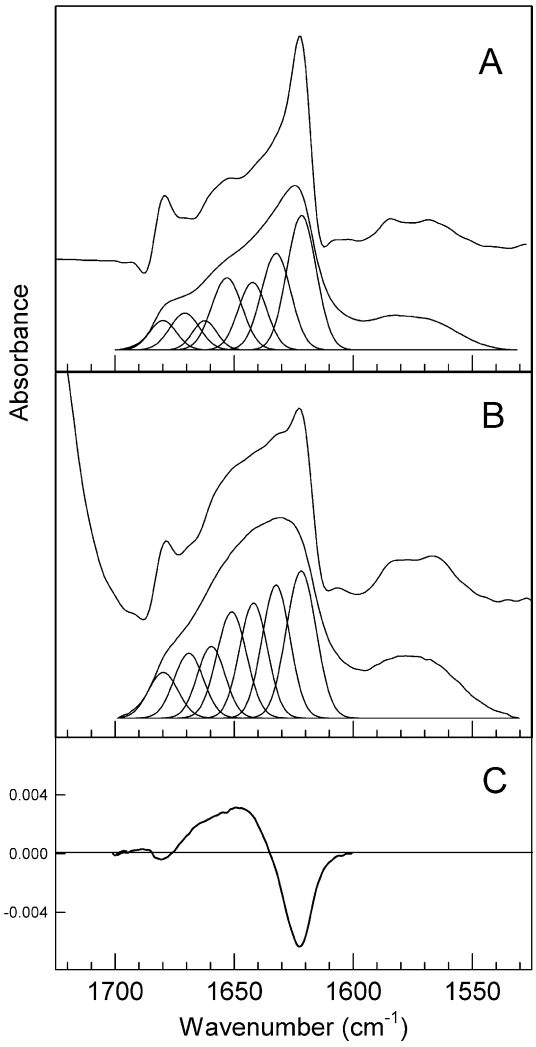

Fig. 1. FT-IR absorbance spectra of chicken Lb-FABP. Panel A: pure Lb-FABP in solution; panel B: Lb-FABP bound to POPG membranes; panel C: difference spectrum obtained by subtracting a normalized spectrum of the free protein from a normalized spectrum of the membrane-bound protein. Upper trace in panel A and B are deconvolved spectra using $K=2$ and FWHH = 18 cm^{-1}; middle trace in each panel is non-deconvolved spectra. Gaussian curves below the middle traces are the spectral components obtained by curve fitting. The positions of the components are displayed in Table 1. The total lipid to protein molar ratio was 88 to 1. Samples also contained 10 mM Tris in D_2O p*D* 7.4.

Table 1
Band position (cm^{-1}) and percentage area (%) of the amide I′ components of Lb-FABP in solution and bound to POPG membranes

Pure protein		Protein bound to POPG	
Band position (cm^{-1})	Percentage area (%)	Band position (cm^{-1})	Percentage area (%)
1622	29	1622	23
1632	21	1632	19
1642	14	1642	16
1653	16	1651	16
1662	5	1660	10
1671	8	1669	10
1680	6	1680	7

protein with a high content of β-sheet structure. Fourier deconvolution of the spectra [16], shown in Fig. 1, and the second derivative (not shown) allow identification of the different band components. Fitting of the components to the original (not deconvolved) spectrum was performed according to the procedure described by Arrondo and Goñi [17] except that peak height, band width, and peak position of the components were allowed to vary one at a time in this order. A Gaussian shape was assumed and the components due to amino acid side-chains (below 1600 cm^{-1}) were included in the fitting. The absorption band due to the lipid carbonyl groups was subtracted before the fitting procedure. For clarity, only the components corresponding to the amide I′ are shown in Fig. 1. When the original spectra of the pure protein in solution were smoothed or a lower line narrowing factor, K, was used for deconvolution, the band at 1632 cm^{-1} (see below) was not resolved. Nevertheless, if this band is not included for the fitting procedure, it is obviously missing and the flanking bands have to shift several cm^{-1} from the original position in order to obtain a good fit.

The proportion of the band components obtained by fitting must be taken with caution. Different mathematical results can be obtained by changing the initial conditions or the order in which the parameters are allowed to vary [17]. Our purpose was not to reveal the secondary structure of Lb-FABP, which is already well known from the crystal structure, but to investigate the conformational changes that occur upon binding to the membrane. To this end, we have applied exactly the same criteria to obtain the initial conditions (band positions, intensity, and width) and to perform the fitting, both for the protein in solution and bound to the membrane. In this way, the differences between the final results must actually reflect the difference between the structures.

In the absence of lipids, seven components were revealed (Fig. 1). Their positions and contributions to the area of the amide I′ band are shown in Table 1. A remarkable feature is the band at 1622 cm^{-1}. This component has been previously observed in concanavalin A [18], lipophilin [19], a peptide with hairpin structure [20], apo B-100 [21], and a lentil lectin [22]. It has been assigned to extended β-chains in which part of the backbone hydrogen bonds is not formed with another β-chain. This happens, for example, in the edges of a β-sheet and in the hairpin motif. In Lb-FABP the candidates that could be responsible for this band are chains E and F and the edge of chain D (see the structure in Ref. [8]). The other bands have been assigned according to the classical work of Byler and Susi [14]. The band centred at 1632 cm^{-1} corresponds to antiparallel β-chains. Unordered structures generate the band appearing at 1642 cm^{-1}. The band at 1653 cm^{-1} can be assigned to the α-helical structure present in Lb-FABP. Turns and bends, including β-turns, are considered responsible for the bands at 1662, 1671, and 1680 cm^{-1}. The bands at 1671 and 1680 cm^{-1} can also be assigned to extended β-structures. To a very good approximation, the areas of the component bands can be considered proportional to the amount of the corresponding secondary structure present in the protein [14]. The total area corresponding to β-bands (1622 and 1632 cm^{-1}) and α-helix (1653 cm^{-1}) sum up to 50% and 16%, respectively, of the amide I′ area. This is in good agreement with the number of residues involved in β-chains (58%) and α-helix (9%) observed for the native protein by X-ray crystallography [8]. The same spectral shape (see Fig. 2), band components and their proportions (not shown) were obtained for the pure protein in solution in high and low ionic strength (0.1 M NaCl or no salt added, respectively), in the presence of POPC, and in the presence of POPG in high ionic strength.

Under the experimental conditions of panel B in Fig. 1, i.e., in the presence of POPG at low ionic strength, Lb-FABP is bound to the anionic lipid membrane. The same bands were obtained for the bound protein and the protein in solution. The large change in the spectral shape, as compared with the protein in solution, is due to a significant change in the relative proportion of several bands: the areas of the bands

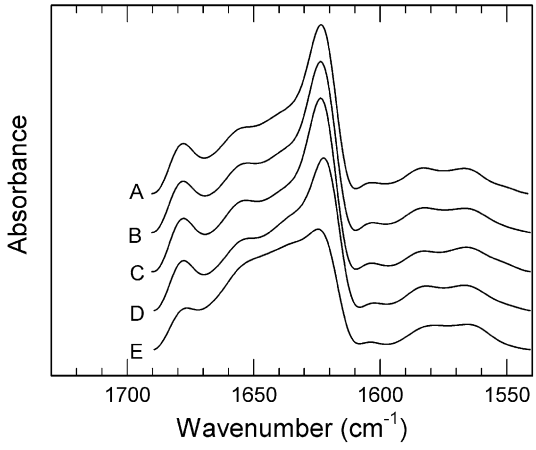

Fig. 2. Deconvolved FT-IR spectra of chicken Lb-FABP. (A) Pure in solution; (B) in the presence of POPG in a medium of high ionic strength; (C) in the presence of POPC in a medium of low ionic strength; (D) incubated with POPG at low ionic strength and subsequently taken to high ionic strength; (E) in the presence of POPG in a medium of low ionic strength. Medium of low or high ionic strength contained 10 mM Tris or 50 mM Tris, 0.1 M NaCl, respectively, in D_2O pD 7.4. Protein concentration was 6.6 mg/ml. The total molar lipid to protein ratio was 88 to 1. Deconvolution parameters were $K=2$, FWHH = 18 cm^{-1}.

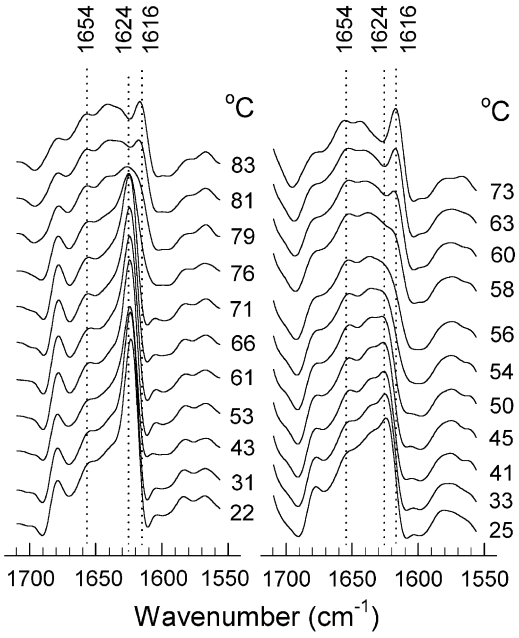

Fig. 3. Deconvolved FT-IR spectra of chicken Lb-FABP as a function of temperature. Left panel: pure protein in solution. Right panel: protein-bound to POPG. Medium contained 10 mM Tris D_2O p*D* 7.4. Protein concentration was 6.6 mg/ml. The total molar lipid to protein ratio was 88 to 1. Deconvolution parameters were $K=2$, FWHH = 18 cm^{-1}.

corresponding to β-edges (1622 cm^{-1}) and β-sheet (1532 cm^{-1}) are reduced from 29% and 21% in the free protein to 23% and 19% in the protein bound to POPG. This decrease is accompanied with a proportional increase in the bands corresponding to unordered structure and turns (see Table 1). This variation in the relative proportions of the bands is indicative of a structural change. Part of the β-components, most probably strands E and F and the β-edges, and part of the β-sheet have acquired a new structure now absorbing at 1642 and 1660 cm^{-1} upon binding to the membrane (see Table 1). On the basis of these observations, we can conclude that the conformational change involves the shift of extended β-structure to turns and unordered structures. A similar conclusion can be obtained from the difference spectrum shown in Fig. 1. After subtraction of the lipid bands, the area of the non-deconvolved spectra was normalized to 1 cm^{-1} between 1700 and 1600 cm^{-1} and the spectrum of the free protein was subtracted from the spectrum of the bound protein. The negative band centred at 1625 cm^{-1} reveals a decrease in the content of β-structures in the membrane-bound protein together with an increase in the bands between 1640 and 1670 cm^{-1} as evidenced by the positive band.

Because all the features of the native spectrum appear to be conserved in the bound protein, it must also be concluded that this conformational change does not correspond to a global unfolding and part of the native secondary structure is conserved in the lipid-bound protein.

The release of Lb-FABP bound to POPG membranes at low ionic strength upon increasing the salt concentration was also followed using FT-IR spectroscopy. Samples were prepared in the conditions of Fig. 1B and, after a 2-h incubation, with the protein bound to the membrane, the ionic strength was increased to 0.1 M by adding a concentrated NaCl solution in D_2O and the FT-IR spectra of the released Lb-FABP were acquired. Fig. 2 shows that the spectrum recovered to a large proportion the shape corresponding to the free protein in solution. This observation strongly supports the conclusion that the conformational change observed when Lb-FABP binds to POPG is reversible and that the protein recovers the native structure after being released from the membrane.

3.3. Thermal dependence of the FT-IR spectra

Fig. 3, left panel, shows the deconvolved spectra of Lb-FABP in solution as a function of the temperature. Between 20 and 76 °C the general shape is conserved. The main change is an increase in the band widths with temperature. Above 76 °C a drastic decrease in the band at 1622 cm^{-1} is observed together with the appearance of a new band at 1618 cm^{-1}. Bands below 1620 cm^{-1} have been attributed to intermolecular contacts and generally appear when unfolded chains are self-aggregated [23–25]. The superposition of normalised spectra reveals an isosbestic point at 1620 cm^{-1} (not shown), indicating an interconversion between the structures responsible for the bands at 1622 and 1618 cm^{-1}. We have taken the ratio between the absorbance at 1654 and 1624 cm^{-1} in the original (non-deconvolved) spectra as a measure of the disappearance of the band attributed to the β-edges. Fig. 4 suggests that the conformational change that leads to this spectral change is a cooperative process. This transition occurs above 76 °C. Because of technical limitations, we have not acquired spectra well above 80 °C and the plateau region at high temperatures was not reached. Consequently, it was not possible to determine the temperature midpoint of the

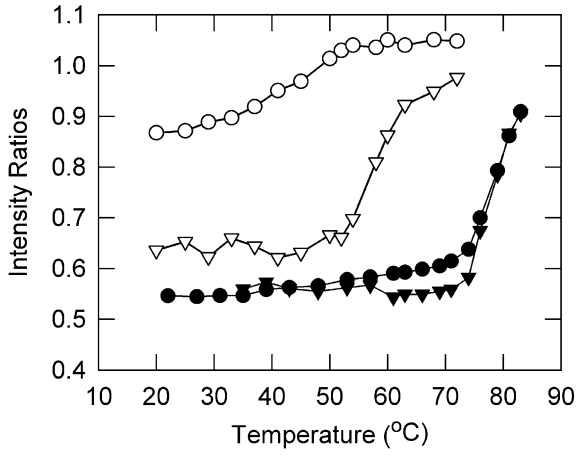

Fig. 4. Ratios of the IR absorbances at different wavenumbers as a function of the temperature. Circles, I_{1654}/I_{1624}; triangles, I_{1616}/I_{1624}; filled symbols are for the pure protein in solution. Open symbols are for the protein bound to POPG. Data were taken from the experiment in Fig. 3.

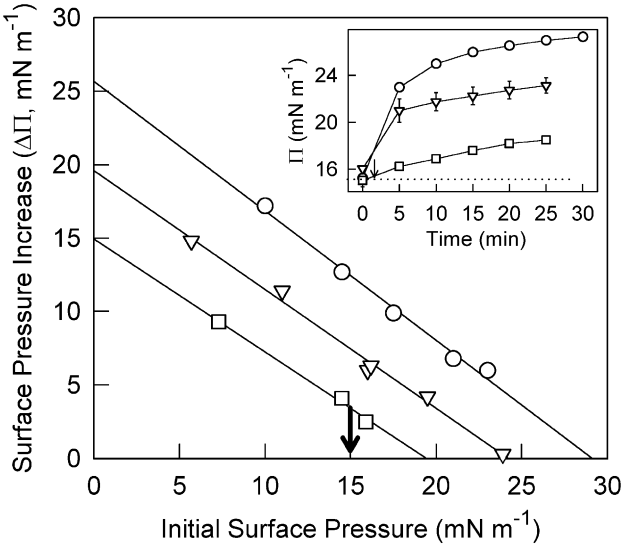

Fig. 5. Increase in the surface pressure of lipid monolayers induced by chicken Lb-FABP as a function of the initial surface pressure. Circles: monolayer of POPG in a subphase of low ionic strength. Triangles: monolayer of POPG on a subphase of high ionic strength. Squares: monolayer of POPC on a subphase of low ionic strength. The arrow indicates the maximal surface pressure reached by the pure protein (see text). The inset shows the time course of the surface pressure after the injection of chicken Lb-FABP under the monolayers at an initial surface pressure of 15 mN m^{-1}.

transition. For the protein bound to POPG (Fig. 3, right panel), the most noticeable changes with the increase in temperature were a decrease of the band at 1622 cm^{-1} and the appearance of the band due to self-aggregation at 1618 cm^{-1}. In this case, the disappearance of the band at 1623 cm^{-1} (I_{1654}/I_{1624} vs. temperature in Fig. 4) occurred as a much broader transition and at lower temperature than for the pure protein. Self aggregation, as revealed by the band at 1618 cm^{-1}, also took place at lower temperature for the bound protein (see I_{1616}/I_{1625} in Fig. 4).

The spectra obtained at high temperatures, where the band at 1622–1623 cm^{-1} has almost disappeared, still show some structure both for the pure protein in solution or bound to POPG. Broad bands at 1678, 1656, and 1644 cm^{-1} can be seen in the upper traces of Fig. 3. This indicates that Lb-FABP is not completely unfolded at high temperatures and some secondary structure elements are still present either in the free or in the membrane-bound protein.

3.4. Interaction of Lb-FABP with lipid monolayers

We have observed that Lb-FABP adsorbs to the air–water interface and reaches a maximal equilibrium surface pressure of 15 mN m^{-1} when it is injected into the subphase at a concentration of 337 nM. The inset in Fig. 5 shows the time course for the increase in surface pressure of monolayers of POPG and POPC at an initial pressure of 15 mN m^{-1} after injection of FABP in the subphase at a final concentration of 337 nM. The final pressure reached due to the insertion of the protein decreased in the order POPG at low ionic strength, POPG in a high ionic strength medium and POPC. Fig. 5 shows the increase in surface pressure ($\Delta\Pi$) of POPG and POPC monolayers (obtained in experiments as shown in the insert) as a function of the initial surface pressure of the pure lipid. The extrapolation to the abscissa axis ($\Delta\Pi = 0$ means no further increase in surface pressure after a protein injection in the subphase) gives the maximum initial pressure for the pure lipid at which the protein can interact with the monolayer [26]. In all cases, this "cut-off" value was above the pressure that the protein reaches by itself in a lipid-free interface, indicating a favourable interaction with the different lipids. The cutoff values obtained were 29, 24, and 19 mN m^{-1} for POPG at low ionic strength, POPG high ionic strength and POPC at low ionic strength, respectively. The conclusion is that Lb-FABP interacts with all the interfaces tested, the stronger interaction being with the POPG interface at low ionic strength.

3.5. X-ray diffraction

The small-angle X-ray diffraction patterns show rather broad intensity peaks that usually show splitting, which suggests the existence of more than one coexisting phase. Fig. 6 shows the intensities measured as a function of the

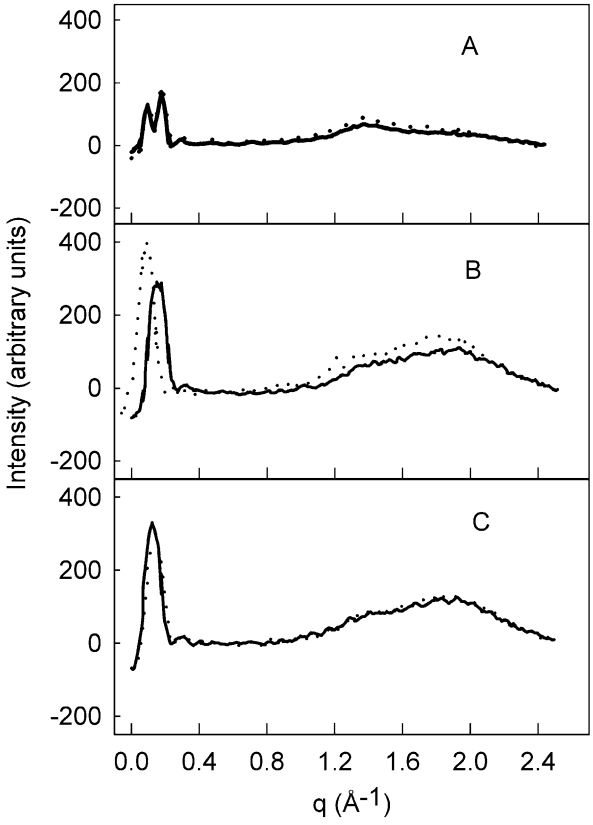

Fig. 6. Wide- and low-angle X-ray diffraction of LUVs. (A) Pure POPC in low ionic strength; (B) POPG in low ionic strength; (C) POPG in high ionic strength. Continuous lines correspond to pure lipid and dotted lines to the lipid in the presence of Lb-FABP.

scattering vector $q = 2\pi s = (4\pi/\lambda)\sin\theta$, where s is the reciprocal spacing, $s = 1/d$ with d as the lattice spacing, θ is half of the scattering angle, and λ the radiation wavelength. For pure POPC reflections in the small angle region correspond to lamellar spacings of 57.86 Å and a broad peak centred at 4.55 Å, characteristic of a disordered chain lattice [27] (the broadest reflection centred at about 3.31 Å occurs in all samples and corresponds to the pure buffer solution). No alteration of the spacings of POPC is caused by Lb-FABP, either in buffer of low or high ionic strength. On the other hand, pure POPG in 10 mM Tris buffer shows small-angle reflections corresponding to lamellar spacings of 57.28 Å and a wide-angle broad reflection at 4.76 Å; the spacings of the pure lipid in 0.1 M NaCl are similar. The interaction of Lb-FABP with POPG at low ionic strength induces a shift of the intensity pattern and increases the low-angle lamellar spacings to 69.12 Å while the chain lattice revealed at wide angle is centred at 5.21 Å. At higher ionic strength (0.1 M NaCl), the lamellar spacing of pure POPG in the presence of Lb-FABP is not altered. Thus, the X-ray studies are in keeping with the preferential interaction of the protein with POPG at relatively low ionic strength. They further indicate that the association of the protein with the lipid induces an increase of the interlamellar spacing and a distortion of the lateral lattice with more disordered, or widely separated, lipid hydrocarbon chains.

4. Discussion

Lb-FABP has an isoelectric point of 9 and, therefore, it is positively charged at pH 7. Its binding to lipid membranes takes place under conditions in which electrostatic interactions are favoured: a membrane composed of an anionic lipid and at low ionic strength. We have not observed an equally strong binding to LUVs made of the zwitterionic lipid POPC nor of the anionic POPG in a medium of relatively high ionic strength. We have observed that the protein can be released by increasing the salt concentration. It is generally accepted that this behaviour corresponds to a peripheral interaction with the membrane, driven only by electrostatic interactions. However, the formation of hydrophobic interactions of the protein with other membrane systems, in which nonpolar domains are more exposed as in the case of micelles or small unilamellar vesicles, cannot be ruled out.

The increase in surface pressure of lipid monolayers at the air–water interface is very sensitive to the interactions established with the molecules present in the subphase. Even when the binding to POPC LUVs is not measurable by separation techniques, it is clear that some interaction with the zwitterionic lipid, as revealed by the penetration in monolayers, may be present. These experiments have also shown that the interaction is favoured by electrostatic forces.

Deconvolution and curve fitting of the FT-IR spectra of the protein in solution revealed a secondary structure in agreement with that determined by X-ray crystallography. Binding to POPG produced a large change in the shape of the IR spectra due to the decrease in the bands that could be assigned to β-edges (chain D and the structural motif formed by the β-strands E and F) and antiparallel β-sheet, and an increase in the bands assigned to turns and unordered structures. Conformational changes have been proposed to be involved in the mechanism of fatty acid release [4]. It is possible that the conformational change observed in Lb-FABP is related to the fatty acid transfer mechanism.

Binding to the anionic membrane also produces a decrease in the thermal stability of Lb-FABP. At least part of the β-structure that remains in the lipid-bound protein unfolds at lower temperature than in the native soluble protein. Nevertheless, lacking direct calorimetric data, we cannot completely ascertain whether this corresponds to the global temperature-induced unfolding. Preliminary results (not shown in the present work) indicate that hydrogen–deuterium exchange, as measured by shifts in the amide I′ and amide II′, is faster in the membrane-bound Lb-FABP than in the native protein in solution. This is indicative of a less compact structure and it is in agreement with a lower denaturation temperature for the membrane-bound protein.

The concentration of H^+ ions near a negatively charged membrane is higher than in the bulk solution [28]. It is possible that changes in protein structure and stability are due to the lower pH in the membrane surface. Nevertheless, the changes in the lipid packing as revealed by the X-ray diffraction and penetration into the lipid monolayers suggest that a direct interaction with the lipids also occurs, which must also have influence on the protein structure and stability.

Several types of soluble proteins can bind and insert into lipid membranes with profound changes in their thermal stability and structure. Bacterial toxins, for example, are partially unfolded and lose their tertiary structure when they are bound to the membrane [29]. In spite of the general decrease in thermal stability, the secondary structure of the domains that insert into the hydrophobic membrane core is increased and results to be more stable as compared with the protein in aqueous solution [30]. Clearly, this is not the case for the stability of the secondary structure of Lb-FABP which seems to be decreased in the membrane-bound protein. This is consistent with the proposal that Lb-FABP establishes a peripheral interaction and does not penetrate into the membrane.

We conclude that the membrane-bound Lb-FABP undergoes a conformational change without losing its general secondary structure; it has lower thermal stability and apparently lower compactness as compared with the protein in solution. This partial unfolding does not lead to irreversible self-aggregation within the membrane environment, at low temperatures, and the native structure can be recovered after protein release from the membrane. It is possible that the partially folded state found in the membrane corresponds to a folding intermediate that could also be present

in solution. This is a point that deserves to be further explored. At present, no data are available to reveal the possible mechanisms of fatty acid transfer for Lb-FABP. Nevertheless, the ability to interact with anionic lipids and the properties that it acquires in the membrane are probably relevant to the physiological role of Lb-FABP.

Lb-FABP was set in a group apart from other FABPs because of its isoelectric point, fatty acid binding properties, and amino acid sequence [31]. Its binding to membranes and the conformational changes described here reflect also differences with other FABPs. In the case of rat liver FABP [4], for example, electrostatic interactions are required to drive binding to the membrane but once this interaction is established the protein cannot be released by high salt concentration, suggesting that electrostatics helps to establish further nonpolar interactions, most probably with the hydrophobic core of the membrane. To the best of our knowledge, the only FT-IR spectra of a membrane-bound FABP that can be compared with our data are those of the adipocyte FABP [10]. In this case the differences with Lb-FABP are noticeable: binding to an anionic membrane does not produce changes in the FT-IR spectra and thermal stability, measured by the changes in the FT-IR spectra.

Understanding the behaviour of Lb-FABP upon binding to membranes is also relevant to the problems of protein folding in the membrane environment. Lb-FABP behaves much like cytochrome *c*, which has been extensively used as a paradigm to study these problems [32,33]. It has been shown that the anionic membrane accelerates the passage from the native conformation to a folding intermediate which is also present in solution [34]. This kind of question cannot yet be answered for Lb-FABP because of the current lack of information on the existence of particular folding intermediates.

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