The ligand-mediated affinity of escort proteins determines the directionality of lipophilic cargo transport

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Abstract Intracellular cargo transport is a highly dynamic process. In eukaryotic cells, the uptake and release of lipophilic ligands are executed by escort proteins. However, how these carriers control the directionality of cargo trafficking remains unclear. Here, we have elucidated the unliganded structure of an archetypal fatty acid-binding protein (FABP) and found that it possesses stronger binding affinity than its liganded counterpart towards empty nanodiscs. Titrating unliganded FABP and nanodiscs with long-chain fatty acids (LCFAs) rescued the broadening of FABP cross-peak intensities in HSQC spectra due to decreased protein-membrane interaction. Crystallographic studies revealed that the tails of bound LCFAs obstructed the charged interfaces of the FABP-nanodisc complexes. We conclude that the lipophilic ligands, by taking advantage of escort proteins with high conformational homogeneity and nanodiscs as the third interaction partner involved in this transport study, participate directly in the control of their own transportation in an irreversible, unidirectional fashion.

Introduction

Intracellular cargo transport is the cornerstone of many biological processes, including cellular metabolism, energy homeostasis, neuron development and organelle function. In eukaryotic cells, a number of escort proteins have evolved to shuttle lipophilic cargos from external or internal environments to distinct subcellular compartments (1-3). Among these escort proteins, the cytosolic fatty acid-binding proteins (FABPs) are the archetypal lipid chaperones for long-chain fatty acids (LCFAs) and certain other energy-rich lipophilic ligands (4-7). The FABP carriage system is essential because 1) it minimizes the potential toxicity of free non-esterified LCFAs; 2) it facilitates the rapid cellular uptake and regulatable transport of LCFAs in concert with metabolic demands; and 3) it permits LCFAs to traverse intracellular space to various sites of utilization, e.g. to the endoplasmic reticulum for transacylation and to the nucleus for transcriptional regulation (8, 9). Upon binding to LCFAs, FABPs themselves may enter the nucleus to activate several transcription factors such as PPARs (peroxisome proliferator-activated receptors) and ERRs (oestrogen-related receptors) for downstream cellular signalling, metabolic reactions and immune responses (10-12).

Considerable evidence suggests that once absorbed by cell membrane phospholipids, LCFAs are

sequestered and delivered by FABPs (13, 14) in a manner probably similar to the substrate-binding proteins (15). Despite substantial efforts to determine the key structural factors contributing to lipophilic cargo binding (16-18), the precise mode of how LCFAs are directionally transported by FABPs from a specific donor membrane to a target acceptor membrane or protein remain largely in dispute (2). One major reason for this ambiguity is inadequate research on the role of the phospholipid membrane in cargo trafficking, in that the uptake and release of lipophilic ligands depend not only on the ligand competition between escort proteins and ligand donors/acceptors but also on the selective affinities of unliganded and liganded escort proteins towards ligand donors/acceptors. Recent NMR and protein engineering studies revealed that the helical portal motif of FABPs for ligand entry was capable of reversibly transferring LCFAs across the plasma and intracellular membranes (19-22). However, owing to the uncertain conformational homogeneity (23, 24) and the lack of appropriate biomembrane mimetics in past research (25, 26), it is still unclear whether lipophilic ligands can be reversibly transported and how escort proteins control the directionality of cargo trafficking.

In this article, we utilized *Drosophila* FABP as a model system to assess the hypothesis of a directionality-controlled mechanism in escort proteins. An improved purification strategy was provided to separate the liganded-like and unliganded forms of FABP. The latter exhibited very high conformational homogeneity, allowing us to verify how different forms of FABP interact differently with lipid bilayer membranes. By employing membrane mimetic nanodiscs for NMR experiments, we were able to increase the tumbling rates of FABP–nanodisc complexes to reveal the residue-based membrane-interacting region of escort protein. Our structural analyses demonstrated that unliganded FABP has a much stronger binding affinity than its liganded counterpart to empty nanodiscs. Crystallographic studies showed that the LCFAs participate in their own transportation by disturbing the charged interfaces between FABPs and lipid membranes with aliphatic tails. Together, these results clearly suggest that the membrane-binding capacity of escort proteins is regulated by their target ligands, ultimately facilitating irreversible unidirectional lipophilic cargo transfer.

Results

Escort proteins with high conformational homogeneity are important for determining the regulatory mechanism of membrane-associated cargo transport

The escort protein represented herein is a *Drosophila* brain-type FABP (dFABP) that exists mainly in glial cells and localizes in both the cytoplasm and the nucleus (27, 28). Its movement from the cytoplasm to the nucleus in response to diurnal regulation and memory consolidation raises the question of how ligands trigger dFABP function. Although earlier biochemical assays of FABPs tolerated the minor heterogeneity in assessing the nature of their ligand binding (29, 30), we believed that studying escort proteins with high conformational homogeneity would illustrate the apparently dynamic lipid transport process. To clarify whether insufficient conformational homogeneity may impede further research on the interaction between FABPs and membrane mimetics, we compared the two apo forms of dFABP isolated by the existing method (denoted as the urea-treated method) and by the newly improved two-stage purification procedure with the use of the reagent GuHCl to induce partial unfolding (denoted as the GuHCl-purification method, see **Supp. Fig. S1**). The results showed that the secondary structures of the two apo forms of dFABP with different conformational homogeneities varied significantly upon heating (**Fig. 1**). The impact of undesired conformational heterogeneity in dFABP on biophysical analysis was observed in the initial far-UV circular dichroism (CD) spectrum. In contrast to the irreversible profile observed for urea-treated dFABP, there exists a partially reversible profile for GuHCl-purified dFABP after the heating process (**Fig. 1A and B**). In addition, the melting curves obtained at 215 nm revealed that the two apo forms of dFABP behave differently upon interaction with the ligand oleic acid (OA; **Supp. Fig. S2A**). This result implies that the secondary structures of the urea-treated sample were altered

Figure 1. Spectroscopic analysis of urea-treated and GuHCl-purified dFABP. The urea-treated (**A**) and GuHCl-purified (**B**) samples were subjected to far-UV CD spectroscopy for secondary thermal stability analysis. Notably, the reversed 25 °C spectrum (denoted re-25 °C and shown as the thinnest line) in the temperature profile of the urea-treated dFABP was found to be irreversible at 215 nm due to protein precipitation (**Supp. Fig. S2**).

by the heat-induced precipitation of some nonprotein contaminants (**Supp. Fig. S3B and E**) that were absent in the GuHCl-purified sample (**Supp. Fig. S2D, S3F**). The undesired contaminants seem to have high molecular weights, as demonstrated by size-exclusion chromatography (denoted as P1 and A in **Supp. Fig. S3B**). Despite the small change upon heating observed for the secondary structures of the apo and holo forms in GuHCl-purified dFABP, the melting temperatures measured through intrinsic fluorescence showed that both samples obtained from the two purification methods underwent tertiary structure denaturation (**Supp. Fig. S2C**).

Improved conformational homogeneity of dFABP benefits the study of membrane interaction by increasing the proportion of the apo-conformer

The pronounced difference observed in the melting curves of CD spectra convinced us to study the ligand binding based on the dFABP structure with different conformational homogeneities. The HSQC spectrum of the OA complex, holo-dFABP, was assigned (BMRB code 27112, **Supp. Table S11**) and used as a template spectrum to manually assist the assignments of conformer-A and conformer-B' (a conformer similar to the holo form) in the spectrum of apo-dFABP (BMRB code 27113, **Supp. Table S12**). Conformer-B' was identified as a liganded-like conformation. The cross-peaks in HSQC spectra indicated a higher proportion of conformer-A in the GuHCl-purified dFABP sample than in the urea-treated sample (**Fig. 2A and B**). Representative residues of conformer-A and conformer-B' showed that the urea-treated apo form of dFABP might be a liganded-like dFABP based on the proportion of conformer-A, which was 3-fold less than that in the GuHCl-purified dFABP on average (**Fig. 2C and D**). Superimposition of the initial and final HSQC spectra upon OA titration revealed that the increasing intensity of conformer-B' in urea-treated dFABP was difficult for comparison to the stoichiometry measured in biochemical assays (**Supp. Fig. S4D**). Although the disappearance of signals in conformer-A was similar to the results observed in GuHCl-purified dFABP (**Supp. Fig. S9**), the larger proportion of conformer-B' in urea-treated dFABP proved to be problematic for subsequent structural analyses on membrane interaction.

Crystallographic structures of dFABP elucidate different potentials for membrane interaction with minimal protein conformational changes

The apo forms of both urea-treated and GuHCl-purified dFABP were crystallized, and their structures were obtained and identified as 5GGE and 5GKB, respectively (**Supp. Table S13**). Unexpectedly, the density maps for the ligand-binding cavity of urea-treated dFABP were incomplete, such that we were uncertain of the liganded state and therefore termed it the liganded-like structure (**Fig. 3A**).

Figure 2. Conformational subpopulations of apo-dFABP determined by HSQC NMR. **(A)** The HSQC spectrum of urea-treated liganded-like dFABP is shown in magenta and is superimposed on that of GuHCl-purified dFABP (light blue). Representative residues for conformer comparison are marked using black boxes. **(B)** The HSQC spectrum of GuHCl-purified apo-dFABP is shown in light blue. Representative residues for conformer comparison are marked using black boxes. **(C)** Representative cross-peaks are enlarged to indicate the different conformational subpopulations in the apo forms of dFABP from the two purification strategies. The residues labelled in gray with the prime symbol represent conformer-B', which is overwhelmingly present in urea-treated liganded-like dFABP, and the dispersion resembles that of OA-liganded holo-dFABP. **(D)** The intensity ratio of conformer-A reveals that the proportion of the apo form is approximately 3 times higher in GuHCl-purified dFABP than in urea-treated dFABP.

Since the region was relatively empty in GuHCl-purified dFABP, containing only three molecules of water, this structure was denoted as apo-dFABP (**Fig. 3B**). The crystal structures presented herein support the importance of conformational homogeneity through the subtle dissimilarities observed between two structures at similar resolution, with different temperature factors observed in the corresponding hypothesized portal (α II, loops of β C-D and β E-F) and gap regions (β D-E). The temperature factor-derived atom distributions of α II and the β C-D loop in the portal region of apo-dFABP were more compact than those in liganded-like dFABP, and those of β E-F in the gap region were expanded during crystallization (**Fig. 3, Supp. Fig. S10**). The larger atom distributions of liganded-like dFABP in the portal region may have resulted from the use of ambiguous restraints for the aliphatic tail of the endogenous unknown ligand, while the internal hydration of water molecules within this region of apo-dFABP may result in more compact atom distributions (31). As shown in the 5GGE liganded-like dFABP structure, crystallization might preserve the slow-moving (gap region β E-F) and promiscuous binding (portal region α II) sites where diverse positions occur.

The altered distribution of ordered waters in liganded-like dFABP is worth comparing to that of apo-dFABP. Despite the presence of eleven water molecules within the binding cavity (blue) in both structures, three water molecules (red) were positioned differently (**Fig. 3**). Because of the existence of an unknown ligand, the three differently positioned water molecules were buried in the lower part of the cavity in the liganded-like dFABP but in the upper part of the cavity in apo-dFABP. In contrast to the possibility that the unknown ligand occupied the space in the upper cavity in the liganded-like dFABP, the allosteric ordered waters associated with apo-dFABP may be related to the rearrangement of the hydrogen networks occurring among the surrounding residues, as explained based on a prior molecular dynamics simulation of human heart-type FABP (32). Residues that are potentially involved in networks with a possible carboxylate group of the unknown ligand (within 3.5 Å) at the bottom of the ligand-binding cavity are also conserved in previous holo-FABP

Figure 3. Distribution of water molecules in the apo structures of dFABP. **(A)** Fourteen water molecules within the ligand binding cavity are defined by PyMOL and shown as spheres. Eleven in similar locations are colored in blue. However, 3 water molecules (shown in red) were observed in different locations in the urea-treated liganded-like dFABP (PDB ID: 5GGE) and the GuHCl-purified apo-dFABP (PDB ID: 5GKB) structures. Both dFABP crystal structures are depicted with the true atom distribution derived from temperature factors, shown in a sausage representation. The discontinuously incomplete electron density maps are shown as red (lower) and blue (upper) meshes. The three residues R105, R125 and Y127, shown as CPK sticks, are within 3.5 Å of the lower part of the unknown ligand-like map and are conserved in FABPs due to their interaction with the carboxylate head of LCFAs. **(B)** The upper part of the cavity in the GuHCl-purified apo-dFABP is filled with 3 water molecules (shown in red) that are located in a different region from those in liganded-like dFABP. Two α -helices are nominated as α I and α II, and the ten β -strands are designated as β A- β J.

144 complex structures (**Fig. 3A, 5C**). Accordingly, the distribution of these water molecules rather than
 145 the unknown ligand near the upper cavity region of apo-dFABP is consistent with the claim of higher
 146 conformational homogeneity in the GuHCl-purified dFABP sample.

147 **Membrane interaction reveals a ligand triggering its own unidirectional transport** 148 **through modulating the dissociation of apo-dFABP from membranes**

149 The GuHCl purification of dFABP enabled a study on the role of conformational relationship in
 150 protein-membrane interactions. We first obtained conventional HSQC spectra to observe the
 151 discrepancies between the apo and holo forms of GuHCl-purified dFABP through titration with
 152 nanodiscs (**Fig. 4A and C**). Next, transverse relaxation-optimized spectroscopy (TROSY) spectra of
 153 apo- and holo-dFABP bound to the 1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt)
 154 /1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPG/DOPC) lipid nanodiscs (lipid NDs) at a 1:1 molar
 155 ratio were acquired to enhance the signal disappearance by virtue of the TROSY effect (**Fig. 4B and**
 156 **D, Supp. Fig. S5A, Supp. Table S14**). Contrary to our expectations, after the addition of the nanodiscs,
 157 holo-dFABP exhibited few changes from the previously acquired spectrum (**Fig. 4A**). In contrast,
 158 the signals of apo-dFABP in both the HSQC and TROSY spectra were clearly broadened after the
 159 addition of nanodiscs, by an order of magnitude after TROSY spectrum, due to a decrease in the
 160 tumbling rate (**Fig. 4B and C**). This result suggested that apo- rather than holo-dFABP interacts with

Figure 4. Interaction of GuHCl-purified apo-dFABP (5GKB) with membranes. **(A)** Superimposition of the HSQC spectra of OA-liganded holo-dFABP in the presence (red) or absence (blue) of DOPG/DOPC lipid nanodiscs (ND) at a molar ratio of 1:1. **(B)** The intensity change ratio in the TROSY spectra were obtained by dividing the final intensity (I_f , with ND) by the initial intensity (I_0 , without ND). The average difference on TROSY spectra between the two states is approximately 10. **(C)** Superimposition of the HSQC and TROSY spectra of apo-dFABP in the presence (red) or absence (blue) of lipid ND at a molar ratio of 1:1. A similar trend was observed for the representative TROSY spectra shown here. **(D)** The thickness of sausage presentationOSY spectra) after interacting with lipid NDs. Residues in the α II, β D-F, and β G-H loops and in α I-II (see Fig. 3B) with larger changes are labelled, and the unassigned residues are colored yellow. The thicker segment above the average fraction of attenuation is further divided into three groups according to a serial lipid ND titration (followed using HSQC). Red indicates that the signal decayed immediately, whereas blue indicates that the signal decayed faster than those of the other residues (labelled in black).

membrane mimetic nanodiscs. The scale of peak broadening in apo-dFABP in the presence of lipid NDs was plotted as the fraction of attenuation and then superimposed on the tertiary structure of apo-dFABP in a sausage model (Fig. 4D).

The diminishing peaks were clustered near the portal and gap regions and near the ligand entry gates (Fig. 4D, 5B). This harmony between membrane and ligand interactions enabled the development of a feasible model in which ligand uptake occurs after the attachment of dFABP to membranes near the β G-H loop, and the mechanism involves sliding to the region that encompasses the α II and β D-F regions. To further decipher the enigmatic membrane interaction, residues with attenuation differences above the average value in the TROSY spectra were selected, and their signal decay rates were analyzed using HSQC lipid ND titration (Supp. Fig. S5A and B). After the integrated evaluation, including signal attenuation and decay rate, four clusters with the following characteristics were defined: 1) signals that decayed more slowly than the average rate (e.g., R30, shown in black); 2) signals that decayed faster than the average rate (e.g., T74, shown in blue); 3) signals that immediately disappeared upon the addition of lipid NDs (e.g., K99, shown in red); and 4) signals that remained detectable (e.g., L51, shown in hollow font) (Supp. Fig. S5B and C). The red and blue clusters are also shown in Fig. 4D. More polar and charged residues (namely, D76, R78, K81, K96, D98, and K99), which are responsible for primary membrane attachment, were observed, followed by successive sliding to integrate nonpolar and aromatic residues (namely, L23, G24, V25, F57, and A61) in the portal and gap regions. Polar residues can be reasonably inferred to engage

Figure 5. Intersection analysis between ligand binding and membrane interaction. **(A)** The chemical shift perturbations of apo-dFABP were measured by ligand OA titration. Residues with values greater than the total average value (0.04 ppm, the lower, more closely spaced dotted line) were recalculated to obtain an average value similar to that of the upper, more widely spaced dotted line (approximately 0.1 ppm). Five of the seven residues above the highest average CSP value (in blue frames) are found to have higher levels of disappearance (fraction of attenuation in TROSY spectra) during lipid ND interaction. **(B)** The effect of lipid NDs on apo-dFABP mapping onto the apo-dFABP structure (5GKB) is shown in sausage representation. The five residues with higher levels of disappearance during lipid ND interaction are shown as sticks and are labelled on the structure; two of these (in squares) are located in the membrane-associated region. The maps of the unknown ligand in liganded-like dFABP (5GGE, red and blue meshes), stearic acid of human heart-type FABP (4WBK, yellow sticks), arachidonic acid of human epidermal-type FABP (4LKT, green sticks) and palmitic acid in human liver-type FABP (3VG7, purple sticks) are shown using the PyMOL structure alignment suit for ligand orientation comparison. **(C)** The effect of lipid NDs on holo-dFABP mapping onto the liganded-like structure (5GGE) is shown in sausage representation. The structure is superimposed with the 5GGE docking ligand using docosahexanoic acid (HXA, cyan sticks) in human B-FABP (1FDQ) and PatchDock, and the two residues interacting with the carboxylate head group of the docked HXA ligand are indicated in CPK with labels. Three aromatic residues of 5GGE, 5GKB and the docking model, which are located in the β C-D loop and β D-E, are labelled in magenta, green and mustard, respectively.

180 the polar heads of biomembranes and nonpolar residues to interact with hydrophobic ligands
181 embedded in lipid bilayers. This interplay of membrane-association regions constructed in space is
182 likely to imitate amphitropic peripheral proteins.

183 A ligand rescue experiment was performed using OA for further analyses of the cross-peak
184 change, and representative residues participating in the membrane interaction are shown in **Supp.**
185 **Fig. S6**. Notably, the signals representing apo-dFABP binding to nanodiscs recovered by rescued
186 ligand OA were more similar for conformer-B', which did not completely superimpose on the spectra
187 of OA-liganded holo-dFABP, implying that the ligand can be internalized into the escort protein
188 and modulate the dissociation of the holo form from the membrane mimetics. In relation to both
189 ligand binding and membrane interaction, four out of five selected residues shown in **Fig. 5B** are
190 polar and charged, and the two residues in squares (R30 and S60) lie at the protein-membrane
191 interface. The unknown ligand map coheres with those of ligands from other similar escort proteins
192 (e.g., heart-type FABP, epidermal-type FABP and liver-type FABP), and the docking complex signifies
193 that the aliphatic tails of ligands around the portal region interfere with the protein-membrane
194 interactions (**Fig. 5C**).

195 According to comprehensive data obtained for these residues, when the apo-dFABP first ap-

Figure 6. Proposed model for ligand-regulated dFABP transport. The mechanistic trafficking model of fatty acid (FA) accompanied by dFABP is shown in detail. A putative ligand transfer model was proposed in conjunction with our study (as shown in the square) as follows: 1) The apo form of the escort protein FABP attaches to the lipid bilayer. 2) Apo-FABP slides to accommodate complementary LCFA. 3) The ligand-bound form, holo-FABP, dissociates from the lipid bilayer. 4) Importin α (PDBID: 1EJL) recognizes the spatial arrangement of the NLS in the holo form of FABPs (Amber-Vitos et al., 2015). 5) Holo-FABP transfers the ligand to PPARs (Armstrong et al., 2014). 6) PPARs trigger gene transcription and produce proteins for lipid metabolism (e.g., β -oxidation) in the mitochondria or through other signaling pathways. The fraction of attenuation is illustrated in sausage representation for apo- and holo-dFABP (Fig. 4D, Supp. Table S14) (5GKB and 5GGE, respectively), and the PyMOL-defined cavity water molecules and incomplete ligand-like maps are shown in spheres and meshes, respectively.

proaches a lipid ND, a rolling motion occurs in the region composing the β G-H β E-F loops and the α I-II turn, which subsequently turns towards the portal and gap regions for ligand accommodation (Fig. 6). In particular, the intensity of conformer-B' remained almost unchanged during lipid ND titration, whereas that of conformer-A of apo-dFABP continued to diminish (Supp. Fig. S6B and S7C). Together with the consistent results showing a barely changed intensity in conformer-B' between apo-dFABP and holo-dFABP during lipid ND interaction, these findings strongly indicate that the ligands escorted by dFABP would favour unidirectional transportation (Fig. 4A and B).

Discussion

Intracellular cargo transport is a complex and highly dynamic process. By analyzing dFABP with enriched conformational homogeneity, we present a mechanistic model for the transport directionality of lipophilic cargo molecules in representative escort proteins, namely, FABPs. FABPs comprise ten β strands, which are organized orthogonally as a clamp appended to a short helix-turn-helix motif as the portal cap region near N terminus (33). Previous studies have reported that the minor open state of the portal region in apo forms of FABPs was responsible for ligand entry (17, 34), while the slow protein conformational exchange was suggested to be irrelevant to the ligand association

(18). A fundamental problem with escort protein-dependent ligand transport, such as the transport of LCFAs by FABPs, is whether the directionality is reversible or irreversible. Initial analysis of the ligand-FABP complex structures in the Protein Data Bank did not reach any conclusion regarding the transport mechanism (35), and the hypothesis that the inward state of the conserved Phe residue in the β C-D loop of FABPs corresponds to the liganded closed form and the outward state to the unliganded open form has not been universally consistent (e.g., 5BVQ, 4I3B). In our view, the potential interactions among several key factors, including the escort protein, the target ligand, and the ligand donor, all need to be considered concurrently for a successful interpretation of the dynamic cargo transport mechanism.

In this article, we provide several lines of compelling evidence, including biophysical analyses and structural comparisons, to support our speculation that the lipophilic ligand facilitates protein-ligand complex dissociation from ligand donor membranes. The level of trace contaminants in the urea-treated sample was unproblematic in our conventional ligand-protein interaction analyses (**Supp. Fig. S4B and C, Supp. Fig. S8**). According to the CD studies shown in **Fig. 1** and **Supp. Fig. S2**, however, some biophysical properties such as thermal stability might yield inappropriate interpretations (36, 37). The decreased T_m of the urea-treated sample in CD experiments might have resulted from an association between the protein and nonprotein membrane-like debris (**Supp. Fig. S3**). The differences between the apo and holo forms of urea-treated dFABP that are seen in far-UV CD spectra may result from the presence of associated impurity because both forms had secondary structures with similar thermal stability in the GuHCl-purified sample (**Supp. Fig. S2A**). Further investigation of the membrane interactions confirmed that the holo form had far fewer interactions with membrane mimetics (**Fig. 4A and B**) and that the apo form dissociated from membranes by an additional ligand binding, as seen in the rescue study (**Supp. Fig. S6C**). Therefore, the ability of OA to cause the urea-treated sample to dissociate from membrane-like debris and adopt the free OA-liganded form would explain the higher secondary thermal stability of the OA-liganded urea-treated dFABP (**Supp. Fig. S2A**). Although the analysis of protein thermal stability based on precipitation using far-UV CD spectra was not applicable (**Supp. Fig. S2A and D**), the proteins unfolded cooperatively and globally, as seen in intrinsic fluorescence experiments (**Supp. Fig. S2C**). The changes in T_m (as determined using intrinsic fluorescence spectra) between the apo and holo forms of urea-treated and GuHCl-purified dFABP were approximately 10 °C and 5 °C, respectively, and both holo forms had identical melting curves, suggesting that the liganded forms of dFABP were consistent (**Supp. Fig. S2C**). Moreover, the difference in stability between the apo and holo forms was confirmed by a chemical denaturation experiment, which showed that the rigidity assisted by the ligand was similar to the thermal stability (**Supp. Fig. S4A**). Altogether, the intrinsic fluorescence experiments on dFABP demonstrated that the findings regarding the difference in thermal unfolding between the apo and holo forms were consistent with those of previous CD studies (37, 38), even if the T_m values from the CD spectra were not readily interpreted.

We discovered through both microscopic and macroscopic analyses that the general heterogeneity of FABPs, including unknown ligands and membrane-like debris, may be a reason for inconsistency in the Phe residue orientation in the β C-D loop (e.g., penguin apo-AFABP, 5BVQ). Based on the crystallization results, the urea-treated liganded-like dFABP exhibited a tertiary structure similar to that of the GuHCl-purified apo-dFABP, except that no ligand-like electron density was calculated inside its binding cavity (**Fig. 3**). Therefore, the difference in temperature factors around the portal and gap regions may be attributed to conformational heterogeneity (**Supp. Fig. S10**). On the basis of a further comparison of the ordered waters in the ligand binding cavity of liganded-like and apo-dFABPs, we conjectured that the residues around ligand entry sites likely have higher temperature factors in the apo form of urea-treated dFABP because of the possible lack of an ordered-water hydrogen networks resulting from the flexible hydrophobic tail of the unknown ligand (**Fig. 3A**). Although the HSQC spectrum of urea-treated liganded-like dFABP exhibited minor perturbation upon ligand binding (**Supp. Fig. S9A**), a high proportion of the holo form left inadequate space for further membrane interaction. The spectra of urea-treated liganded-like

dFABP and OA-dFABP, especially in the region from β D to E, are indistinguishable from ligand entry to membrane interaction (**Fig. 2D and 4D**).

Consequently, GuHCl-purified apo-dFABP was used to study the relationship between proteins and membranes in the presence and absence of ligands. The broadened apo-dFABP signals resulting from membrane interaction were superimposed on the unliganded 5GKB structure, and the residues were grouped by decay features corresponding to the order of membrane attachment (**Fig. 4D, Supp. Fig. S5**). By carefully dissecting the clusters in terms of ligand binding and membrane interaction, a harmonious coupling was found to explain the interactions among lipophilic ligands, escort proteins, and bilayer membranes as follows: 1) based on the crystallographic temperature factors, we assumed that β E-F controls ligand entry; and 2) membrane attachment was described as being initiated by attaching to the β G-H loop and rolling towards the region involving the α II and β D-E because the signals for the β G-H and β E-F loops decayed faster than those for the portal and gap regions (α II/ β D-E) (**Fig. 6**). The ligand is assumed to participate in the interface between the protein and the membrane, and liganded dFABP tended to dissociate from the membranes after binding to the ligand, as reported by ligand OA rescue analysis (**Supp. Fig. S5**).

Numerous studies have suggested that positively charged residues in the helix region control membrane interactions (39-41). More recent research has demonstrated that these residues are part of a spatially arranged nuclear leading sequence (42-44). Although residues in α II, such as R30, were disturbed by ligand and membrane titration (**Supp. Fig. S6A and B**), they were neither the main cause of nor independent contributors to the membrane attachment. In addition to allowing further study of membrane interaction, the enriched conformational homogeneity of dFABP provides a promising means of distinguishing membrane interactions from other interactions and demonstrates that the conformer-A signals of residues that participate in membrane interaction tend to disappear when the interaction occurs (**Supp. Fig. S6B, S7C**). Residues that do not strongly contribute to membrane interaction and those facing the ligand binding cavity (e.g., Y127) do not fully lose intensity or show no chemical shift perturbation after membrane titration (**Supp. Fig. S7D**), in contrast to how ligand titration transforms conformer-A into the OA-liganded holo form (**Supp. Fig. S7B**).

According to our findings, the greatly decreased membrane affinity of liganded dFABP determines ligand transport directionality. Not only does the orientation of ligand binding inside the protein affect repulsion in the protein-membrane interaction (**Fig. 7B and C**), but also the ligand donor membranes may induce the open state of the escort protein to enable ligand uptake (34). Considering that lipophilic LCFAs are physiologically embedded in membranes and that high affinity for FABPs is observed in vitro, the combination of these two scenarios may further explain why not all ligands with similar binding affinities can trigger FABP nuclear translocation. First, ligands that can be uploaded complementarily into the escort protein may repel either the α II of FABPs (45) or the charged interface of FABP-membrane due to their aliphatic tails and thereby force FABPs to dissociate from the membrane (**Fig. 5**). In contrast, ligands that do not fold properly into the binding cavity of FABPs would leave a tail below the membrane surface inside the hydrophobic environment, thus preventing the protein from dissociating. Moreover, the spatial distribution of charged residues in β C-F and the conserved Phe residue in the β C-D loop seems to facilitate the attachment of FABPs to membranes. These findings might be crucial, necessitating the re-evaluation of previous studies on the interactions among FABPs, complementary ligands and membranes based on molecular dynamic simulations, which have mostly focused on explaining how LCFAs enter FABPs or how holo-FABPs transport ligands to membranes (46-48). The relationship between liganded FABPs and membranes provides an alternative interpretation: only through a specific orientation of the ligand in the protein can the aliphatic tail modulate protein-membrane dissociation and act as activator ligands to control protein-ligand complex release for nucleocytoplasmic translocation (44). The use of blockers targeting holo-FABPs instead of competitors for apo-FABPs may be able to interrupt the pathways for entering the nucleus or for interacting with effectors. Through illuminating the unidirectional transport by dFABP, this study advances research on FABPs, particularly on their

relationships with complementary LCFAs and membranes, which will enable further investigation of their roles in the nucleus. The mechanism generalizes a model, obtained by clarifying the working patterns of FABPs, in which the complementary lipophilic ligand is the decisive factor in regulating its own transport directionality in membrane-associated escort proteins.

Materials and Methods

Cloning and expression of dFABP

The second spliced isoform (CG6783-B) of the dFABP locus (CG6783) in the *Drosophila* genome was found to share the highest homology to mammalian brain-type FABP, and its 130-residue peptide sequence was expressed and purified for subsequent structural studies (49). In brief, the open reading frame coding for dFABP isoform B was chemically synthesized with E. coli-optimized codons (Eurofins Scientific, Luxembourg), and inserted into the pET23a(+) expression vector (Merck, Darmstadt, Germany) at the NdeI/XhoI sites. The dFABP-pET23a plasmid was subsequently transformed into E. coli BL21-Gold (DE3) competent cells (Thermo Fisher Scientific, MA, USA) for recombinant protein expression. Individual colonies were selected, and an aliquot of host cells harboring the correct insert was inoculated into 20 mL of Luria broth (LB) containing 100 µg/mL ampicillin. The broth was incubated at 37 °C with 180 rpm agitation for 12 h as a condensed starting culture. The 20-mL cell culture was either poured into 800 mL of LB or precipitated and then resuspended in 1 L of uniformly isotopes-labelled M9 medium, which consisted of 1 g of ¹³C-glucose/1 g ¹⁵N- NH₄Cl (Cambridge Isotopes Laboratories, MA, USA) with 20 µg/mL ampicillin. Subsequently, dFABP expression was induced with 0.2 mM isopropyl β-D-1-thio-galactopyranoside (IPTG) when the optical density at 600 nm reached 0.6, and the cultures were incubated for an additional 6 h at 37 °C.

Purification of dFABP with higher conformational homogeneity

Supp. Fig. S1 represents the flowchart for dFABP purification, by our improved new strategy with the combination of 8 M urea and the partially unfolding reagent 2 M GuHCl, depending on the denaturation analysis (**Supp. Fig. S4A**). Briefly, a batch of 800 mL LB broth or 1 L isotopes-labelled of the protein expression culture was harvested by 12,000 × g centrifugation at 4 °C for 20 min. Cell pellets were resuspended collectively in 30 mL of lysis buffer (20 mM Tris pH 8.0, 2 mM EDTA, 1 mM β-ME, 0.05% NaN₃) and frozen at -20 °C before further purification. The thawed cells were lysed by sonication and centrifuged at 38,500 × g for 15 min at 4 °C. The pellet was solubilized in 5 mL of 8 M urea at 4 °C for 3 h and then centrifuged again at 38,500 × g at 4 °C for 15 min to remove insoluble debris. The supernatant was dialyzed against 1 L of the sample buffer (20 mM Tris and 1 mM β-ME, pH 8.0) using 3-kDa molecular weight cutoff dialysis tubing (Orange Scientific, Braine-l'Alleud, Belgium) and stirred at 4 °C for 6 h. The precipitates were removed by centrifugation at 38,500 × g at 4 °C for 15 min, and the supernatant was mixed with an equal volume of buffer B (1 M (NH₄)₂SO₄ in 20 mM Tris, pH 8.0) and concentrated to 10 mL for hydrophobic interaction chromatography (5-mL HiTrap phenyl HP column, GE Healthcare, IL, USA). Subsequently, a 2-mL elution of hydrophilic flow-through was incubated in the absence (denoted as urea-treated dFABP) or presence (denoted as GuHCl-purified dFABP) of 2 M GuHCl preincubated at room temperature for 30 min for the next size-exclusion chromatography. The urea-treated dFABP was obtained and validated by twice injection of S-200 size-exclusion chromatography (HiPrep 16/60 Sephacry S-200 HR, GE Healthcare), and the GuHCl-purified dFABP underwent two rounds of S-200 size-exclusion chromatography, with final validation by S-75 size-exclusion chromatography (Superdex 75 10/300 GL, GE Healthcare). All chromatography samples had a volume of 5 mL after the removal of precipitates by 0.22-µm membrane filters. The crude quality of the desired dFABP protein after each step was assessed using SDS-PAGE. The polished samples at the final purification step were further determined through ESI UPLC Q-TOF mass spectrometry (Impact HD, Bruker, MA, USA) and the yield of 1 L of culture was approximately 15 mg, as measured by Bradford and BCA assays. The

361 final dFABP samples were stored in 500 μ M in the sample buffer at 4 °C until use in 6 months.

362 **Spectroscopic characterization of dFABP**

363 A 25- μ M sample was prepared in sample buffer using 0.1-cm quartz cuvette for an Aviv Model 202
364 CD spectrometer (Aviv Biomedical, NJ, USA) and a 2- μ M sample was used for intrinsic fluorescence
365 measurement (F-7000, HITACHI, Tokyo, Japan). The mean residue ellipticity (MRE) was calculated
366 based on the initial sample concentration in temperature-dependent far-UV CD experiments. As
367 a result of the decrease in soluble concentration during the heating process in the urea-treated
368 sample, the CD melting curves from 25 °C to 95 °C measured at a wavelength of 215 nm were
369 expressed in millidegrees instead of MRE. Holo-dFABP was combined with OA (100 mM stock in 250
370 mM KOH, Avanti Polar Lipids, AL, USA) to achieve a molar ratio of 1:2 in the CD and fluorescence
371 spectroscopic experiments. The excitation wavelength of intrinsic fluorescence was set at 275 nm
372 because there were four tyrosines instead of tryptophan in the dFABP sequence and detected
373 at an emission wavelength of 303 nm. Each temperature of the stepwise melting experiments
374 was equilibrated for 10 s before manual detection. The CD melting curve was automatically set at
375 increments of 2 °C from 24 °C to 96 °C and equilibrated for 1 s with 0.5-s data averaging. The assay
376 results were fitted using the Sigmoidal model in GraphPad Prism 6 software (GraphPad Software,
377 Inc. La Jolla, CA, USA).

378 **Crystallographic characterization of dFABP**

379 The dFABP samples were concentrated to approximately 8 mg/mL in 20 mM Tris buffer (pH 8.0)
380 for crystallization. Crystals were screened either manually or by using the automatic protein
381 crystallization robot Oryx8 (Douglas Instruments, Hungerford, UK) with Wizard crystallization
382 screens (Rigaku, WA, USA). The urea-treated dFABP (denoted as 5GGE) was crystallized through
383 vapour diffusion in hanging-drop format, and a 1- μ L protein sample was mixed with a 1- μ L drop of
384 reservoir solution against 200 μ L of reservoir solution in 24-well Linbro plates (Flow Laboratories,
385 VA, USA). The GuHCl-purified dFABP (denoted as 5GKB) was crystallized through vapour diffusion
386 in sitting-drop format. The microbatch screening system was applied under the crystallization
387 conditions of Emerald Wizard II-F7 (0.1 M phosphate citrate, 1.6 M NaH₂PO₄/0.4 M K₂HPO₄, final pH
388 5.2), and 0.3 μ L of GuHCl-purified dFABP was mixed with 0.3 μ L of reservoir solution against 60 μ L
389 of crystallization solution in 96-well crystallization plates. To acquire X-ray diffraction data, crystals
390 were grown for 2 months at 20 °C, and data acquisition was performed at the National Synchrotron
391 Radiation Research Center, Taiwan. The data were processed and scaled using HKL2000 software,
392 and the molecular replacement program MOLREP in CCP4 was applied for phase determination (50).
393 The initial models for 5GGE and 5GKB were 1FTP and 5GGE, respectively. Further model building,
394 the incorporation of the ligand citrate in 5GGE, and both structural refinements were performed
395 using the PHENIX suite (51). The structure of liganded-like dFABP (5GGE) was subjected to the
396 docking program PatchDock for ligand docking (docosahexanoic acid, HXA of 1FDQ).

397 **NMR HSQC spectroscopy analysis**

398 Samples for NMR were buffer exchanged into 50 mM phosphate buffer (pH 6.8) with the addition
399 of 10% D₂O and trace 4,4-dimethyl-4-silapentane-1-sulfonate as a calibration standard for spectra
400 collected at 298K. An Avance-600 MHz spectrometer was used for the OA titration of HSQC spectra,
401 and an Avance-850 MHz spectrometer (Bruker, Germany) equipped with a cryoprobe was used
402 to acquire the spectra for nanodisc interactions. The holo form of the OA-dFABP sample was
403 prepared at a 1:3 molar ratio of protein to OA, and the 3D spectra (HNCA, HNCOC, HNCACB,
404 CBCACONH, HNCO and HNCACO) for sequential assignments were acquired on the Avance-850
405 MHz instrument. Identical 3D NMR experiments for assignments of apo-dFABP were recorded for
406 back-calculation from the OA-dFABP assignments (Supp. Table S11, S12). The data were processed
407 using NMRPipe (52) and autoassigned by MANI PINE server v.2.0 (53), with manual inspection
408 through NMR assignment and Sparky integration software (Goddard TD & Kneller DG, 2008, SPARKY

3. University of California, San Francisco, CA, USA). Chemical shift perturbations (CSPs) from the initial conformer-A to the final holo form for dFABP were analyzed by weighting the averaged ^1H and ^{15}N chemical shift changes with the equation $\Delta\delta = [(\Delta\delta_{\text{HN}})^2 + (0.17\Delta\delta_{\text{15N}})^2]^{1/2}$.

Membrane interaction of dFABP with lipid bilayer nanodiscs

The scaffold protein MSP1D1 was expressed and purified for the self-assembly of lipid bilayer nanodiscs with DOPG/DOPC (1:1) as a membrane mimetic (54). GuHCl-purified dFABP was prepared at 100 μM and 70 μM for lipid ND titration, and HSQC and TROSY spectra were obtained, respectively. Holo-dFABP was mixed with 2.5-fold OA to ensure ligand saturation before interaction with lipid NDs. A final 1:1 ratio of protein to lipid NDs was used for the TROSY and HSQC spectra. The settings for the TROSY spectra were based on the pulse sequence method described previously (55) in order to optimize the power of water suppression by gradient-tailored excitation. The data calculation for the fraction of attenuation was weighed by dividing the initial intensity by a factor 10, depending on the diminishing effect in TROSY spectra, to enlarge the difference in the broadened signals after the addition of lipid NDs to apo-dFABP.

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Additional information

Author contributions

Y.Y.C. designed the study, executed all experimental work, data interpretation and drafted the manuscript. Y.F.H. collaborated in improving the protein purification processes. H.H.L. contributed to cloning and crystal collection of urea-treated wild-type dFABP. W.S.C. and P.C.L. contributed to project supervision and assisted manuscript preparation.

Competing interests

The authors declare that no competing interests exist.

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588 **Supplementary Fig. S1. Scheme of the purification procedures for urea-treated and GuHCl-**
589 **purified dFABP**

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591 **Supplementary Fig. S2. Comparison of thermal stability and sample solution after heating**
 592 **(A)** CD thermal melting curves of urea-treated and GuHCl-purified samples were compared in the
 593 presence or absence of ligand OA. No apparent secondary conformational change was detected
 594 at 215 nm for the GuHCl-purified dFABP with or without the ligand OA. **(B)** SDS-PAGE analyses of
 595 the urea-treated and GuHCl-purified samples before and after heating in CD Tm experiments. The
 596 urea-treated sample showed solid precipitates after heating, whereas the GuHCl-purified sample
 597 showed a clear solution, with both supernatants displaying bands at higher molecular weights. **(C)**
 598 The unfolding curves derived from the intrinsic fluorescence spectra of urea-treated and GuHCl-
 599 purified dFABP in the presence or absence of the ligand OA were measured to determine the tertiary
 600 thermal stability. **(D)** The sample solution of GuHCl-purified apo-dFABP remained transparent after
 601 heating, whereas that of urea-treated apo-dFABP formed white solid precipitates.

Supplementary Fig. S3. Comparison of the purification profiles of urea-treated and GuHCl-purified dFABP

(A) The first round of S200 size-exclusion chromatography (SEC) for HIC eluted samples is presented with (solid line) and without (dotted line) the 2 M GuHCl treatment. With the treatment, two groups of components were separated and denoted eluent P1 (1) and eluent P2 (2 and 3). (B) Eluent P2 of the GuHCl-purified dFABP from the first S200 SEC in the presence (solid-line) or absence (dotted line) of repeated 2 M GuHCl treatment is compared for the second round of S200 SEC. This step resulted in the separation of four components, denoted as eluents A, B, C and D. (C) Eluent D was finally confirmed by analytical S75 chromatography to be a single elution peak. The sample was preincubated with 2 M GuHCl before injection. (D) Eluents of the first round of S200 SEC from urea-treated and GuHCl-purified samples were analyzed using SDS-PAGE and denoted as 1 (P1), 2 and 3 (P2). (E) SDS-PAGE analysis of the eluents in the second round of S200 SEC involving GuHCl-purified samples. Four isolated eluents (denoted A, B, C and D) were separated by repeated preincubation with 2 M GuHCl before the injection. (F) SDS-PAGE determination of the final fraction of S75 chromatography from the GuHCl-purified sample. The arrow indicates protein bands corresponding to the representative escort protein dFABP.

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Supplementary Fig. S4. Biochemical properties of dFABP

(A) Urea-treated dFABP was titrated with GuHCl (0 M to 3 M) and monitored by far-UV CD. Both apo- and holo-dFABP were unfolded in 2 M GuHCl, and this concentration was used in the GuHCl purification. **(B)** OA binding assay based on the intrinsic fluorescence of urea-treated dFABP (5GGE): 6 μ M protein was mixed with OA at a final molar ratio of 1:2. Data were collected using an excitation wavelength of 280 nm and an emission wavelength of 302 nm. The K_d was calculated to be approximately 2.4 μ M. **(C)** ANS competition assay; 1 μ M GuHCl-purified dFABP (5GKB) presaturated with 5 μ M ANS was mixed with protein in competition with the ligand OA at a final molar ratio of 1:2, and ANS fluorescence (detected at 495 nm) was measured to enable the calculation of K_d calculation (approximately 1.4 μ M). **(D)** GuHCl-purified dFABP at a concentration of 170 μ M was subjected to iTC200 and titrated with OA at 30°C. The derived K_d is approximately 1.6 μ M.

Supplementary Fig. S5. Membrane interaction pattern of apo-dFABP

(A) The fraction of attenuation was derived from TROSY spectra of 5GKB in the presence or absence of lipid ND (Fig. 4C). The average change is indicated by the dotted line. Residues labelled in red are decayed immediately; others above the average value were analyzed further by lipid ND titration using a series of HSQC spectra. Residues shown in yellow are not assigned. **(B)** Residues with attenuation fractions above the average in the TROSY spectra were selected, and their decay rates were analyzed using lipid ND HSQC titration. The signals of selected residues were fit to the one-phase decay model in GraphPad, and those above the average K (2.88 M^{-1}) were defined as faster decaying residues. **(C)** The residues were classified according to a combination of the attenuation fraction and signal decay rate, and representative residues appended with A indicate conformer-A. Residues with consistent intensities bracketed as L51-A are depicted in a thin sausage representation, as shown in Fig. 4D. A single-exponential decay function was applied to all TROSY-selected residues, and three representative residues, namely, R30, T74 and K99, are plotted and colored according to their decay patterns.

Supplementary Fig. S6. Comparisons of ligand binding and membrane interaction as analyzed in the signal recovery study

(A) Four representative ratios in the ligand titration illustrated by R30. The gray arrow indicates the disappearance of conformer-A upon ligand titration, and in the final spectrum, the OA-liganded cross-peak in labelled with the representative residue in brackets. (B) Four representative ratios in the lipid ND titration are illustrated by R30. The signal for conformer-B' of R30 in apo-dFABP changes very little (black arrow), whereas the signal for conformer-A continues to broaden (gray arrow). (C) Four representative residues with higher levels of disappearance, as seen in Fig. 4D, in an OA rescue experiment. The signals of conformer-A (gray arrow) of apo-dFABP that disappeared after interaction with membrane mimetics are recovered as conformer-B' (gray dotted arrow) after the addition of ligand OA. The final comparison panel plots the preceding spectra together with the OA-liganded spectrum (light magenta), and a dotted arrow links the initial (green) and the rescued (red) signals.

Supplementary Fig. S7. Comparison of residues in different regions of dFABP in terms of their effects on ligand binding and membrane interaction

(A, B) Four representative ratios used in the ligand titration of GuHCl-purified apo-dFABP are presented. The ligand OA titration for E69 and Y49, which are located near the membrane attachment (β E) and ligand entry (β B) sites, respectively, shows that conformer-A disappeared sooner during ligand binding. The ligand titration of Y127, which controls binding to the carboxylate heads of ligands, indicates that CSP occurred upon ligand binding (the arrow indicates CSP). (C, D) Four representative ratios used in the lipid ND titration of GuHCl-purified apo-dFABP are presented. The lipid ND titrations for E69 and Y49 show that conformer-A disappeared more slowly during lipid ND titration (dashed arrows) than during ligand titration. In contrast, the conformer-B' of both residues was nearly unchanged (arrows) during membrane attachment. The lipid ND titration of Y127 indicates few CSP changes upon lipid interaction.

681

682 **Supplementary Fig. S9. Comparison of NMR HSQC spectra in the presence and absence of**
683 **ligand OA in samples with different conformational homogeneity**

684 **(A)** The final holo form (magenta) is superimposed with the initial apo form (cyan) to compare the
685 cross-peak dispersion of OA titration in urea-treated dFABP through HSQC. **(B)** The final holo form
686 (magenta) is superimposed with the initial apo form (cyan) to compare the cross-peak dispersion
687 of OA titration in GuHCl-purified dFABP through HSQC. Residues labelled with a symbol prime
688 represent conformer-B', and residues colored in gray exhibit a relatively low proportion compared
689 to conformer-A.

690

691 **Supplementary Fig. S10. Temperature values of dFABP crystal structures**

692 Each C α was plotted as a solid circle (5GGE) and an empty circle (5GKB) with the y-axis on the left
693 and right. Higher values of residues in the gap region (β E and β F) of 5GKB are highlighted in gray
694 stripes. Dashed and dotted lines are the average values of 5GGE and 5GKB, respectively.

Residue	P(H-N)	¹ H ^N	¹⁵ N	¹³ CO	¹³ C ^α	¹³ C ^β	Residue	P(H-N)	¹ H ^N	¹⁵ N	¹³ CO	¹³ C ^α	¹³ C ^β
MET ¹	0						LEU ⁶⁶	1	9.238	123.25	179.82	55.8	40.4
SER ²	0						GLY ⁶⁷	1	9.428	108.6	173.42	46.3	
PHE ³	0.483	7.896	128.22	175.58	57.1	39.8	VAL ⁶⁸	0.005					
VAL ⁴	1	7.184	124.03	177.8	64.2	31.1	GLU ⁶⁹	1	9.152	133.56	175.29	57.3	31.1
GLY ⁵	1	9.5	115.56	174.26	45.5		PHE ⁷⁰	1	9.319	123.3	172.79	55.5	
LYS ⁶	1	7.534	119.92	174.81		33.9	ASP ⁷¹	1	8.446	119.62	174.97	54.4	41.9
LYS ⁷	1	8.416	123.11	175.55	55.7	34.6	GLU ⁷²	1	9.066	126.84	174.28	55.1	
TYR ⁸	1	8.946	121.23	174.69	56.6	42	GLU ⁷³	1	8.58	128.41	177.82	54.2	30.1
LYS ⁹	1	9.466	124.19	176.88	54.9	36.6	THR ⁷⁴	1	9.324	119.45	178.24	62	70.7
LEU ¹⁰	1	8.096	130.36	174.28	57.8	42.1	LEU ⁷⁵	1	9.435	122.69	177.44	58.4	43.6
ASP ¹¹	1	9.69	127.96		55.1		ASP ⁷⁶	1	9.379	110	175.41	53.1	39.7
LYS ¹²	1	7.853	115.61	174.27	55.3	36.2	GLY ⁷⁷	1	7.988	108.7	175.5	45.8	
SER ¹³	1	8.594	116.48	173.42	57	64.4	ARG ⁷⁸	1	7.631	119.23	175.57	55.8	31.6
GLU ¹⁴	1	8.824	128.5	176.08	55.3	32.7	ASN ⁷⁹	1	8.497	120.65	175.12	53.5	39.8
ASN ¹⁵	1	9.432	121.5			38.3	VAL ⁸⁰	1	8.951	117.05	174.9	58.4	35.7
PHE ¹⁶	1	8.801	120.19	177.88	59.1	39	LYS ⁸¹	1	7.846	120.53		54.3	33.8
ASP ¹⁷	1	8.815	119.97	178.31	58.9	41.9	SER ⁸²	1	8.792	120.94	172.16	56.7	67.6
GLU ¹⁸	1	8.417	117.92	179.06	60.1	28.6	ILE ⁸³	1	8.064	118.22	174.23	61.5	42
TYR ¹⁹	1	7.624	120.28	175.79	62.7	38.9	ILE ⁸⁴	1	9.904	130.4	175.15	59.9	38.7
MET ²⁰	1	8.261	115.97	178.71		34.4	THR ⁸⁵	1	8.81	117.18	172.41	59.7	72.2
LYS ²¹	1	8.649	120.11	180.82	60.3	32.2	LEU ⁸⁶	1	8.785	123.61	175.04	54	47.6
GLY ²²	1	7.836	121.8	178.13	58.3	29	ASP ⁸⁷	1	8.607	127	176.83	53.1	42.7
LEU ²³	1	7.296	117.39	177.3	55.3	43.6	GLY ⁸⁸	1	9.129	116.81	173.61	47.9	
GLY ²⁴	1	7.673	106.58	174.88	45		ASN ⁸⁹	1	8.729	126.85	173.41	52.5	38.4
VAL ²⁵	0.004						LYS ⁹⁰	1	8.006	119.22	175.63	55.3	34.2
GLY ²⁶	0						LEU ⁹¹	1	9.528	131.67	177.44	53.2	45.4
LEU ²⁷	1	8.332	121	178.89	59.4	42.4	THR ⁹²	1	9.589	128.51	171.44	64.3	69.4
VAL ²⁸	1	8.392	118.12	178.65	66.8	31.3	GLN ⁹³	1	9.207	129.12	173.69	53.4	30.6
THR ²⁹	1	7.511	117.93	176.47	999	67.5	GLU ⁹⁴	1	8.811	127.39	176.09	999	31.1
ARG ³⁰	1	8.672	123.07	178.4	59.8	30.1	GLN ⁹⁵	1	8.637	122.38	176.39	54.7	30.2
LYS ³¹	1	8.062	118.69	180.62	59.8	31.8	LYS ⁹⁶	1	8.903	124.15		55.6	32.9
MET ³²	1	7.328	117.88	179.26	57.7		GLY ⁹⁷	1	7.666	109.05	173.34	44.7	
GLY ³³	1	8.921	107.81	175.52	48		ASP ⁹⁸	0.006					
ASN ³⁴	1	8.458	116.95	175.91	55.2	39.5	LYS ⁹⁹	0.009					
SER ³⁵	1	7.688	113.04	174.57	59.2	65	PRO ¹⁰⁰	0					
LEU ³⁶	1	7.233	120.84		54.9	44.3	THR ¹⁰¹	1	8.196	115.34	171.05	59.5	70.6
SER ³⁷	0						THR ¹⁰²	1	7.83	122.73	173.35	61.7	71.1
PRO ³⁸	0						ILE ¹⁰³	1	9.423	126.16	176.16	61.1	41.1
THR ³⁹	1	8.648	111.04	174.14	60.4	71.6	VAL ¹⁰⁴	1	9.616	132.61	174.28	62.2	33.6
VAL ⁴⁰	1	9.387	124.32		61.2	36	ARG ¹⁰⁵	1	8.856	127.68	173.01	54.8	32.5
GLU ⁴¹	1	8.437	123.82	175.07	55.5	35.3	GLU ¹⁰⁶	1	8.635	124.46	174.97	54.7	31.7
VAL ⁴²	1	10.288	129.77	175.45	60.8	33.3	PHE ¹⁰⁷	1	8.493	124.97	176.88	58.5	41.8
THR ⁴³	1	9.431	118.68	172.32	60.1	71.6	THR ¹⁰⁸	1	9.154	115.43	174.88	60.2	71.9
LEU ⁴⁴	1	8.161	121.39	174.77	54.5	46.2	ASP ¹⁰⁹	1	8.889	119.54	176.62	57.7	40.3
GLU ⁴⁵	1	8.083	127.64	176.25	55.1	31.2	ASN ¹¹⁰	1	8.292	111.8	175.66	53.7	40.8
GLY ⁴⁶	1	8.968	117.96	173.4	47.6		GLU ¹¹¹	1	8.184	121.41	172.05	55.7	34.4
ASP ⁴⁷	1	8.377	126.68	175.17	52.9	41.1	LEU ¹¹²	1	8.643	125.81	179.73	52.8	43.4
THR ⁴⁸	1	8.093	116.46	174.6	62.5	69.9	ILE ¹¹³	0.928	7.479	121.06	179.86	60.6	39.1
TYR ⁴⁹	1	9.034	133.31	176.99	57.6	42	THR ¹¹⁴	1	9.855	127.53	172.91	61.4	70.5
THR ⁵⁰	1	8.815	114.98	173.07	61.8	71.6	THR ¹¹⁵	1	9.612	124.57	174.56	61.7	69.5
LEU ⁵¹	1	9.35	129.98	176.52	54.5	45.3	LEU ¹¹⁶	1	9.652	130.4	175.62	54.8	42.8
THR ⁵²	1	9.392	127.74	174.31	62.7	69.7	THR ¹¹⁷	1	8.841	114.04	175.89	60.2	71.5
THR ⁵³	1	9.473	128.16	179.26	62	999	ILE ¹¹⁸	1	8.914	127.38	174.15	60.6	40.4
THR ⁵⁴	0						GLY ¹¹⁹	1	9.19	118.27	175.42	47.3	
SER ⁵⁵	0						ASN ¹²⁰	1	9.017	125.6	175.12	52.9	38.6
THR ⁵⁶	0						VAL ¹²¹	1	8.83	123.38	174.37	63.4	32.5
PHE ⁵⁷	1	7.309	119.54	174.64	58.3	41.8	LYS ¹²²	1	8.661	128.65	174.84	54.8	35.1
LYS ⁵⁸	1	6.965	116.43	174.81	56.3	36.7	CYS ¹²³	1	9.431	128.66	172.96	55.7	28.8
THR ⁵⁹	1	8.602	121.99	173.87	61.7	71.3	VAL ¹²⁴	1	8.415	121.98	177.01	57.0	
SER ⁶⁰	1	9.171	121.22	172.2	56.5	65.8	ARG ¹²⁵	1	7.896	118.06	173.88	54.6	34.9
ALA ⁶¹	1	8.66	127.49	177	51.3	21.9	VAL ¹²⁶	1	8.986	124.09	175.55	61.8	34
ILE ⁶²	1	8.335	117.09	174.7	59.4	41.6	TYR ¹²⁷	1	10.002	128.25		55.8	
SER ⁶³	1	8.472	119.27	173.23	57.2	65.3	LYS ¹²⁸	0.486	7.896	128.22	176.54	54.6	35.4
PHE ⁶⁴	1	8.684	119.41	172.66	55.7	40.4	ALA ¹²⁹	1	8.161	126.91		53	18.7
LYS ⁶⁵	1	9.19	119.14	178.03	54.2	34.4	VAL ¹³⁰	1	8.198	125.39		63.5	33.3

695

696 **Supplementary Table S11. Sequential chemical shift assignments of ¹⁵N/¹³C OA-dFABP (BMRB**
697 **ID: 27112)**

698 Autoassignment was assisted with MANI PINE server v.2.0. The MANI PINE reports were manually
699 inspected through Sparky and subsequently removed, as summarized here. The assignments of ¹H,
700 ¹⁵N, and ¹³C chemical shifts were deposited into the BioMagResBank database under the accession
701 number 27112.

Residue	P(H-N)	¹ H ^N	¹⁵ N	¹³ CO	¹³ C ^α	¹³ C ^β	Residue	P(H-N)	¹ H ^N	¹⁵ N	¹³ CO	¹³ C ^α	¹³ C ^β
MET ¹	0						LEU ⁶⁶	1	9.169	123.13	169.21	53.2	
SER ²	0.987	7.629	113.18	174.28	56.6	62.6	GLY ⁶⁷	1	9.289	108.50	175.53	43.6	
PHE ³	0.948	8.371	119.08	173.45	54.3	37.3	VAL ⁶⁸	1	7.562	120.74	172.18	59.1	
VAL ⁴	1	7.109	123.76	171.13	61.4	28.6	GLU ⁶⁹	0.998	9.102	132.58	174.04		
GLY ⁵	1	9.396	115.33	174.71	42.9		PHE ⁷⁰	0.716	8.974	126.71	172.00	60.2	39.3
LYS ⁶	1	7.437	119.74	174.11	52.7	31.4	ASP ⁷¹	0.998	8.728	114.74	174.54		
LYS ⁷	1	8.275	122.65	171.09	56.4	36.4	GLU ⁷²	0.997	8.246	120.76	174.05	56.7	
TYR ⁸	0.525	8.730	119.79				GLU ⁷³	0.697	7.386	120.78		60.2	29.4
LYS ⁹	0						THR ⁷⁴	0.694	8.119	115.28			
LEU ¹⁰	0.534	8.552	125.62	174.68	55.2	39.3	LEU ⁷⁵	0.377	9.000	118.75	171.58	55.7	
ASP ¹¹	1	9.578	127.66	173.97	52.5	43.5	ASP ⁷⁶	1	9.333	109.25	173.62	50.5	
LYS ¹²	1	7.742	115.53	174.64	52.6	33.5	GLY ⁷⁷	1	7.812	108.34	173.41	43.3	
SER ¹³	1	8.476	116.08	175.53	54.4	61.7	ARG ⁷⁸	1	7.537	119.08	173.39	55.2	29.0
GLU ¹⁴	1	8.754	128.24	172.87	52.6	30.1	ASN ⁷⁹	1	8.398	120.37	173.82	50.8	37.2
ASN ¹⁵	1	9.357	121.37	172.89	51.6		VAL ⁸⁰	1	8.885	117.01			
PHE ¹⁶	0.995	8.652	120.31	171.20	57.0		LYS ⁸¹	0.337	8.264	115.87	173.67	59.6	31.5
ASP ¹⁷	0.917	8.682	119.82		56.1		SER ⁸²	1	8.560	111.31	172.02	57.8	
GLU ¹⁸	0						ILE ⁸³	1	9.766	127.08	174.98	58.5	
TYR ¹⁹	1	7.534	120.13		60.0	36.3	ILE ⁸⁴	1	9.928	129.73	173.81	57.3	36.0
MET ²⁰	1	8.163	115.72	170.32	57.5	31.8	THR ⁸⁵	1	8.729	117.00	176.57	57.0	69.5
LYS ²¹	1	8.556	119.93	168.5	57.5	29.6	LEU ⁸⁶	1	8.697	123.47	173.88	51.3	44.9
GLU ²²	1	7.786	121.75	170.82	55.6	26.4	ASP ⁸⁷	1	8.523	126.75	172.16	50.5	40.0
LEU ²³	1	7.222	117.23	171.70	52.7	41.2	GLY ⁸⁸	1	9.037	116.64	175.30	45.3	
GLY ²⁴	1	7.568	106.16		42.2		ASN ⁸⁹	1	8.637	126.62	175.54	49.8	35.8
VAL ²⁵	0.243	8.040	107.64				LYS ⁹⁰	1	7.922	118.99	173.32	52.5	31.5
GLY ²⁶	0.367	8.044	107.60	171.99	48.5		LEU ⁹¹	1	9.438	131.36	172.51	51.9	42.8
LEU ²⁷	0.996	8.252	116.89	170.03	56.7	39.7	THR ⁹²	1	9.252	127.43	174.65	59.9	67.0
VAL ²⁸	1	8.300	117.98	170.27	64.0	28.7	GLN ⁹³	1	9.387	127.89		57.0	27.5
THR ²⁹	1	7.435	117.82		63.8		GLU ⁹⁴	0.998	7.959	118.67		58.0	
ARG ³⁰	0.111	7.752	122.68	173.88	59.0		GLN ⁹⁵	1	9.365	124.19	172.58	52.1	27.8
LYS ³¹	0.999	8.639	116.79	168.38	57.0		LYS ⁹⁶	1	8.832	124.04	173.10	52.8	30.3
MET ³²	1	7.256	117.67	169.59	55.0		GLY ⁹⁷	1	7.569	109.98	175.60	40.0	
GLY ³³	1	8.942	107.84	173.32	45.2		ASP ⁹⁸	1	8.339	121.93	171.93	54.3	38.0
ASN ³⁴	1	8.371	117.23	173.10	52.6	36.8	LYS ⁹⁹	1	7.786	117.76		50.0	30.6
SER ³⁵	1	7.590	112.91	174.44	56.4	62.3	PRO ¹⁰⁰	0			172.59	60.5	
LEU ³⁶	1	7.153	120.80	172.08	52.3	41.6	THR ¹⁰¹	0.296	8.111	115.25	177.92		
SER ³⁷	0						THR ¹⁰²	0.613	7.83	122.73	173.35	61.7	71.1
PRO ³⁸	0			177.48	61.5		ILE ¹⁰³	1	9.338	126.08	172.77	58.3	38.5
THR ³⁹	1	7.513	117.86	172.23	63.5		VAL ¹⁰⁴	1	9.537	132.46	173.13	59.6	31.0
VAL ⁴⁰	1	8.498	123.13		58.2		ARG ¹⁰⁵	0.208	8.540	124.29	174.00	52.0	29.1
GLU ⁴¹	0.987	8.395	123.95	173.80	52.8		GLU ¹⁰⁶	1	8.635	124.46	174.97	54.7	31.7
VAL ⁴²	1	10.093	129.76	173.51	58.0	30.7	PHE ¹⁰⁷	1	8.409	124.77	172.08	55.7	39.2
THR ⁴³	1	9.343	118.57	176.62	57.3	68.9	THR ¹⁰⁸	1	9.059	115.38	174.03	57.5	69.1
LEU ⁴⁴	1	8.063	121.27	174.19			ASP ¹⁰⁹	1	8.805	119.39	172.34	55.0	37.6
GLU ⁴⁵	1	7.993	127.54	172.69	52.3	28.6	ASN ¹¹⁰	1	8.206	111.67	173.72	50.8	38.2
GLY ⁴⁶	1	8.878	117.79	175.55	44.9		GLU ¹¹¹	0.529	9.003	118.75	173.68	54.5	28.4
ASP ⁴⁷	1	8.377	126.68	175.17	52.9	41.1	LEU ¹¹²	0.996	9.225	123.22	176.20		
THR ⁴⁸	1	7.974	116.11	174.33	59.8	67.2	ILE ¹¹³	0.682	8.338	119.23	177.28	56.8	
TYR ⁴⁹	1	9.019	133.10		54.9		THR ¹¹⁴	0.623	7.874	122.38	176.09	58.6	67.8
THR ⁵⁰	0						THR ¹¹⁵	0.656	9.525	124.40	174.42	59.0	66.8
LEU ⁵¹	0.627	8.887	120.25	176.17			LEU ¹¹⁶	0.971	9.595	130.19	173.39	52.0	40.1
THR ⁵²	0.743	9.283	119.43	171.07			THR ¹¹⁷	1	8.778	113.83	173.48	57.4	68.8
THR ⁵³	0.792	9.219	119.35			68.0	ILE ¹¹⁸	1	8.797	127.07	174.80	57.6	
THR ⁵⁴	0.273	9.524	124.39	175.01	59.3	67.1	GLY ¹¹⁹	1	9.060	117.79	173.93	43.4	
SER ⁵⁵	0.416	9.385	122.63		53.8	63.1	ASN ¹²⁰	0.911	9.615	119.63	173.82	50.2	36.0
THR ⁵⁶	0.658	8.576	127.40	173.88	61.6	65.7	VAL ¹²¹	1	8.748	123.24	174.58	60.7	29.9
PHE ⁵⁷	1	7.220	119.06	174.21	55.5	39.0	LYS ¹²²	1	8.570	128.45		52.2	32.4
LYS ⁵⁸	1	6.924	116.38	174.16	53.6	33.8	CYS ¹²³	1	9.367	128.63	176.00	53.0	26.1
THR ⁵⁹	1	8.532	122.04		59.0	68.5	VAL ¹²⁴	1	7.816	128.11	177.40	58.7	
SER ⁶⁰	0.182	8.127	116.17	177.50	56.3	64.3	ARG ¹²⁵	1	9.531	130.47			
ALA ⁶¹	1	9.077	128.98	175.31	50.7		VAL ¹²⁶	1	8.908	124.26	174.17	59.0	
ILE ⁶²	1	8.736	127.27	175.12	59.3		TYR ¹²⁷	0.996	9.890	128.01	173.60		
SER ⁶³	0.999	8.999	121.71	176.83	54.0	65.1	LYS ¹²⁸	0.996	8.857	120.66	172.46	52.1	
PHE ⁶⁴	0.998	7.977	118.04	174.68	58.7	39.3	ALA ¹²⁹	1	8.126	126.97	171.52	50.4	16.1
LYS ⁶⁵	0.900	9.833	130.26			31.8	VAL ¹³⁰	1	8.038	125.17			

702

703 **Supplementary Table S12. Sequential chemical shift assignments of ¹⁵N/¹³C apo-dFABP (BMRB**
704 **ID: 27113)**

705 The ¹H, ¹⁵N, and ¹³C chemical shifts of apo-dFABP were assigned through comparison to rule out
706 the sequential assignments of liganded OA-dFABP and deposited in the BioMagResBank database
707 under the accession number 27113.

Dataset	Monomer	Dataset	Monomer
Protein sample	dFABP ₂₋₁₃₀ (5GGE)	Protein sample	dFABP ₂₋₁₃₀ (5GKB)
Beamline	NSRRC_BL13B1	Beamline	NSRRC_BL13C1
Wavelength (Å)	0.97622	Wavelength (Å)	0.97622
Resolution (Å)	27.05-1.86 (1.93-1.86)	Resolution (Å)	30.00-2.04 (2.11-2.04)
Space group	I422	Space group	I422
<i>Unit cell dimensions</i>		<i>Unit cell dimensions</i>	
a, b, c (Å)	61.70, 61.70, 137.32	a, b, c (Å)	61.60, 61.60, 137.06
a, b, g (°)	90	a, b, g (°)	90
Unique reflections	11518	Unique reflections	8811
Total observations	79527	Total observations	81557
Redundancy	6.9 (6.2)	Redundancy	9.3 (9.1)
Completeness (%)	99.5	Completeness (%)	99.9
Mean I/S (I)	27.277 (3.2)	Mean I/S (I)	26.900 (6.1)
Solvent content (%)	41.6	Solvent content (%)	45.1
R merge	0.068	R merge	0.100
Refinement		Refinement	
Resolution range (Å)	27.052-1.861	Resolution range (Å)	28.091-2.039
No. of reflections	26.98-1.86 (1.93-1.86)	No. of reflections	28.091-2.04 (2.11-2.04)
No. of protein atoms	2229	No. of protein atoms	2146
No. of water molecules	139	No. of water molecules	128
Average B-factors (Å ²)	30	Average B-factors (Å ²)	24
R work/R free	0.179/0.246	R work/R free	0.186/0.241
<i>Ramachandran plot statistics (%)</i>		<i>Ramachandran plot statistics (%)</i>	
Most favored regions	99.21	Most favored regions	98.41
Additional allowed regions	0.85	Additional allowed regions	2.59
Generously allowed regions	0.79	Generously allowed regions	1.59
Disallowed regions	0.00	Disallowed regions	0.00
Rmsd bond length (Å)	0.56	Rmsd bond length (Å)	0.77
Rmsd bond angle (°)	0.69	Rmsd bond angle (°)	0.83

708

709 **Supplementary Table S13. Structure statistics of crystallographic data**

Residue	Apo I_i	Apo + ND I_i	Fraction of attenuation $I_i - 0.1 I_i / 0.1 I_i$	Holo I_i	Holo + ND I_i	Fraction of attenuation $I_i - I_i / I_i$
V4	10205656	10236169	0.003	57453128	78385112	0.364
G5	47288044	4389232	0.072	41196720	45754608	0.111
K6	100498408	10004599	0.005	59538982	76520176	0.284
K7	51352068	3754745	0.269	47363384	47638208	0.008
Y8	37831528	2500352	0.341	29671190	41380316	0.395
K9	50540088	2442100	0.517	25914096	31040130	0.198
L10	13167488	408376	0.690	26716432	13971254	0.477
D11	30579616	2758910	0.098	22290676	27510230	0.234
K12	40388696	4984648	0.235	31912974	45327876	0.358
S13	44587316	4090012	0.083	41674232	44828176	0.076
E14	29433458	1028791	0.650	32607412	28187700	0.135
N15	41519732	2707875	0.348	33378808	45184864	0.254
F16	53035452	4486050	0.152	35610288	64464056	0.815
D17	53287656	4486050	0.161	42432080	64464056	0.524
E18	41125312	2578051	0.373	41762300	23567050	0.436
Y19	36330800	1371478	0.623	32587724	27295032	0.162
M20	28291586	2986226	0.020	36352580	34012964	0.054
K21	42373052	2913828	0.312	38084528	44066804	0.157
E22	30949432	4408471	0.258	38224716	39344004	0.029
L23	33140102	859221	0.747	38366136	17484900	0.544
G24	29811718	616473	0.794	29110736	19676654	0.530
V25	36547724	1081359	0.704	32851128	27120450	0.154
G26	11901384	10465228	0.121	86537120	98805128	0.142
L27	61610444	6636268	0.077	42693872	53271368	0.239
V28	87984644	7387486	0.163	50030808	64820552	0.256
T29	34683780	3003827	0.134	47456168	33107848	0.302
R30	38688728	2064465	0.466	39371616	37461168	0.049
K31	69153184	2006797	0.710	40640920	47087452	0.159
M32	30240604	678983	0.710	41387144	20751034	0.499
C33	15516145	462039	0.704	26655456	16986488	0.397
N34	16984862	1304392	0.232	33334536	31296824	0.061
S35	69028184	6711601	0.028	48304140	69155264	0.416
L36	54521812	5152728	0.055	47688664	53381026	0.152
T39	19079124	554021	0.710	34238864	15300431	0.553
V40	17638696	568693	0.679	34396800	16594798	0.518
E41	31686684	1954089	0.383	48951380	41617796	0.114
V42	25888518	1114666	0.569	24238218	23384872	0.047
T43	131238840	8450780	0.280	50211528	69131064	0.357
L44	58651200	282944	0.491	54715776	68216064	0.247
E45	116395264	9518416	0.182	57113520	70489568	0.234
G46	76307096	5877092	0.122	39582288	48717744	0.216
D47	111530906	9165391	0.178	68461184	69591800	0.017
T48	131807480	11380262	0.137	65622736	89784752	0.221
Y49	37456908	1730016	0.538	36364664	29917378	0.177
T50	11901384	10465228	0.121	87620264	98805128	0.128
L51	8794472	7345301	0.158	42929506	53885616	0.434
T52	30508848	2651447	0.131	35977680	32356112	0.020
T53	36691204	863502	0.765	42559682	24186334	0.431
F57	29181638	624810	0.718	41251852	13048280	0.684
K58	33433942	994915	0.702	45543336	20005960	0.548
T59	50378792	1533512	0.695	44407864	32388802	0.250
S60	53569444	1630639	0.696	43041948	30692192	0.287
A61	74485616	2240313	0.699	63687116	58144560	0.087
B62	37038456	1181830	0.681	40685156	30337076	0.257
S63	48617392	4541397	0.066	38503808	52424112	0.362
F64	94833368	18430593	0.111	53576840	68018424	0.270
K65	38234856	1266239	0.669	31107304	20010692	0.357
L66	52828548	5393597	0.021	50284720	52472796	0.044
O67	44384024	2588282	0.415	35573708	35188952	0.011
V68	68566104	3765098	0.451	62578320	54556180	0.128
E69	54343688	1591521	0.707	42313528	35723884	0.156
F70	36086284	10885294	0.698	35014896	17478464	0.500
D71	62591720	3772711	0.397	36621320	52424112	0.432
E72	39360696	767073	0.805	40538884	18460140	0.545
E73	23224158	701332	0.698	38203740	14825069	0.612
T74	26381516	1060854	0.598	36210448	22756350	0.372
L75	13825667	2254867	0.637	32764628	12336290	0.620
D76	22039212	524594	0.762	35148544	13759992	0.609
G77	34137036	1750480	0.487	34579872	31837884	0.079
R78	33101106	1444323	0.564	38926456	28467212	0.263
N79	49918880	3188134	0.361	39882056	40763016	0.022
V80	29951526	601823	0.799	38883726	13408120	0.653
K81	47073376	2266050	0.519	40590356	35672176	0.121
S82	19473498	536052	0.725	33815956	64464056	0.912
B83	72643200	6348858	0.138	39825588	55495020	0.303
B84	30014670	873856	0.709	32257152	18717910	0.420
T85	53430632	2142209	0.589	41659336	36488804	0.112
L86	89898416	8625142	0.082	5160872	65560348	0.282
D87	104176040	8883612	0.147	55742000	64100584	0.150
C88	94040080	8603070	0.085	49562720	61382044	0.238
N89	117720616	8670496	0.263	55389068	59818144	0.080
K90	103189568	8865503	0.141	10084400	80725416	0.197
L91	35024116	820591	0.765	29620446	15817540	0.466
T92	36035668	3124339	0.133	34300976	39211132	0.143
O93	37242656	1985000	0.468	37094592	30337892	0.166
E94	70416464	2186693	0.689	41776472	3284780	0.214
Q95	50378792	2394817	0.525	40154224	37457740	0.067
K96	34855556	805114	0.769	31116952	20573766	0.336
G97	52757160	663074	0.874	51532752	20294048	0.606
D98	32107684	895124	0.721	32017790	22732204	0.290
K99	41775636	791836	0.808	54960448	29052502	0.562
T101	37201828	853889	0.770	40533828	23583416	0.418
T102	35625700	11971465	0.664	34010848	22831480	0.329
I103	45388664	4589105	0.011	32553186	46857676	0.439
V104	69372168	6400458	0.077	37719076	44913548	0.207
R105	33822664	5437836	0.608	32311088	39383844	0.219
E106	80123480	6929281	0.135	40432288	50036664	0.238
F107	57228840	5689551	0.004	38271188	47136516	0.231
T108	88254464	8131306	0.077	40781024	6275320	0.527
D109	56897220	4780132	0.157	28568392	37258604	0.304
N110	96074048	6283920	0.138	52676776	59186136	0.105
E111	89593432	7141574	0.203	54715776	69202832	0.265
L112	30031082	925762	0.692	31693778	23929902	0.245
T114	15038651	395350	0.752	29232296	12655336	0.568
T115	42106652	1215790	0.711	42548540	24772392	0.418
L116	31003632	823370	0.734	29535744	2968078	0.005
T117	30536778	1186350	0.611	36614776	29863504	0.346
I118	63035652	5815133	0.086	35726864	39794324	0.114
G119	49945288	4162145	0.167	42161864	46365216	0.100
N120	81048224	7139675	0.119	48838724	53944472	0.152
V121	112570056	9940398	0.116	55603628	75957352	0.366
K122	91884104	8060704	0.123	41176568	61103848	0.484
C123	39359176	3922857	0.003	30511132	44030192	0.443
V124	67938072	6448454	0.051	40573196	53789428	0.304
V126	42441488	3743733	0.118	35075312	43388184	0.237
Y127	44466216	3999029	0.101	26752718	30630126	0.145
A129	29458280	3353948	0.138	32795734	45550688	0.388
V130	34709404	3012004	0.132	38839656	38506668	0.009

710

711 **Supplementary Table S14. Intensity changes during lipid ND titration**