Binding of Recombinant Rat Liver Fatty Acid-Binding Protein to Small Anionic Phospholipid Vesicles Results in Ligand Release: A Model for Interfacial Binding and Fatty Acid Targeting†

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ABSTRACT: A number of intracellular proteins bind to negatively charged phospholipid membranes, and this interfacial binding results in a conformational change that modulates the activity of the protein. Using a fluorescent fatty acid analogue, 11-[5-(dimethylamino)naphthalenesulfonyl]undecanoic acid (DAUDA), it is possible to demonstrate the release of this ligand from recombinant rat liver FABP in the presence of phospholipid vesicles that contain a significant proportion of anionic phospholipids. The ligand release that is observed with anionic phospholipids is sensitive to the ionic strength of the assay conditions and the anionic charge density of the phospholipid at the interface, indicating that nonspecific electrostatic interactions play an important role in the process. The stoichiometric relationship between anionic phospholipid and liver FABP suggests that the liver FABP coats the surface of the phospholipid vesicle. The most likely explanation for ligand release is that interaction of FABP with an anionic membrane interface induces a rapid conformational change, resulting in a reduced affinity of DAUDA for the protein. The nature of this interaction involves both electrostatic and nonpolar interactions as maximal release of liver FABP from phospholipid vesicles with recovery of ligand binding cannot be achieved with high salt and requires the presence of a nonionic detergent. The precise interfacial mechanism that results in the rapid release of ligand from L-FABP remains to be determined, but studies with two mutants, F3W and F18W, suggest the possible involvement of the amino-terminal region of the protein in the process. The conformational change linked to interfacial binding of this protein could provide a mechanism for fatty acid targeting within the cell.

The primary function of lipid binding and transfer proteins is to deliver a nonpolar lipid from one membrane site to another. Where the lipid forms a stable aggregate in the cell membrane or lipoprotein, as is the case with phospholipids and cholesterol, the monomer concentration of the ligand within the aqueous phase is minimal. As a result, transfer of lipid from one membrane compartment to another compulsorily requires the docking of the transfer protein with the aggregate interface to allow uptake and subsequent release of the lipid.

In the case of proteins that bind and transport fatty acid, the ability of long chain fatty acids to rapidly equilibrate between membrane compartments via the aqueous phase reduces the requirement for protein binding to the membrane surface. It has been proposed that the rate of dissociation of normal long chain fatty acids from phospholipid bilayers is sufficiently rapid not to require a protein-mediated event (*1*). In this case, a soluble fatty acid-binding protein could then take up such an aqueous phase fatty acid. In contrast, the transfer of a long chain fatty acid from a complex with a binding protein back to a membrane environment may be less favorable, due to a high affinity of the binding protein for the long chain fatty acid. This transfer process may be facilitated by a collisional mechanism involving an interaction of the binding protein with the membrane interface that could involve a receptor (reviewed in ref *2*).

Fatty acid-binding proteins (FABPs)[1] belong to a family of structurally related small (14−15 kDa) cytosolic lipid-binding proteins that include liver, intestinal, heart (muscle), and adipocyte FABP (for recent reviews, see refs *2*−*4*). The main endogenous ligands are fatty acids with chain lengths of 16−22 carbons, although in the case of L-FABP other lipids such as acyl coenzyme A and lysophospholipids bind with lower affinity (*5*−*8*). The exact physiological role of these proteins is unclear, though it is generally thought that they may have a potential role in the uptake and targeting of fatty acids to various intracellular organelles and metabolic pathways (*2*−*4*). A process involving transitional membrane

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[1] Abbreviations: FABP, fatty acid-binding protein; L-FABP, liver fatty acid-binding protein; DAUDA, 11-[5-(dimethylamino)naphthalenesulfonyl]undecanoic acid; DOPC, dioleoylphosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; DOPA, dioleoylphosphatidic acid; DOPS, dioleoylphosphatidylserine; PI, phosphatidylinositol; dansyl DHPE, *N*-[5-(dimethylamino)naphthalene-1-sulfonyl]-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; cmc, critical micellar concentration; SUV, small unilamellar vesicle.

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binding has been advocated for muscle and adipose FABP where model fluorescence studies have led to the proposal of a collisional mechanism for explaining the FABP-mediated transfer of fatty acids between phospholipid membranes and vesicles (*9*−*16*). However, such a process was not observed for the mechanism of L-FABP which appeared to operate by an aqueous diffusion mechanism not requiring interaction of protein with membrane surfaces (*12*, *14*).

In this paper, we have investigated the ability of L-FABP to interact with phospholipid interfaces containing anionic phospholipids. We demonstrate that the interaction with small phospholipid vesicles results in a rapid and complete loss of ligand due to a presumptive conformational change on interfacial binding, and the molecular basis of the phenomenon is investigated. The process is an attractive model for studying interfacial binding and could provide a mechanism for fatty acid targeting within the cell.

MATERIALS AND METHODS

Materials. DAUDA and dansyl DHPE were obtained from Molecular Probes (Junction City, OR). Phospholipids were obtained from the following manufacturers: DOPS from Alexis Corp. (Nottingham, U.K.), DOPC from Lipid Products (Redhill, Surrey, U.K.), and DOPG, DOPA, and cardiolipin from Sigma Chemical Co. (Poole, Dorset, U.K.). The cardiolipin contained >80% polyunsaturated fatty acid as primarily linoleic acid, as per the supplier. DNA restriction enzymes were obtained from Promega Corp. (Madison, WI), and *pfu* DNA polymerase was from Stratagene Ltd. (Cambridge, U.K.). All other chemicals were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.).

Molecular Biology. A synthetic gene of rat liver L-FABP (*17*) was cloned into the pET-11a vector and transformed into competent BL21(DE3) cells using standard techniques (*18*). A *Nde*I site was mutated into the previously used plasmid (*17*) using the following sense oligonucleotide to introduce the *Nde*I site at the 5′-end of the insert (5′-CCTAGGAGGTGAGCTCATATGAACTTCTCTGG-3′), while the following oligonucleotide was used as a primer for amplification from the 3′-end of the insert (5′-AAAAAAAGCTTCTATTAGATACGTTTAGAAACACGT-3′) (Oswel DNA Service, Southampton, U.K.). PCR temperature cycling conditions were as follows. The samples were heated to 95 °C for 5 min before addition of native DNA *pfu* polymerase. Twenty-five cycles were used with the following temperatures: denaturation for 30 s (95 °C), annealing for 1.5 min (55 °C), and elongation for 2 min (68 °C). The resulting amplified DNA fragment was isolated from a 0.8% agarose gel using a DNA cleanup system (Promega Corp.). The purified DNA fragment and pET-11a plasmid (Novagen Inc., Madison, WI) were digested with the restriction enzymes *Nde*I and *Hin*dIII, extracted from a 0.8% agarose gel, and the gene fragment was ligated into the cut pET-11a vector. Following transformation into BL21-(DE3) cells (Novagen Inc.), several clones were examined by restriction digests, DNA sequencing, and induction with 1 mM IPTG. DNA sequencing was performed with DNA plasmids isolated using a QIAprep Spin miniprep kit (QIAGEN GmbH, Hilden, Germany) using a Thermo Sequenase fluorescently labeled primer cycle sequencing kit with 7-deaza-dGTP according to the manufacturer's instructions (Amersham Life Science, Buckinghamshire, England). The sequences were analyzed on a Li-Cor model 400 DNA sequencer (Lincoln, NE). The preparation of the tryptophan-containing mutants, F3W and F18W, has been described previously (*19*) except that the genes were cloned into the pET-11a vector, as described above for the wild-type expression system.

Proteins. Recombinant L-FABP was purified from sonicated bacterial suspensions by chromatography of a 60% ammonium sulfate suspension on (naphthoylaminodecyl)-agarose (*20*) and delipidated on Lipidex-1000 (*21*). The L-FABP purity was confirmed by SDS−PAGE, and the protein concentration was determined by the dye-binding assay of Bradford using bovine serum albumin as the standard. The Bradford assay overestimates the L-FABP concentration by 1.69-fold (*22*).

Fluorescence. All fluorescence measurements were performed on a Hitachi F-4500 fluorimeter using a 10 or 50 mM Hepes/NaOH (pH 7.5) buffer. DAUDA was made up in methanol ($\epsilon_{335} = 4400\ M^{-1}$) and fluorescence measured at an excitation wavelength of 335 nm and emission at 500 nm (slit width of 10 nm). Dansyl DHPE was made up in methanol ($\epsilon_{335} = 4500\ M^{-1}$), dissolved by sonication, and then mixed at 5 mol % with DOPG prior to vesicle preparation.

Phospholipids. Phospholipids were prepared by drying down chloroform suspensions in a desiccator and resuspension in methanol at concentrations of 10 or 2 mg/mL as appropriate. The phospholipids were added to the buffer solutions by methanol injection (*23*). Because DAUDA will partition into phospholipid vesicles with an increase in fluorescence, all titrations involving addition of phospholipid to a highly fluorescent FABP/DAUDA system were corrected for this by performing control titrations in the absence of FABP. Typically, the fluorescence enhancement of DAUDA/FABP relative to DAUDA in buffer was about 20-fold, while addition of DOPG or DOPC gave a linear increase in fluorescence with increasing phospholipid concentration over the range used (approximately 1.4- and 2.0-fold at 20 μg/mL DOPG and DOPC, respectively). The control titrations for other anionic phospholipids gave results very similar to those with DOPG. Titrations involving Triton X-100 were corrected for a control titration involving DAUDA/FABP treated with methanol (no phospholipid) followed by Triton X-100 to correct for the effect of Triton-X-100 on DAUDA binding to L-FABP.

HPLC. Gel filtration HPLC was performed on a 7.8 mm internal diameter × 30 cm TSK G2000SWXL column (Anachem, Luton, U.K.) equilibrated with 50 mM Hepes/NaOH (pH 7.5) at a flow rate of 1 mL/min. To L-FABP samples (50 μL) was added 2.5 μL of a 10 mg/mL phospholipid solution (DOPG or DOPC), and after incubation at room temperature for a few minutes, an aliquot (40 μL) was loaded onto the HPLC column with detection at 220 nm. Column fractions were analyzed for DAUDA binding by fluorescence and L-FABP using SDS−PAGE.

RESULTS

Anionic Phospholipids Cause Loss of Protein-Enhanced DAUDA Fluorescence. We have used a fluorescent fatty acid analogue, DAUDA, to study the binding and structural

properties of recombinant rat L-FABP (*24*−*26*). DAUDA binds to L-FABP with high affinity ($K_d \sim 0.2$ μM), and this is accompanied by a considerable enhancement of fluorescence and a blue shift in the fluorescence emission maximum, consistent with the probe being located in a nonpolar environment within this protein (*24*, *26*). The difference in fluorescence intensity of free and L-FABP-bound DAUDA is now the basis of a variety of fluorescence displacement enzyme assays that measure the amount of released fatty acid, particularly assays involving phospholipids and membranes as substrates (*27*−*29*). During the course of the investigations described here, we observed that addition of SUVs of the anionic phospholipid, DOPG, to the highly fluorescent complex of DAUDA and L-FABP, under conditions of low ionic strength, resulted in an immediate decrease in DAUDA fluorescence intensity to a value that was identical to that for DAUDA in buffer in the absence of L-FABP. This result, which was consistent with release of DAUDA from L-FABP, was not a problem using various higher-ionic strength assay conditions reported previously (*27*−*29*) and does not compromise such assays. In view of the considerable interest in the binding of proteins and peptides to anionic interfaces (see the Discussion), the molecular mechanisms responsible for the rapid loss of fluorescence were investigated.

The ability of DOPG to cause a loss of fluorescence is clearly seen in Figure 1A where DOPG is titrated into an assay containing the L-FABP−DAUDA complex. This figure shows an almost stoichiometric loss of fluorescence with added phospholipid, which is also dependent on the amount of L-FABP present (Figure 1A). This phenomenon is not seen when DOPC provides the phospholipid interface (Figure 1A). The stoichiometry of fluorescence quenching by phospholipid was linearly related to the total mass (micrograms) of protein (Figure 1B), and one explanation would be that the protein binds quantitatively to the phospholipid interface under these assay conditions. The molar ratio of DOPG to L-FABP at saturation is approximately 30 (calculated from Figure 1), and it is noteworthy that this ratio is similar to that obtained when the 14 kDa phospholipase A_2 coats the surface of anionic phospholipid vesicles (*30*), consistent with the 14 kDa L-FABP binding to the phospholipid surface in an equivalent manner.

The experiments described above involve titration of an anionic phospholipid such as DOPG into a fluorescent L-FABP−DAUDA complex. The same phenomenon is observed if the titration is reversed and the L-FABP−DAUDA complex is added to DOPG. As shown in Figure 2, whereas an expected linear increase in fluorescence is seen when the fluorescent complex is added to buffer or DOPC, when the L-FABP−DAUDA complex is added to vesicles containing DOPG a nonlinear response is observed in terms of increased fluorescence. This initial lack of fluorescence increase is consistent with the FABP binding to the phospholipid vesicles with loss of DAUDA until the FABP is in stoichiometric excess. The stoichiometry of this lack of response, i.e., 10 μg (12.5 nmol of DOPG) of phospholipid binds about 0.4 nmol of FABP, gives a DOPG:FABP ratio of about 30 and is very similar to that obtained when DOPG is titrated into a L-FABP−DAUDA complex (Figure 1).

Stopped-Flow Kinetic Analysis of Ligand Release Induced by DOPG. Stopped-flow analysis of DAUDA release from L-FABP upon addition of excess DOPG exhibits single-exponential kinetics with a pseudo-first-order rate constant of approximately 22 s^{-1} (Figure 3). This rate remained unchanged with the addition of either 10 and 20 μg/mL DOPG vesicles to L-FABP and DAUDA (22.2 $\pm$ 1.2 and 22.7 $\pm$ 0.8 s^{-1}, respectively), confirming that the DOPG was present in excess as expected from the stoichiometry demonstrated in Figure 1. This greatly enhanced rate involving binding to the anionic interface is consistent with displacement of DAUDA as a result of a significant change in protein conformation. Although DAUDA in not a fatty acid, the ability of DAUDA to be very rapidly displaced by long chain fatty acids in a variety of fluorescent displacement assays for lipases and phosphosplipase A_2 (*27*−*29*) strongly suggests that DAUDA is a good analogue of a normal long chain fatty acid in these systems.

Is There Anionic Headgroup Specificity for Promoting FABP Binding and Release of DAUDA? To investigate the

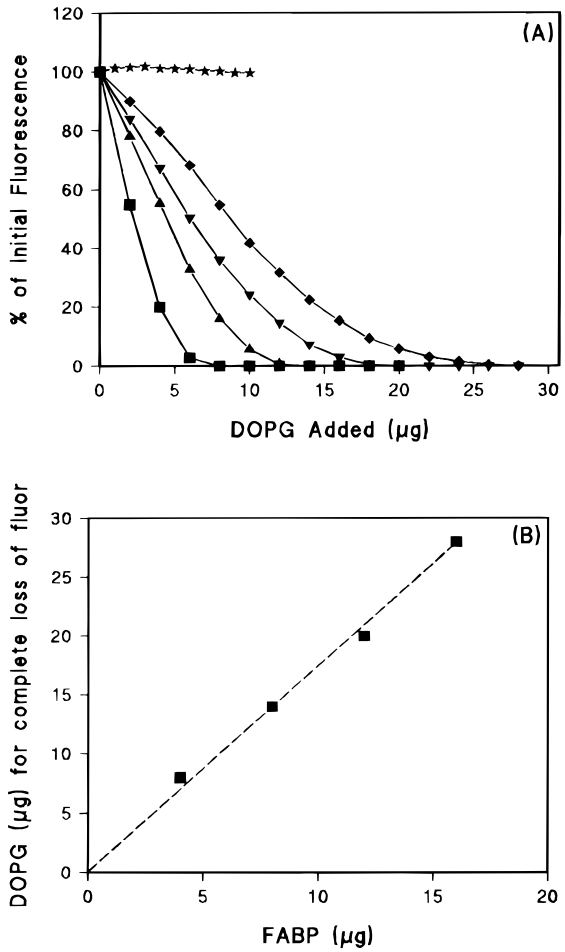

FIGURE 1: Dependence of L-FABP−DAUDA fluorescence on the concentration of DOPG. (A) To samples of L-FABP was added DOPG (10 mg/mL in methanol) by titration. Samples contained 4 (■), 8 (▲), 12 (▼), and 16 μg of L-FABP (◆). The data for the addition of DOPC to 4 μg of L-FABP−DAUDA are also shown (★). (B) A plot of the relationship between the amount of L-FABP (micrograms) and the amount of DOPG (micrograms) required for the complete loss of L-FABP−DAUDA fluorescence. The assay was carried out in 10 mM Hepes/NaOH buffer (pH 7.5), and 1 μM DAUDA was used in all cases. DAUDA fluorescence was measured as described in Materials and Methods. Control titrations were performed in the absence of FABP. The % initial fluorescence after each addition is corrected for the small increase in fluorescence as DAUDA partitions into the added phospholipid vesicle. All values are the means of titrations performed in triplicate.

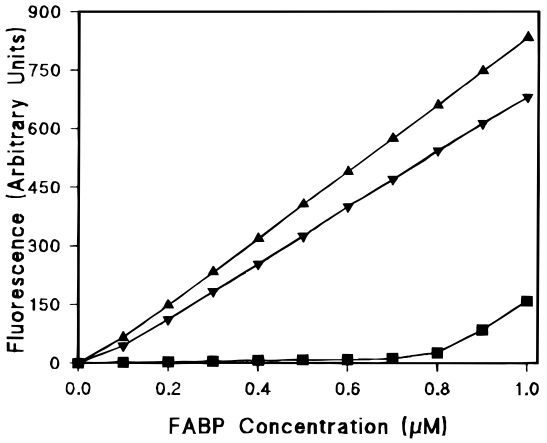

FIGURE 2: Effect of adding a fluorescent L-FABP−DAUDA complex to DOPG vesicles. L-FABP and DAUDA in a 0.6:1 molar ratio were added to 10 μg of DOPG and to controls of DOPC or buffer alone, and the increase in fluorescence was monitored: (■) DOPG, (▲) DOPC, and (▼) 10 mM Hepes buffer control. All values are the means of titrations performed in triplicate.

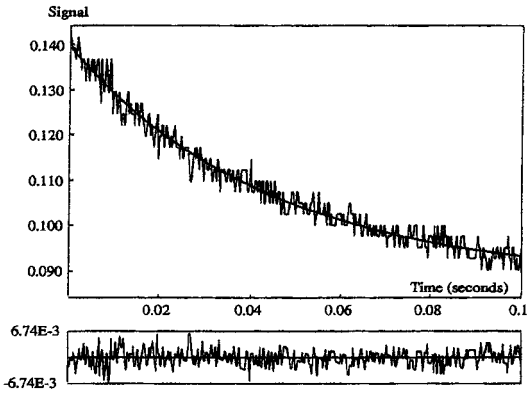

FIGURE 3: Stopped-flow analysis of the fluorescence decrease resulting from the addition of DOPG to L-FABP−DAUDA. Separate sample syringes contained 10 mM Hepes/NaOH (pH 7.5) with 16 μg of L-FABP and 2 μM DAUDA mixed together in one syringe with a second syringe containing the same buffer with 40 μg/mL DOPG. Fluorescence was measured at 18.5 °C using an Applied Photophysics SX17 stopped-flow spectrometer equipped with fluorescence detection with excitation set at 335 nm and a 490 nm cutoff filter used for the emission. The solid line is for a first-order decay process, and the residuals are shown in the lower trace.

molecular specificity of the phospholipid effect, a variety of phospholipids were prepared as vesicles by the solvent injection method (*23*). To minimize differences in gross physical properties of vesicles with different compositions, each anionic phospholipid was presented as a 20 mol % mixture with DOPC. The results of titrating such mixed vesicles into a highly fluorescent L-FABP−DAUDA complex are shown in Figure 4 and dramatically highlight the effectiveness of cardiolipin in causing loss of fluorescence from the L-FABP−DAUDA complex. In contrast, DOPG was somewhat less effective under these conditions, and similar decreases were seen using DOPS and PI. DOPA gave a curve very similar to that of DOPG (data not shown for clarity) when titrated in the presence of EGTA; however, the DOPA was consistently less effective than the other anionic phospholipids when titrated in the normal assay buffer containing residual calcium. This difference may reflect the higher sensitivity of PA vesicles to calcium. No loss of DAUDA fluorescence was seen by the addition of

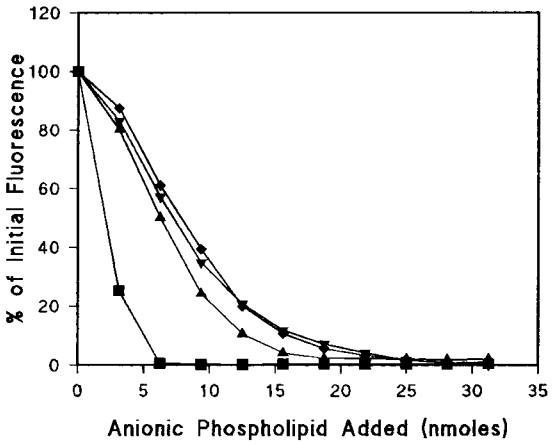

FIGURE 4: Phospholipid specificity of the fluorescence decrease seen with different phospholipids in the presence L-FABP−DAUDA. Phospholipids at a 20% molar ratio were mixed with DOPC (80 mol %) in methanol and injected into samples containing 10 mM Hepes/NaOH (pH 7.5) with 5 μg of L-FABP and 1 μM DAUDA. The data for anionic phospholipids, at 20 mol % relative to DOPC, are shown for DOPG (▲), cardiolipin (■), PI (◆), and DOPS (▼). DAUDA fluorescence was measured as described in Materials and Methods. All values are the means of three separate titrations, each from two separate preparations of phospholipid. Control titrations were performed for all phospholipid mixtures in the absence of FABP to correct for small increases in fluorescence as DAUDA partitions into the added phospholipid vesicles. All titrations were performed in triplicate, but error bars are not shown for clarity. The curves for DOPG, DOPS, and PI were not significantly different.

DOPE which will be essentially neutral at pH 7.4 or with the control of 100% DOPC. It should be noted that the effectiveness of cardiolipin (diphosphatidylglycerol) is consistent with its structure as it may be assumed to be equivalent to two molecules of DOPG in terms of charge density.

In all cases with the exception of cardiolipin and PI, the anionic phospholipid was presented as the dioleoyl derivative. Although PG is not an abundant anionic phospholipid in mammalian membranes, it is known to behave like PS in the binding of basic peptides (*31*) while it a less complex phospholipid than cardiolipin. For this reason, much of the work described in this paper was performed with DOPG which is a bilayer forming phospholipid that mixes well with DOPC.

Importance of Phospholipid Charge Density and Electrostatic Interactions in the Loss of L-FABP−DAUDA Fluorescence. When phospholipid vesicles were prepared from DOPG and DOPC mixed in various molar proportions, a threshold (sigmoidal) relationship was observed between increasing % DOPG and % fluorescence, with no effect at 10 mol % DOPG, suggesting that a minimum charge density was required for the type of interaction that results in ligand release (Figure 5). This sigmoidal relationship is characteristic of basic peptides and other proteins binding anionic vesicles as a result of multiple electrostatic interactions (*32*, *33*). When this experiment was repeated using cardiolipin, the system was more sensitive and a significant loss of fluorescence was seen at 10 mol % cardiolipin that was not seen at 10 mol % DOPG, consistent with the structure of cardiolipin. No effect was seen with cardiolipin at 5 mol % (Figure 5).

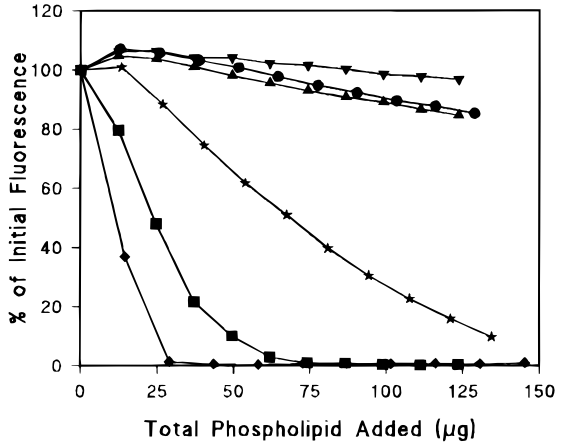

FIGURE 5: Dependence of L-FABP−DAUDA fluorescence on the DOPG:DOPC and cardiolipin:DOPC molar ratios. To samples of 1 μM DAUDA and 8 μg of L-FABP in 10 mM Hepes/NaOH buffer (pH 7.5) was added up to 145 μg of total phospholipid at various DOPG:DOPC or cardiolipin:DOPC ratios. The fluorescence intensity is plotted as the percentage of initial fluorescence and is corrected for control titrations without FABP: 20 mol % cardiolipin in DOPC (◆), 20 mol % DOPG in DOPC (■), 10 mol % cardiolipin in DOPC (★), 10 mol % DOPG in DOPC (▲), 5 mol % cardiolipin in DOPC (●), and 5 mol % DOPG in DOPC (▼). All values are the means from titrations performed in triplicate.

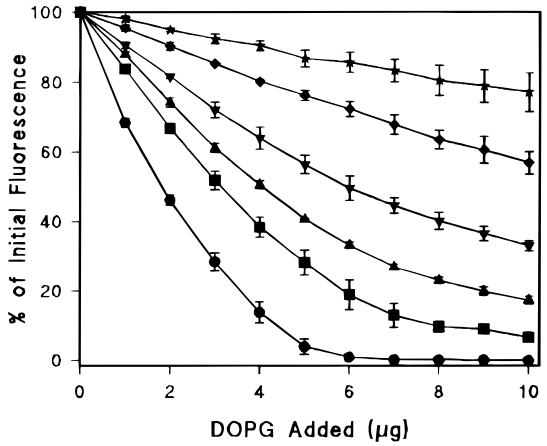

FIGURE 6: Effect of ionic strength on the ability of DOPG to cause a loss of L-FABP−DAUDA fluorescence. Samples contained the following buffers: (●) 10 mM Hepes/NaOH (pH 7.5) and (◆) 50 mM Hepes/NaOH (pH 7.5). The remaining curves used 50 mM Hepes/NaOH (pH 7.5) containing NaCl at the following concentrations: (▲) 20, (▼) 50, (◆) 100, and (★) 200 mM. The concentrations of DAUDA and L-FABP were 1 μM and 5 μg/mL, respectively, and DAUDA fluorescence was measured as described in Materials and Methods and in the legend of Figure 1. All values are the means from titrations performed in triplicate.

Since the loss of fluorescence was dependent on the concentration of DOPG in the membranes, and thus the presence of negative charge at the phospholipid interface, the role of potential electrostatic charge interactions was investigated by varying the ionic strength of the buffer. Increased NaCl concentrations had a strong effect in preventing the release of DAUDA from L-FABP by DOPG (Figure 6), indicating a major role for electrostatic interactions in the overall phenomenon. Though this interpretation could be complicated by the effect of high salt concentrations on electrostatic interactions between protein and DAUDA, we have shown that increased NaCl concentrations do not affect oleate displacement of DAUDA from L-FABP (*34*). Moreover, the binding of DAUDA to FABP under these assay conditions was not significantly affected by the presence of 200 mM NaCl. The K_d values for DAUDA were 0.20 ± 0.04 and 0.16 ± 0.03 μM in the absence and presence of 200 mM NaCl, respectively, while the corresponding values for maximum fluorescence (arbitary units) were 595 ± 44 and 486 ± 35.

It should be noted that Ca^{2+} provides a very effective counterion for the anionic phosphate of the phospholipid interface, and loss of fluorescence from the DAUDA−FABP complex by adding DOPG required only 5 mM Ca^{2+} for complete inhibition (data not shown). Overall, these results highlight the importance of electrostatic binding in the initial interaction of liver FABP with the anionic interface and a requirement for a minimum charge density consistent with multiple electrostatic interactions.

Mechanisms for Explaining the Loss of DAUDA Fluorescence. The most obvious, and physiologically interesting, explanation for the loss of DAUDA fluorescence is that binding of the protein to the anionic phospholipid interface induces a conformational change in the protein and release of DAUDA. Other explanations are also possible: (i) quenching of protein-bound DAUDA fluorescence as a result of the presence of the phospholipid, (ii) displacement of DAUDA by monomeric phospholipid binding to the FABP, and (iii) differential partitioning of DAUDA between proteins and membranes.

(i) Fluorescence quenching of DAUDA fluorescence by phospholipids is unlikely as it is hard to imagine a major structural change within the binding cavity of the protein that might cause quenching (e.g., change in polarity) but not release of DAUDA. (ii) A direct displacement by monomeric anionic phospholipids under conditions of low ionic strength is unlikely as (a) there is no evidence for phospholipids as ligands for L-FABP (*35*, *36*) and (b) the monomer concentration of the phospholipids that were used would be too low ($<10^{-9}$ M) to allow significant binding unless the K_d for phospholipid binding was similarly low ($<10^{-9}$ M), in which case the protein would be primarily a phospholipid binding protein. (iii) The differential partitioning of DAUDA between the lipid binding protein and the phospholipid vesicles will vary according to the nature and concentration of the phospholipid aggregate to produce a change in fluorescence (see Materials and Methods). All results are corrected for the effect of DAUDA partitioning into the phospholipid in the absence of FABP, and it should be noted that this partitioning is not significant in the presence of anionic phospholipids under these assay conditions.

Binding of L-FABP to DOPG: Indirect Fluorescence Evidence. A mechanism of L-FABP binding to phospholipid membranes resulting in release of ligand was therefore investigated in more detail. Interfacial binding was demonstrated by using phospholipid vesicles containing a polarity-sensitive phosphatidylethanolamine analogue labeled with a dansyl moiety at the headgroup (dansyl DHPE). When L-FABP was titrated into vesicles of DOPG containing 5 mol % dansyl DHPE, there was a saturable increase in fluorescence (Figure 7) indicative of interfacial binding by a peripheral membrane-binding protein (*37*). A similar experiment using vesicles prepared from DOPC containing 5 mol % dansyl DHPE did not show a fluorescence increase, even when the protein concentration was increased to 70 μg/

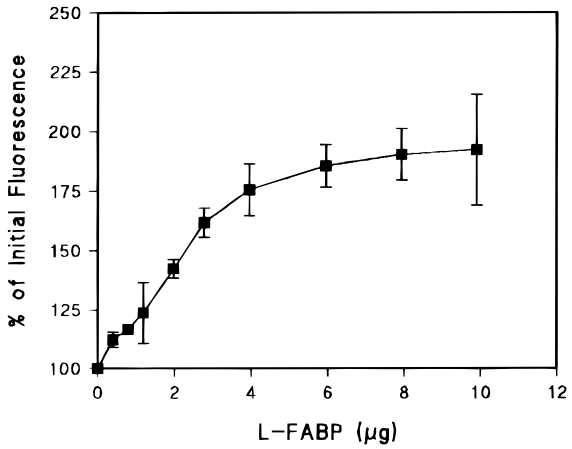

FIGURE 7: Enhancement of dansyl DHPE fluorescence by L-FABP in the presence of DOPG. Samples contained 10 μg/mL DOPG with 5 mol % dansyl DHPE in 50 mM Hepes/NaOH (pH 7.5), to which aliquots of L-FABP were added. Dansyl DHPE fluorescence was measured as described in Materials and Methods. All titrations were performed in triplicate.

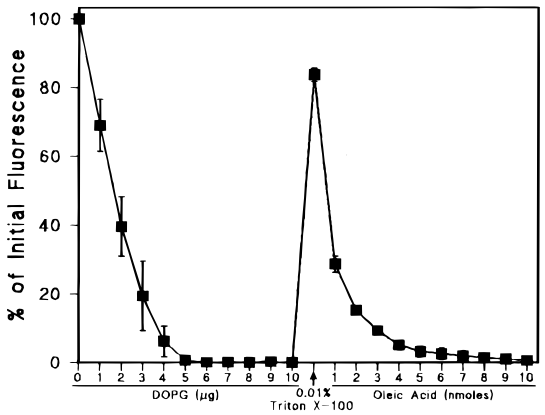

FIGURE 8: Ability of Triton X-100 to restore the ability of L-FABP to bind DAUDA. This binding is competitive with oleic acid. To a sample of L-FABP (4 μg) was added excess DOPG (10 mg/mL in methanol) by titration. Triton X-100 was then added to give a final concentration of 0.01% (v/v) to release L-FABP from the phospholipid vesicle. The recovery of fluorescence due to released L-FABP rebinding to the DAUDA is calculated as a percentage of the fluorescence seen under the same conditions in the absence of added DOPG to correct for the effect of Triton X-100 on DAUDA binding to L-FABP. This fluorescence value for the control is the percentage of that seen before the addition of Triton X-100. Finally, oleic acid (1 mM in methanol) is titrated into the system to confirm that the increased fluorescence was due to the L-FABP binding the DAUDA. All titrations were performed in triplicate.

mL (data not shown). The binding to DOPG did not require the presence of ligand, essentially identical titration curves being obtained in the presence or absence of oleic acid (data not shown).

Binding of L-FABP to DOPG: Direct Evidence from Gel Filtration. HPLC gel filtration was used to separate phospholipid vesicles from liver FABP. On incubation of DOPC with L-FABP and subsequent HPLC separation, it could be shown by SDS−PAGE that liver FABP did not elute with the vesicle fraction. In contrast, with DOPG it was shown that liver FABP eluted in two fractions, one of which corresponded to the DOPG vesicle fraction and the second of which eluted in the same position as the FABP (data not shown). However, when functional analysis of the FABP after HPLC was performed by addition of DAUDA to the column fractions under conditions of high ionic strength, no significant binding activity could be detected for the FABP eluting with the DOPG vesicles. This result would suggest that further changes in protein structure occur after initial electrostatic binding to the anionic phospholipid interface (see below).

Nature of the Interaction between L-FABP and the Phospholipid Interface. The results described above strongly suggest that L-FABP binds to the anionic phospholipid interface of small phospholipid vesicles. This binding results in a conformational change that reduces the affinity of DAUDA for the L-FABP, resulting in the release of DAUDA into the environment where it is much less fluorescent. The effects of ionic strength and anionic charge density indicate a major role for electrostatic interactions, at least in initial complex formation with the interface. Therefore, it would be anticipated that increasing the ionic strength of the assay medium would allow release of FABP from the phospholipid surface and hence rebinding of DAUDA and recovery of fluorescence.

The contribution of electrostatic interactions was determined by studying the effect of addition of salt to the nonfluorescent complex liver FABP with anionic phospholipid vesicles. Although addition of high salt (200 mM or 1 M NaCl) or Ca^{2+} ions (5 mM $CaCl_2$) produced a modest increase in fluorescence consistent with release of FABP from the phospholipid interface, this increase at best accounted for about 25% of the expected increase if all the FABP bound DAUDA. In contrast, the initial presence of such high-salt conditions would have greatly reduced the loss of fluorescence on titration in the phospholipid (Figure 6). Control experiments confirmed that the addition of high salt did not affect the fluorescence of DAUDA either in the absence or in the presence of FABP or DOPC.

The inability of high salt to release completely L-FABP from the anionic phospholipid interface suggested that a secondary rapid membrane insertion or equivalent event had occurred, after the formation of an electrostatic complex. This membrane insertion event presumably involves hydrophobic interactions that could not be reversed by high salt. To distinguish between a complex of FABP with the phospholipid vesicles involving hydrophobic interactions and irreversible denaturation of FABP, the vesicle structure was denatured by the addition of Triton X-100, a nonionic detergent. The addition of 0.01% detergent did not significantly affect the binding of DAUDA to FABP, assessed by fluorescence enhancement, or the fluorescence of DAUDA in buffer. However, this addition resulted in >85% recovery of fluorescence which, presumably, reflected release of native liver FABP from the phospholipid surface. The subsequent addition of high salt did not produce a further increase in fluorescence. The fact that the recovery of fluorescence was due to rebinding of DAUDA to L-FABP was confirmed by addition of oleic acid when there was immediate loss of fluorescence due to competitive displacement of DAUDA from the L-FABP. The overall sequence of events is illustrated in Figure 8 using 100% DOPG followed by Triton X-100 and then oleic acid.

The fluorescence recovery pattern with high salt was greater (~50%) using 20 mol % DOPG for cardiolipin, however, recovery was not affected by the length of the exposure of the L-FABP to the phospholipid. The same

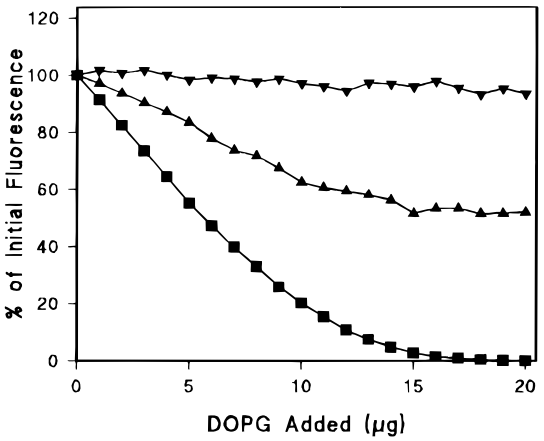

FIGURE 9: Effect of added DOPG vesicles on the tryptophan fluorescence of the F3W and F18W mutants of L-FABP. To a sample of F3W or F18W L-FABP (14 μg) in 10 mM Hepes/NaOH buffer (pH 7.5) was added excess DOPG (10 mg/mL in methanol) by titration. The change in tryptophan fluorescence was monitored at 330 nm after excitation at 280 nm. In parallel experiments, the same solution of DOPG was added to F3W L-FABP−DAUDA and the decrease in fluorescence intensity at 500 nm was monitored. An F18W L-FABP−DAUDA titration gave the same result and is not shown for clarity: (▲) F3W tryptophan titration, (▼) F18W tryptophan titration, and (■) F3W DAUDA titration. All values are the means of titrations performed in triplicate.

results were obtained on addition of salt immediately after addition of the phospholipid or after a delay of several minutes. The cumulative evidence would suggest that membrane insertion (or an equivalent hydrophobic interaction) and ligand release may be the same event, showing a single first-order rate constant of 23 s^{-1} (Figure 3). Confirmation of such a proposal will require a method which allows a measurement of the rate of membrane insertion which is not possible at this time. However, the fact that the first-order rates of ligand release from FABP at two different concentrations of DOPG are the same indicates that ligand release is after initial (electrostatic) binding of FABP to the phospholipid interface.

Use of Tryptophan Mutants To Demonstrate Conformational Changes in L-FABP on Binding to Anionic Vesicles. To identify the molecular events that result in ligand release from L-FABP on binding to anionic vesicles, we have used two tryptophan-containing mutants of L-FABP (F3W and F18W) that have been described previously (*19*). These were mutants in which the inserted tryptophans were located at strategic positions in the protein, at the amino terminus and the α-helical region. These mutants show minimal perturbation in terms of their ligand binding properties (*19*).

When DOPG vesicles were titrated into these mutants, characteristic changes in tryptophan fluorescence were obtained. In the case of the F3W, a very significant fall in fluorescence was observed with binding to the vesicle that exactly paralleled DAUDA release as monitored in separate experiments (Figure 9). The F18W mutant failed to show a significant change in tryptophan fluorescence on addition of DOPG (Figure 9) even though, in a separate experiment, the characteristic loss of DAUDA fluorescence was observed (not shown). For both tryptophan-containing mutants, the changes in tryptophan fluorescence on addition of DOPG were the same when titrations were performed in the absence or the presence of ligand (oleic acid).

The considerable fall in fluorescence of the F3W is consistent with the tryptophan being exposed to a more quenching environment. The fluorescence emission maximum wavelength did not change significantly, being 333.0 ± 0.7 and 333.6 ± 1.7 nm before and after addition of DOPG, respectively. Such a conformational change might bring the tryptophan residue from the more nonpolar hydrophobic environment of the protein core to a more polar environment nearer the membrane interface.

DISCUSSION

The ability of proteins and peptides to interact with anionic interfaces is well-known, and the properties of numerous examples have been studied. Cytochrome *c* has become a useful model for investigations involving the interfacial binding of such proteins, and it is now well established that anionic phospholipids such as cardiolipin, phosphatidylglycerol, and phosphatidylserine are particularly effective in promoting such interfacial binding (*38*−*40*). The initial binding of cytochrome *c* is presumed to involve electrostatic interactions as it is sensitive to both the ionic strength of the medium and anionic charge density of the interface. However, subsequent conformational changes and resulting nonpolar interactions with the phospholipid surface are seen (*41*).

Interfacial binding to anionic phospholipids has been shown to be fundamental to the modulation of a number of proteins that translocate to the interface. In the case of the membrane association of Src (*42*) and basic peptides such as charybdotoxin (*32*), a model involving electrostatic binding accurately describes the properties of the system, particularly the effects of salt concentration and the mol % of anionic phospholipid. In this paper, we have clearly demonstrated that under conditions that promote electrostatic interactions, L-FABP can bind to small anionic phospholipid interfaces. This interfacial binding results in a conformational change that is identified by the release of the fluorescent ligand DAUDA into the medium with a decrease in fluorescence intensity to the background levels seen for this probe in buffer. The rapid rate of ligand release associated with fluorescence loss (23 s^{-1}) is similar to the rate (33 s^{-1}) seen for the partial unfolding of cytochrome *c* in the presence of a micellar concentration of sodium dodecyl sulfate (*43*). Therefore, the rate is consistent with such a partial unfolding of L-FABP at the anionic interface. The fact that the DAUDA had been released from the protein was supported by the observation that subsequent addition of excess FABP to the assay resulted in complete recovery of fluorescence (*44*).

Overall, the phenomenon exhibits well-defined physical characteristics consistent with initial electrostatic interactions but involving a subsequent membrane insertion or equivalent hydrophobic interaction. The stoichiometry of binding is consistent with the protein coating the surface of the phospholipid vesicles that would also allow an opportunity for protein−protein interactions. However, loss of fluorescence was observed even at high phospholipid:L-FABP ratios and could not be reversed by high salt, suggesting that protein−protein interactions do not make a significant contribution to conformational changes resulting in ligand loss.

It would be an attractive mechanism if the FABP released its ligand on interaction with membrane surfaces within the

cell, thus allowing further metabolism of ligand (fatty acid) by membrane-bound enzymes such as the acyl CoA synthetases. Anionic phospholipids are a characteristic of the internal membranes of the mammalian cell, and although membrane binding and DAUDA release in vitro are only observed to a significant extent under conditions of low ionic strength, such interactions will be facilitated in vivo. This is because the concentration of L-FABP (*45*) in liver cytosol (0.2−0.4 mM) is nearly 3 orders of magnitude higher than that used in vitro, and this higher concentration would favor interfacial binding. Hence, it is possible that a proportion of cytosolic L-FABP could bind to the interface with ligand release, thus enhancing the concentration of free ligand at the interfacial surface.

It is possible that the in vitro membrane-binding phenomenon is modeling the binding of the FABP to a membrane-associated protein with a resulting conformational change that would allow facile dissociation of ligand. An association between bovine mammary gland FABP and CD36 was observed at the milk fat globule membrane (*46*). Recently, a direct protein−protein interaction has been identified between adipocyte FABP and triglyceride lipase, presumably facilitating uptake of released fatty acid into the FABP as TG hydrolysis proceeds. In this system, it is proposed that ligand binding results in a dissociation of the ALBP from the lipase (*47*).

Storch et al. have used a fluorescence energy transfer approach to show that the movement of anthroyloxy-labeled fatty acid analogues from L-FABP to small unilamellar vesicles, in particular phosphatidylcholine vesicles, is mediated by aqueous diffusion and the rate-limiting step is the dissociation of the ligand from L-FABP (*12*, *15*). It should be noted that the rate of release of an anthroyloxy fatty acid analogue from L-FABP to phospholipid vesicles under conditions involving aqueous transfer is about 1000-fold slower (*15*) than that seen for DAUDA release from L-FABP, indicating that different phenomena are being studied. In contrast, similar experiments with intestinal, heart, and adipocyte FABP demonstrate that transfer of the anthroyloxy fatty acids requires collision of the FABP with the membrane vesicles (*9*−*16*). Interestingly, in the case of adipocyte FABP, Wootan and Storch showed that cardiolipin increased transfer rates relative to those with phosphatidylcholine (*48*), while a strong interaction is directly demonstrated to anionic phospholipid such as phosphatidylserine and cardiolipin using FTIR spectroscopy (*49*). It should be noted that cardiolipin, which is found almost exclusively associated with mitochondrial membranes (*50*), was the most effective anionic phospholipid in promoting release of DAUDA. However, detailed analysis of the mitochondrial membrane has indicated that the cardiolipin is located on the inner mitochondrial membrane while the outer membrane has a minimal amount of this phospholipid (*51*).

The general structure of intracellular FABPs consists of a hydrophobic β-clam ligand-binding cavity with two α-helices forming part of a postulated portal domain (*52*, *53*). The most common secondary structure in the interfacial phospholipid-binding domains of peripheral proteins and peptides is an α-helix (*54*), and therefore, it could be postulated that the FABP α-helical domain would be involved in ligand release induced by interactions with phospholipid membranes. This has been clearly demonstrated for heart and adipocyte FABP where the α-helical domain and lysine residues are required for the electrostatic binding of the FABP to phospholipid membranes (*13*, *55*). Cistola et al. have also shown that the absence of the α-helices affected ligand dissociation from intestinal FABP and concluded that the α-helices may control ligand transfer within the cell (*56*), while the use of this mutant has highlighted the importance of the helical domain in membrane interactions and fatty acid transfer (*57*). This view is supported by NMR data that have highlighted the mobility of this region and the implications for fatty acid targeting (*58*). Detailed kinetic analysis by Richieri et al. has also emphasized the importance of the portal region in ligand binding (*59*).

The accumulative evidence has identified the α-helical region and the adjacent substrate entry portal as being the primary site of interaction between the heart, adipocyte, and intestinal FABP and the membrane surface. Therefore, the lack of fluorescence change on binding to DOPG vesicles with the F18W mutant was surprising as this mutation is located in the α-helical region of the protein. In contrast, the data from our F3W mutant suggest the possibility of a significant conformational change in the amino-terminal region which could generate an exit portal. Visual inspection of the crystal structure of L-FABP suggests a potential portal in this region, although such a functional portal could only be generated by significant conformational changes involving the amino terminus. It should be noted that a similar visual inspection of the intestinal FABP crystal structure (*60*) also highlighted a potential portal in this region of the protein, but again a functional site of ligand release would require significant changes in protein structure. The ability of tryptophan to partition into the headgroup region of the phospholipid bilayer is recognized (*61*), and dramatic effects of such tryptophans on the interfacial binding of phospholipases A_2 have been highlighted (*28*) and discussed (*62*). If binding of the F3W to the interface was being facilitated by the presence of this tryptophan, then this additional hydrophobic interaction should reduce the sensitivity of the interfacial binding to ionic strength. However, the sensitivity of the displacement of DAUDA from the F3W interactions to NaCl concentration was very similar (not shown) to that described for the wild-type L-FABP (Figure 6), thus indicating that this tryptophan was not penetrating the phospholipid interface and stabilizing interfacial binding.

In conclusion, we have demonstrated electrostatic interactions can result in the binding of L-FABP to small anionic phospholipid vesicles, and this process also involves hydrophobic interactions and release of ligand. This FABP system, because of its small size and because it is composed mostly of a rigid β-clam structure, will be a useful model for studying the important phenomenon of the interfacial binding of proteins to anionic phospholipids interfaces. Further studies involving site-directed mutagenesis are required to demonstrate if changes in the α-helical region of L-FABP are directly implicated in interfacial binding and ligand release or if other regions of the protein are involved. It remains to be established if the anionic membrane binding exhibited by L-FABP has major structural differences with respect to the membrane interactions compared to other members of the FABP family.

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REFERENCES

1. Zhang, F. L., Kamp, F., and Hamilton, J. A. (1996) *Biochemistry 35*, 16055−16060.
2. Glatz, J. F. C., and Vandervusse, G. J. (1996) *Prog. Lipid Res. 35*, 243−282.
3. Banaszak, L. J., Winter, N., Xu, Z., Bernlohr, D. A., and Jones, T. A. (1994) *Adv. Protein Chem. 145*, 90−148.
4. Coe, N. R., and Bernlohr, D. A. (1998) *Biochim. Biophys. Acta 1391*, 287−306.
5. Burrier, R. E., Manson, C. R., and Brecher, P. (1987) *Biochim. Biophys. Acta 919*, 221−230.
6. Hubbell, T., Behnke, W. D., Woodford, J. K., and Schroeder, F. (1994) *Biochemistry 33*, 3327−3334.
7. Burrier, R. E., and Brecher, P. (1986) *Biochim. Biophys. Acta 879*, 229−239.
8. Thumser, A. E. A., and Wilton, D. C. (1995) *Biochem. J. 307*, 305−311.
9. Kim, H. K., and Storch, J. (1992) *J. Biol. Chem. 267*, 20051−20056.
10. Storch, J., and Bass, N. M. (1990) *J. Biol. Chem. 265*, 7827−7831.
11. Wootan, M. G., Bernlohr, D. A., and Storch, J. (1993) *Biochemistry 32*, 8622−8627.
12. Hsu, K. T., and Storch, J. (1996) *J. Biol. Chem. 271*, 13317−13323.
13. Herr, F. M., Aronson, J., and Storch, J. (1996) *Biochemistry 35*, 1296−1303.
14. Kim, H. K., and Storch, J. (1992) *J. Biol. Chem. 267*, 77−82.
15. Storch, J. (1993) *Mol. Cell. Biochem. 123*, 45−53.
16. Storch, J., Herr, F. M., Hsu, K. T., Kim, H. K., Liou, H. L., and Smith, E. R. (1996) *Comp. Biochem. Physiol. 115*, 333−339.
17. Worrall, A. F., Evans, C., and Wilton, D. C. (1991) *Biochem. J. 278*, 365−368.
18. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
19. Thumser, A. E. A., and Wilton, D. C. (1996) *Biochem. J. 320*, 729−733.
20. Wilton, D. C. (1989) *Biochem. J. 261*, 273−276.
21. Glatz, J. F. C., and Veerkamp, J. H. (1983) *Anal. Biochem. 132*, 89−95.
22. Lowe, J. B., Strauss, A. W., and Gordon, J. J. (1984) *J. Biol. Chem. 259*, 12696−12704.
23. New, R. R. C. (1990) in *Liposomes: A Practical Approach* (New, R. R. C., Ed.) pp 33−104, Oxford University Press, Oxford, U.K.
24. Wilkinson, T. C. I., and Wilton, D. C. (1987) *Biochem. J. 247*, 485−488.
25. Wilton, D. C. (1990) *Biochem. J. 270*, 163−166.
26. Thumser, A. E. A., Worrall, A. F., Evans, C., and Wilton, D. C. (1994) *Biochem. J. 297*, 103−107.
27. Wilton, D. C. (1990) *Biochem. J. 266*, 435−439.
28. Baker, S. F., Othman, R., and Wilton, D. C. (1998) *Biochemistry 37*, 13203−13211.
29. Koduri, R. S., Baker, S. F., Snitko, Y., Han, S. K., Cho, W., Wilton, D. C., and Gelb, M. H. (1998) *J. Biol. Chem. 273*, 32142−32153.
30. Jain, M. K., and Berg, O. G. (1989) *Biochim. Biophys. Acta 1002*, 127−156.
31. Kim, J., Mosior, M., Chung, L. A., Wu, H., and McLaughlin, S. (1991) *Biophys. J. 60*, 135−148.
32. Ben-Tal, Honig, B., Miller, C., and McLaughlin, S. (1997) *Biophys. J. 73*, 1717−1727.
33. Denisov, G., Wanasaki, S., Luan, P., Glaser, M., and McLaughlin, S. (1998) *Biophys. J. 74*, 731−744.
34. Thumser, A. E. A., Voysey, J. E., and Wilton, D. C. (1995) *Biochem. J. 314*, 943−949.
35. Bass, N. M. (1985) *Chem. Phys. Lipids 38*, 95−114.
36. Zanetti, R., and Catalá, A. (1991) *Mol. Cell. Biochem. 100*, 1−8.
37. Sankaram, M. B., and Marsh, D. (1993) *Protein−Lipid Interactions* (Watts, A., Ed.) pp 127−162, Elsevier Science Publishers, Amsterdam.
38. Heimburg, T., and Marsh, D. (1995) *Biophys. J. 68*, 536−546.
39. Rytomaa, M., and Kinnunen, P. K. J. (1995) *J. Biol. Chem. 270*, 3197−3202.
40. Watts, A. (1997) *Biochem. Soc. Trans. 25*, 1119−1124.
41. Pinheiro, T. J. T., Elove, G. A., Watts, A., and Roder, H. (1997) *Biochemistry 36*, 13122−13132.
42. Murray, D., Hermida-Matsumoto, L., Buser, C. A., Tsang, J., Sigal, C. T., Ben-Tal, N., Honig, B., Resh, M. D., and Mclaughlin, S. (1998) *Biochemistry 37*, 2145−2159.
43. Das, T. K., Mazumdar, S., and Mitra, S. (1998) *Eur. J. Biochem. 254*, 662−670.
44. Wilton, D. C. (1998) *Biochem. Soc. Trans. 26*, 378−383.
45. Burnett, D. A., Lysenko, N., Manning, J. A., and Ockner, R. K. (1979) *Gastroenterology 77*, 241−249.
46. Spitsberg, V. L., Matitashvili, E., and Gorewit, R. C. (1995) *Eur. J. Biochem. 230*, 872−878.
47. Shen, W.-J., Sridhar, K., Bernlohr, D. A., and Kraemer, F. B. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 5528−5532.
48. Wootan, M. G., and Storch, J. (1994) *J. Biol. Chem. 269*, 10517−10523.
49. Gericke, A., Smith, E. R., Moore, D. J., Mendelson, R., and Storch, J. (1997) *Biochemistry 36*, 8311−8317.
50. Hardeman, D., Versantvoort, C., van den Brink, J. M., and Van den Bosch, H. (1990) *Biochim. Biophys. Acta 1027*, 149−154.
51. de Kroon, A. I. P. M., Dolis, D., Mayer, A., Lill, R., and de Kruijff, B. (1997) *Biochim. Biophys. Acta 1325*, 108−116.
52. Sacchettini, J. C., Gordon, J. I., and Banaszak, L. J. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7736−7740.
53. Thompson, J., Winter, N., Terwey, D., Bratt, J., and Banaszak, L. (1997) *J. Biol. Chem. 272*, 7140−7150.
54. Epand, R. M., and Epand, R. F. (1991) in *The Structure of Biological Membranes* (Yeagle, P., Ed.) pp 573−601, CRC Press, London.
55. Herr, F. M., Matarese, V., Bernlohr, D. A., and Storch, J. (1995) *Biochemistry 34*, 11840−11845.
56. Cistola, D. P., Kim, K., Rogl, H., and Frieden, C. (1996) *Biochemistry 35*, 7559−7565.
57. Corsico, B., Cistola, D. P., Frieden, C., and Storch, J. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 12174−12178.
58. Hodsdon, M. E., and Cistola, D. P. (1997) *Biochemistry 36*, 2278−2290.
59. Richieri, G. V., Ogata, R. T., and Kleinfeld, A. M. (1996) *J. Biol. Chem. 271*, 31068−31074.
60. Sacchettini, J. C., Gordon, J. I., and Banaszak, L. J. (1989) *J. Mol. Biol. 208*, 327−339.
61. Yau, W.-M., Wimley, W. C., Gawrisch, K., and White, S. H. (1998) *Biochemistry 37*, 14713−14718.
62. Gelb, M. H., Cho, W., and Wilton, D. C. (1999) *Curr. Opin. Struct. Biol. 9*, 428−432.

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