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Insight into the Interaction Between the Peroxisome Proliferator-Activated Receptor Gamma (PPARγ) and Adipocyte Fatty Acid-Binding Protein (A-FABP)

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INSIGHT INTO THE INTERACTION BETWEEN THE PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA (PPARγ) AND ADIPOCYTE FATTY ACID-BINDING PROTEIN (A-FABP)

by

QIAN WANG

A dissertation submitted to the Graduate Faculty in Biochemistry
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This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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THE CITY UNIVERSITY OF NEW YORK
ABSTRACT

Insight into the interaction between the peroxisome proliferator-activated receptor gamma (PPARγ) and adipocyte fatty acid-binding protein (A-FABP)

by

QIAN WANG

Advisor: Dr. Ruth E. Stark

The Adipocyte Fatty Acid-Binding Protein (AFABP) is mainly expressed in fat cells. It can bind fatty acids and other lipophilic substances such as eicosanoids and retinoids. The peroxisome proliferator-activated receptor γ (PPARγ) is a nuclear receptor protein that requires ligand binding to regulate the specific gene transcription. PPARγ is expressed at extremely high levels in adipose tissue, macrophages, and the large intestine, where it controls lipid adipogenesis and energy conversion. Moreover, it has been found that AFABP and PPARγ can form a complex in vivo. It is proposed that AFABP carries the ligand and enters into the nucleus where it transfers the ligand to PPARγ by binding and macromolecular interaction.

The goal of this project is to study the interaction between these two proteins in vitro, including their binding affinity, the location of the binding interface on the AFABP protein, and the dependence of these phenomena on specific ligands. New protocols were developed to obtain pure monomeric AFABP protein in a delipidated state. The formation of a disulfide-linked dimer connected through an N-terminal cysteine was demonstrated and minimized by the exclusion of oxygen, though it is not known whether this dimer exists under physiological conditions. A
corresponding protocol was developed to isolate delipidated PPARγ and to optimize conditions for the acquisition of its high-resolution NMR spectra.

Making sequence-specific backbone NH assignments and exploiting the local changes in the chemical environment that accompany interactions between the AFABP and PPARγ proteins, TROSY-HSQC NMR was used to determine which AFABP amino acid residues are located at their interface by investigating the perturbation of backbone NH NMR chemical shifts. The perturbed residues are located primarily in the portal region; weak binding between AFABP and PPARγ (Kd ~ 500 μM) is estimated from chemical shift changes during titration. Fluorescence competition assays also provide insight on protein-ligand dissociation constants and show that the ligand prefers to bind AFABP rather than PPARγ. Nonetheless, titration of PPARγ with holo-AFABP demonstrates the partial transfer of the ligand from AFABP to PPARγ, which could be sufficient for the regulation of targeted gene transcription.
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LIST OF ABBREVIATIONS

ADIFABP, Acrylodan labeled Intestinal Fatty Acid Binding protein
AFABP, Adipocyte Fatty Acid-Binding Protein
APC, Adenomatous Polyposis Coli
ALA, α-Linolenic Acid
BFABP, Brain Fatty Acid Binding Protein
BMRB, Biological Magnetic Resonance Data Bank
CREBBP, cAMP-Response Element Binding Protein -Binding Protein
DAUDA, 11-((5-dansyl)amino)undecanoic acid
ESI, Electrospray Ionization
EDTA, Ethylenediaminetetraacetic Acid
EFABP (KFABP), Epidermal Fatty Acid Binding Protein
FFAR, Free Fatty Acid Receptor
FID, Free Induction Decay
FPLC, Fast Protein Liquid Chromatography
GF, Gel-filtration
GPCRs, G-protein Coupled Receptors
HFABP, Heart Fatty Acid Binding Protein
HIC, Hydrophobic Interaction Chromatography
HPLC, High-performance liquid chromatography
HSL, Hormone-sensitive Lipase
HSQC, [$^1$H-$^{15}$N] heteronuclear single quantum correlation
IPTG, Isopropyl β-D-1-thiogalactopyranoside
IFABP, Intestinal Fatty Acid Binding Protein
KFABP (EFABP), Keratinocyte Fatty Acid-Binding Protein
LB, Luria-Bertani Medium
LCFA, Long-Chain Fatty Acid
LC-MS, Liquid chromatography–mass spectrometry
LCPUFA, Long Chain Polyunsaturated Fatty Acid
LOLA, Linoleic Acid
LFABP, Liver Fatty Acid-Binding Protein
MALDI-TOF, Matrix-Assisted Laser Desorption/Ionization, Time of Flight Mass Spectrometry
MS, Mass Spectrometry
NLS, Nuclear Localization Signal
NMR, Nuclear Magnetic Resonance
OLA, Oleic Acid
PCR, polymerase chain reaction
PMSF, Phenylmethane Sulfonyl Fluoride
PUFA, Polyunsaturated Fatty Acid
PPAR, Peroxisome Proliferator-Activated Receptor
RXR, Retinoid X Receptor
SDS-PAGE, Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis
SPR, Surface Plasmon Resonance
SRC-1, steroid receptor co-activator-1
TCEP, tris(2-carboxyethyl)phosphine
TEV, Tobacco Etch Virus
TROSY, Transverse Relaxation-Optimized Spectroscopy
1. INTRODUCTION

1.1. Preamble.

This introduction will begin with previously published studies of long-chain fatty acids (LCFA). By choosing several common and essential fatty acids, their structure and function will be summarized as well as their closely related lipid metabolism and human health implications. Prior studies have offered clues that LCFAs coordinate and regulate lipid metabolism through contacts and interactions with a variety of membranous, cytosolic and nuclear receptors. Two groups of these interaction candidates, fatty acid-binding proteins (FABPs) and peroxisome proliferator-activated receptors (PPARs), will be highlighted in this Introduction. The discovery and importance of these two protein families in biological and biomedical areas will be described, especially the signaling pathways and regulation of correlated gene transcription and downstream lipid metabolism phenomena. Since the PPARs are activated or inhibited by binding various ligands, this introduction will present previous studies of their structures and how ligands are thought to mediate their regulation of gene transcription. According to the literature in recent decades, PPARs have been shown to interact, bind and cooperate with many other proteins, including several members of the FABP family. The FABPs carry the lipids and transfer them in both the cytosol and nucleus. Thus it is interesting to identify how these two lipid-binding proteins interact with each other, with a view toward understanding lipid metabolism and related human health problems, and to augment vital knowledge in the pharmaceutical field.
1.2. What are LCFA’s?

The long-chain fatty acids (LCFA) comprise a large family of carboxylic acids connected to a long aliphatic chain, which typically contains 14-28 aliphatic carbon segments that are either saturated or unsaturated (Figure 1) in nature. In the saturated subgroup, all of the carbon atoms are connected by single bonds (-C-C-) without any double bonds (-C=C-). The most common examples found in living organisms are palmitic acid (16:0) and stearic acid (18:0), which possess 16 and 18 carbons, respectively. By contrast, the polyunsaturated fatty acids contain one or more carbon-carbon double bonds in the constituent aliphatic carbon chains. According to the position of the final carbon-carbon double bond, the polyunsaturated fatty acids are designated as ω-3, ω-6, ω-7, ω-9 and so on. Some of the essential unsaturated fatty acids are common in nature, e.g., α-linolenic acid (ALA), which is 18:3 (n-3), and oleic acid (OLA), which is 18:1 (cis-9). The well-known “trans fats” belong to the unsaturated fatty acid family. For example, elaidic acid, 18:1 (trans-9), an isomer of OLA, is commonly produced industrially for use in fried fast food, snack food and margarine. Although the trans fats are edible, clinical studies have raised concerns because of their correlation with coronary heart disease.

![Molecular structures of common fatty acids.](image-url)
1.3. Physiological Significance of LCFA’s.

The Western dietary tradition has been extensively investigated in order to elucidate the possible relationships between food intake and human health. The traditional Greenland diet is mainly based on fish, seal and whale. Mediterranean food includes not only fish but also vegetables, fruits and whole grain. Although their dietary menus overlap, the latter diets are both low in saturated fat intake, which as noted above is a risk factor for cardiovascular diseases.7

A substantial body of research has considered the importance of LCFA in cardiovascular diseases, aging and neurodegeneration. Several reports showed that n-3 polyunsaturated fatty acids (PUFA) protect against cardiovascular disease by decreasing blood pressure8. For instance, the blood pressure was decreased -5.5/-3.5 mm Hg by giving >3 g/d of omega-3 fatty acids9. Supplementation with PUFA is thought to be helpful to reduce the risk of cardiologic diseases such as hypertension and atherosclerosis.10

Besides inflammation and cardiovascular health, LCFA are also very important in the construction of neuronal membranes. PUFA are critical in the functioning and development of the visual and brain systems.11 During pregnancy the infant brain starts to accumulate docosahexaenoic acid (DHA, 22:6 n-3),12 which supports development and rapid growth. A deficiency of n-3 PUFAs has been reported to lead to a variety of human neurological diseases such as depression, schizophrenia, autism spectrum disorders and anxiety.13 The long-chain polyunsaturated fatty acid (LCPUFA) concentration in the human and rodent brain decreases with age. Cognitive impairment and inflammation have been studied and reported as tightly correlated with the amount of LCPUFA.14

Since LCFA are quite important in maintaining human health and are implicated in a variety of diseases, researchers have tried to understand how they function at the molecular level. It has been
clearly established that fatty acids are good energy storage candidates that play an important role in energy metabolism. It is also recognized that these molecules bind and interact with many enzymes or other proteins which are very important for the regulation of gene transcription and lipid metabolism. The LCFAs are able to bind with peroxisome proliferator-activated receptors (PPARs), which will be introduced in more detail below. The LCFAs bind and activate almost all subtypes (PPARα, PPARβ/δ, and PPARγ), displaying dissociation constants $K_d \sim 1-5 \mu M$ that indicate moderately tight LCFA-binding interaction and association.\(^{15}\) The reported physiological concentrations of non-esterified LCFAs are ~0.1-0.5 mM in liver tissue,\(^{16}\) which is much more larger than the $K_d$ value. However, the intracellular concentration of free LCFAs is approximately equal or lower than $K_d$.\(^{17}\) Meanwhile, there is another protein type, the fatty acid-binding proteins (FABPs), which binds the LCFA tightly and is present at levels of at least 1 mM in liver and adipose tissues.\(^{18}\)

Finally, there are many other nuclear receptors which can bind the LCFAs, such as liver-X receptor (LXR), farnesoid-X receptor (FXR), vitamin D receptor (VDR), retinoic acid receptor (RAR) and thyroid hormone receptor (TR).\(^{19}\) LCFAs can bind the hepatocyte nuclear factor 4α (HNF4α), which is a very important coactivator of nuclear receptors.\(^{20}\)

### 1.4. Biological function and subtypes of FABPs

Fatty acid-binding proteins (FABPs) belong to the intracellular lipid-binding protein (iLBP) family and are involved in binding long chain fatty acids and other water insoluble hydrophobic ligands reversibly. FABPs are also used in trafficking and targeting LCFAs into the nucleus, where nuclear receptors e.g. the PPAR family bind and activate them. Previous research has found that FABPs play an important role in signal transduction pathways, cell growth, cell cycle and
differentiation, by regulating the concentrations of FAs in the nucleus and ultimately affecting the interactions with various nuclear hormone receptors.

FABPs are low molecular weight (~15kDa) fatty acid carrier proteins, which contain variable members, each of which has between 20% and 70% identity in their amino acid sequence. These subtypes are named for the organ in which they were first discovered and identified, including liver- (L-FABP), heart- (H-FABP), intestine- (I-FABP), adipocyte- (A-FABP), epidermal- (E-FABP or K-FABP), ileal- (Il-FABP), brain- (B-FABP), myelin- (M-FABP) and testis-FABP (T-FABP). It is important to be aware that both the AFABP and KFABP (EFABP) are primarily expressed in the adipocyte which could be candidates to interact with the PPARγ that will be discussed later in 1.11.

An understanding of the requirements for import of AFABP to the nucleus is important in understanding the mechanism of how the ligand transfer is transferred to PPARγ. The AFABP cannot enter through the nucleus membrane unless it binds an activating ligand e.g. linoleic acid (LOLA) to reveal the so-called Nuclear Localization Signal (NLS) sequence. Then, it can be transported into the nucleus and pass its bound ligand to target proteins such as PPARγ. When the AFABP is in apo form or binds a non-activating ligand such as oleic acid (OLA), the AFABP’s NLS will be hidden and the protein will remain in the cytosol.

1.5. Structures of FABPs

According to the X-ray and NMR derived structures (Figure 2), these proteins have shown a common β-clam tertiary structure, which contains two α-helices and ten antiparallel β-sheets. The N-terminus of FABPs contains a helix-loop-helix motif which forms a cap-like structure that may
be related to the entering of the fatty acid ligand. A transient conformational change around the helix-loop-helix area and their close loops is proposed to occur for some family members, thereby allowing the ligand to enter or exit the binding region\(^{27}\).

Liver FABP (L-FABP) is one of the subtypes of FABP family members. Unlike the other FABPs, L-FABP can bind two FAs at maximum. The first ligand binds to L-FABP through the carboxyl group interacting with R122, S124 and S39 in the internal binding cavity. The second interacts with K31 and S56 which are located at the portal region of the L-FABP\(^{28}\). Binding constants between the second ligand and protein are variable. If the second binding ligand is saturated FAs, then the protein binds more weakly, in contrast to the indistinguishable binding constants for unsaturated FAs. For instance, the second binding site of saturated chain palmitate is indistinguishable from the first site with dissociation constant \(K_d \sim 23 \pm 2\) nM\(^{29}\). In contrast, for the unsaturated linolenic acid the second binding site is much weaker (1\(^{st}\) site \(K_d \sim 69 \pm 12\) nM, 2\(^{nd}\) site \(K_d \sim 1000 \pm 500\) nM)\(^{29}\). The binding constant of AFABP-linoleic acid is 1.96±0.39 nM\(^{30}\). Although the \(K_d\) values were reported from different laboratory so that they are not quite comparable, it is valuable to be aware of the \(K_d\) of FABPs binding the fatty acids in the nano molar range.

For the apo form of rat LFABP, Stark et al. have reported 109 out of 124 nonprolyl residue (88%) of the backbone amide \(^1\)H, \(^{15}\)N NMR assignments\(^{31}\). Fifteen missing backbone resonances could be attributed to local flexibility (BMRB accession code 4098). Similarly, the \(^1\)H, \(^{15}\)N resonances of Locusta migratoria FABP were partially assigned (88%) as well by Hamilton et al.\(^{32}\) (BMRB accession code: 5541). Six of these unobservable residues (G45, T53, S56, M74, G106, and D107) appeared when LFABP was bound to two molecules of OLA\(^{28}\) (BMRB accession code 15429) published by Stark et al. An intermediate state designated as the preholo-LFABP was observed by stepwise titration of apo-LFABP by OLA. It has been demonstrated that the LFABP can bind up
to two fatty acids by isothermal titration calorimetry (ITC) and solution NMR spectroscopy. An “adaptation” of the singly ligand-binding LFABP to the entry of the second OLA into the ligand-binding cavity was suggested. The residues R122L/S124A have been mutated to study the binding property. The titration result shows that the mutant structure is similar to the wild type, but it can only accommodate just one ligand at an intermediate site between the apo and doubly-holo forms in the binding cavity.

A computational study of the “portal regions” reported that there could be an alternative portal for the inner ligand to dissociate from the L-FABP binding cavity. In this research, the highly favorable egress of an inner ligand from the second site is claimed to be a specific feature of L-FABP, contrasting with I-FABP. This portal (Portal II) is related to βE/F loop and α-helix cap (Figure 2a), while the anterior portal region (Portal I) is delimited by α-helix II and the βC/βD loop (Figure 2b). The inner ligand OLA129 can dissociate from the protein through Portal II predominantly, whereas the outer OLA128 still remains bound in the cavity. This newly proposed portal region (Portal II) has been reported to release not only linear ligands but also compounds with rigid rings i.e. 8-Anilinonaphthalene-1-sulfonic acid (ANS). Portal II may be the main portal for the inner ligand dissociation. It is important to consider different portal regions, especially to anticipate discussions about L-FABP interacting with other proteins and transfer of FA ligands.
Figure 2 Tertiary structure of L-FABP (2JU8) is represented in cartoon by Pymol. Each helix and strand (yellow) is indicated with two oleic acid ligands as well, which have been labeled as OLA128 and OLA129, respectively. Portal I and Portal II have been marked by blue and red unfilled arrows, respectively. b) Expanded view of the ligand-binding pocket containing several amino acid side chains that have been labeled. Hydrogen bonds between the polar side chains and the oleic acid head group are indicated by yellow dashed lines. Water molecules contributing to the hydrogen bond networks have been colored by green dots.
1.6. Physiological Significance of PPARs

PPARs were first discovered in Xenopus frogs, where they induce the proliferation of peroxisomes in cells.\textsuperscript{35} Then they were proven to be the target of hepatic peroxisome proliferator and cloned by Issemann and Green\textsuperscript{36}. So far it has been discovered that the PPAR protein superfamily contains three isoforms: PPAR\textsubscript{α}, PPAR\textsubscript{β/δ} and PPARγ.

The first member of the family that was isolated and cloned was murine PPAR\textsubscript{α}\textsuperscript{36}. Subsequently, the frog, rat, rabbit and human forms were discovered and cloned\textsuperscript{37}. PPAR\textsubscript{α} can be expressed in many metabolic tissues such as liver, adipose, heart, kidney and skeletal muscle.\textsuperscript{38} PPAR\textsubscript{α} is a potent lipid sensor in the nucleus and an important regulator of cellular lipid metabolism, including cellular uptake and \textit{β}-oxidation of fatty acids\textsuperscript{39}. PPAR\textsubscript{α} has been found to be a target for drug design to cure the dyslipidemia, type 2 diabetes and atherosclerosis\textsuperscript{40}, in which activation causes the lowering of triglyceride levels by cooperation with fibrate therapeutic drugs\textsuperscript{41}. PPAR\textsubscript{α} is also a modulator of inflammation: the activation of PPAR\textsubscript{α} inhibits the inflammatory action of the eicosanoids\textsuperscript{42}.

PPAR\textsubscript{β/δ} is another subtype of the PPAR family that was subsequently cloned from human, Xenopus, mouse and rat sources.\textsuperscript{43} It has been found to be expressed in brain, adipose and skin tissues.\textsuperscript{44} As for PPAR\textsubscript{α}, PPAR\textsubscript{β/δ} has been determined to be related to dyslipidemia. The PPAR\textsubscript{β/δ} agonist GW-501516 can lead to an increase in HDL-cholesterol levels as well as a reduction in triglycerides in serum.\textsuperscript{45} PPAR\textsubscript{β/δ} could also play a role on cancer: it has been reported as a target of Adenomatous Polyposis Coli (APC), which is a tumor suppressor in colorectal cancer cells\textsuperscript{46}. Additionally, PPAR\textsubscript{β/δ} has been found to display the highest expression levels in embryonic brain. This latter observation might indicate that PPAR\textsubscript{β/δ} is related to the differentiation of cells within the central nervous system (CNS)\textsuperscript{47}. 
The third member of the family is PPARγ, which is expressed in a broad range of tissues including heart, skeletal muscle, colon, kidney, small intestine and adipose types. Previous research has found that PPARγ is required to differentiate the adipocyte cells.\textsuperscript{48} Moreover it has been found as a antidiabetic target to increase the insulin sensitivity for type 2 diabetes mice.\textsuperscript{49} The PPARγ agonists can stimulate insulin action.\textsuperscript{50} In addition, previous results from cancer studies show that PPARγ ligands inhibit cell proliferation when adipocyte differentiation is induced.\textsuperscript{51} It has been found that PPARγ expression is increased above the normal level in cancer cell lines.\textsuperscript{52} Some researchers found that treatment with high doses of the drug troglitazone produces a small increase in tumor growth,\textsuperscript{53} but others found that treating mice with troglitazone can inhibit the growth of transplanted human tumors.\textsuperscript{54} Although it is still uncertain how the PPARγ functions in tumor proliferation, it appears to display a significant influence on cancer mechanisms.

\textbf{1.7. Molecular Structure of PPARs.}

Although these protein subtypes express and accumulate in different tissues, they share very similar conserved tertiary structure and functional domains. PPARs contain three functional domains (Figure 3). The N-terminus is designed as the AF-1 (A/B) domain, which is a ligand-independent functional domain. It is followed by a DNA binding domain (DBD) that binds PPAR Response Elements (PPRE) of specific targeted gene sequences. After a hinge connection, there is a ligand binding domain (LBD), which contains an AF-2 (ligand-dependent activation functional domain) located at the C-terminus of the polypeptide\textsuperscript{55}. 
Figure 3 Conserved functional domains of the PPAR family. They include an AF-1(A/B) domain at the N-terminus (red rectangle), a DNA binding domain (DBD, marked as a blue rectangle) and a ligand-binding domain (LBD, marked as moss green) at the C-terminus, where there is a activation function domain (AF-2) belonging to the LBD. There is a short hinge between the DBD and LBD.

PPARs, as transcriptional factors, usually work by forming a heterodimer with nuclear hormone receptors such as the RXRs, combined with zinc ions and their functional ligands (Figure 4). This heterodimer recognizes and binds the specific DNA regions termed PPREs, which contain consensus direct repeats of AGGTCA or TGACCT that are recognized and bound by the PPAR-RXR heterodimer\textsuperscript{56} (Figure 4). For instance, Acyl-CoA oxidase (TGACCTTTGTCCT) or Acyl-CoA synthetase (TGACTGATGCCCT) have been implicated. Generally this sequence occurs mainly in the promoter region of the gene. When the PPAR binds its ligands, the subsequent transcription will be regulated, either activated or inhibited, depending on the ligand binding.

When natural ligands or agonists bind, the conformation of the PPAR, namely the AF-2 (helix 12, H12) direction and position, is changed and stabilized, thereby initiating the recruitment of transcriptional coactivators (Figure 4, yellow ribbon).\textsuperscript{57} Antagonists that prevent the PPARs from recruiting the coactivators force AF-2 to occupy the coactivator binding site.\textsuperscript{57} For example, leukotriene B4 (LTB4) was found to be a PPAR\textsubscript{\alpha} ligand,\textsuperscript{58} which was proposed to inhibit inflammation by augmenting the expression of hepatic enzymes.
1.8. Structure of the PPAR ligand-binding domain

According to X-ray crystallographic studies, the structure of the hPPARα LBD is very similar to the hPPARβ LBD and hPPARγ LBD. As noted above, the PPARα ligand binding domain is composed of 12 antiparallel helices packing a 3- antiparallel β sheet core (some references consider that there is one more sheet between H5 and S2 composed of amino acid residues MSKD in PPARα LBD, VNKD in PPARδ LBD, MNKD in PPARγ LBD) (Figure 5). The greatest deviation among these three sub-types occurs for residues 231-265, which is referred to as a loop. Residues 449-457 belong to the C-terminal AF-2 helix, which also varies among these three subtypes.
The ligand binding site is located at the very center core of the LBD, which is highly hydrophobic. Most of the residues in the binding cavity are conserved across the three subtypes. It is interesting to notice that there are four conserved polar residues which are part of the hydrogen-bond network involving the carboxylate group of the FA and eicosanoic acid ligands\(^3\) (Figure 5b). The interacting residues are Ser280 (H4), Tyr314 (H6), His440 (H11) and Tyr464 (H12) of PPAR\(\alpha\) (respectively Thr289, His323, His449 and Tyr473 of PPAR\(\beta\), and Ser289, His323, His449 and Tyr473 of PPAR\(\gamma\)). The entrance to the ligand binding site is between H3, H4, the N-terminus of H6 and the three antiparallel \(\beta\) strands.

Figure 5 Crystal structure of PPAR\(\alpha\) LBD (2GWX) represented by Pymol. a) (H1-navy, H2-royal blue, H3-teal blue, H4-mint, H5-emerald, H6-spring green, H7-lime green, H8-light yellow, H9-marigold, H10- brown, H11-orange, H12 (AF-2)-red). b) Expansion of the ligand binding cavity. Each of the related residues and their side chains are labeled and separated by various colors. The hydrogen bond interactions are indicated by yellow dashed lines involving to Tyr464, His440, Tyr314 and Ser280\(^3\).
1.9. PPAR-binding ligands

PPARα can be activated to dissociate from corepressors and recruit coactivators, which associate with histones of DNA and thus allow RNA polymerase to transcribe mRNA, by binding a broad range of fatty acid ligands, including palmitic acid, oleic acid, linoleic acid, and arachidonic acid\textsuperscript{60}. The activating ligands include linoleic acid (18:2)\textsuperscript{61} and linolenic acid (18:3)\textsuperscript{62}. There are some non-activating ligands which can bind the PPARα LBD as well, such as palmitic acid. (16:0)\textsuperscript{63} The dissociation constants are shown in Table 1 below.

Since both the PPARs and FABPs bind lipids tightly, it is necessary to choose an appropriate measurement method. The most commonly used methods to measure the binding dissociation constant are AcryloDan labeled Intestinal Fatty Acid Binding protein (ADIFABP), fluorometric displacement assay and fluorescence competition titration. ADIFABP is an indicator developed by Dr. Alan Kleinfeld that can bind the free fatty acid in solution. Detection of FFA by ADIFAB is based on a change in the position of the acrylodan fluorophore relative to the nonpolar binding pocket of the protein when it becomes occupied by a fatty acid (FA). All of the methods are quite sensitive to reach $K_d \sim 1 \mu M$. Specifically, the ADIFABP assays can reach $K_d \sim 1$ nM on basis of the reports in Table 1. Notably, the values for $K_d$ obtained from different methods are widely different. For example, the $K_d$s for L-FABP binding oleic acid are determined as 9 nM and 1.8 $\mu$M using ADIFABP assay and Lipidex separation, respectively. Since the experiments are not measured by the same instrument or performed by the same person, the values are not comparable. Thus, if it is required to compare the binding affinity between FABPs and PPARs, it is necessary to perform the experiment using the same instrument and under comparable conditions (e.g. buffer condition and ligand batch). For instance, according to the table 1, only the binding affinities to cis-Parinaric acid ligand are comparable because they are measured by the same methods from the
same laboratory. According to this measurements, it is known that the binding constants of AFABP-\textit{cis} Parinaric acid and PPAR\textit{γ}-\textit{cis} Parinaric acid are in the same magnitude ~40 nM. The PPAR\textit{γ}-\textit{cis} Parinaric acid constant is slightly bigger than the AFABP-\textit{cis} Parinaric acid constant.
Table 1. LCFA Ligands and their binding constants with PPARγ and A-FABP proteins

<table>
<thead>
<tr>
<th>Ligand</th>
<th>A-FABP / $K_d \pm S.E.$ nM</th>
<th>PPARγ / $K_d \pm S.E.$ nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic acid</td>
<td>3.36 ± 1.64$^a$</td>
<td>156000 ± 4720$^e$</td>
</tr>
<tr>
<td></td>
<td>7.7 ± 0.15$^b$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.54 ± 0.11$^c$</td>
<td></td>
</tr>
<tr>
<td>Oleic acid</td>
<td>1.85 ± 3.5$^a$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.61 ± 2.5$^b$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.19 ± 0.19$^c$</td>
<td></td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>1.01 ± 0.12$^b$</td>
<td>1300 ± 84$^e$</td>
</tr>
<tr>
<td></td>
<td>3.22 ± 0.10$^c$</td>
<td>4900 ± 2100$^e$</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>0.76 ± 0.15$^b$</td>
<td></td>
</tr>
<tr>
<td>Docosahexaenoic acid</td>
<td>1.07 ± 0.21$^b$</td>
<td></td>
</tr>
<tr>
<td>Linolenic acid</td>
<td>1.96 ± 0.39$^b$</td>
<td></td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>1.39 ± 0.28$^b$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.80 ± 0.26$^c$</td>
<td></td>
</tr>
<tr>
<td>cis - Parinaric acid</td>
<td>31.7 ± 2.3$^d$</td>
<td>49.6 ± 1.2$^d$</td>
</tr>
<tr>
<td></td>
<td>7800 ± 240$^e$</td>
<td></td>
</tr>
<tr>
<td>Troglitazone</td>
<td></td>
<td>274 ± 0.142$^e$</td>
</tr>
</tbody>
</table>

$^a$ 1,8-ANS (8-anilino-1-naphthalene-sulfonic acid) displacement assay$^{64}$

$^b$ ADIFAB assay$^{65}$

$^c$ ADIFAB assay$^{66}$

$^d$ Fluorescence competition titrations$^{67}$

$^e$ SPR biosensor method$^{68}$
The molecules shown in Table 1 are PPARα ligands based on previously published research.\textsuperscript{69} Most of the molecules share a similar long alkyl chain.\textsuperscript{70,71,72,73,74} An activating ligand is understood to be a molecule that increases the expression of a gene, which has been targeted and is regulated by PPARα. This process is known as transactivation. Transactivation assays are applied to cells that have been transfected with a vector expressing PPARα as well as a second vector containing a PPRE and a reporter gene, which encodes an enzyme such as luciferase.\textsuperscript{75} The activity of a ligand will then correlate to the resultant luciferase activity.

1.10. PPAR Functional Partners

1.10.1. Secondary and tertiary structure

PPARs have been observed to interact with many other polypeptides, such as the steroid receptor co-activator-1 (SRC-1) and CREB-binding protein (CREBBP), which are two co-activators\textsuperscript{76}. The recruitment of these co-activators depends allosterically on the conformation of the AF-2 domain, which is stabilized by binding an activating ligand such as rosiglitazone. When PPARs bind the activating ligands, the AF-2 domain will be stabilized and exposed to the hydrophobic surface, where the LXXLL helix of the co-activator can interact and form a complex, for example PPARγ/rosiglitazone/SRC-1\textsuperscript{3}.

When antagonists are bound to PPARs, they push the AF-2 helix away from the agonist bound position. This causes the AF-2 domain to pack loosely against helix 3, instead of occupying the co-activator-binding groove. As a result of removing H12 from the active surface, there is more space for binding the co-repressor motif, LXXXIXXXL\textsuperscript{3}. The latter binding motif prevents the AF-2 from moving back to an activating conformation.
Another important receptor protein, RXRα, is found to form a heterodimer with PPARγ LBD. By interacting with the RXRα, the complex can function as a transcriptional regulator that activates or inhibits the target gene transcription. The interface occurs between the Lys431 of RXRα and Tyr477 of PPARγ. This arrangement can be strengthened by another interaction pair (PPARγ Tyr477 and RXRα Arg348). This can stabilize the PPAR AF-2 in a position that allows the co-activators to bind and function even without binding agonists.

Figure 6. The residues and structures of PPARγ involved in activating function. (A) PPARγ residues involved in a hydrogen-bond network stabilizing the AF-2 domain in a ligand-independent manner (2PRG [1]). Hydrogen bonds are shown as thin green lines. AF-2 helix H12 is colored in orange. (B) Interactions between residues Leu469, Tyr473, Leu476 and Tyr477 (in dark ball and stick) and some neighboring residues of PPAR that stabilize the active conformation of AF-2 helix H12 (colored in neon). Rosiglitazone is also shown (magenta in red box).
1.10.2. **Open questions about the PPARs delipidation**

In none of the previous studies on PPARs have they been demonstrated to be unliganded (apo) before the subsequent research. It has never been claimed that the PPARs are treated to empty the binding cavity, which usually contains some lipids from host cell lines e.g. *E. coli*. The expressed and purified proteins are assumed to be in “apo form” without adding ligand artificially, but this unverified assumption can lead to misinterpretations. For instance, the reported ligand binding constant will be a “replacing $K_d$” that is related not only to the titrated ligand but also to the remaining ligand from the host cell lines. Even worse, if the titrated ligand binding is weaker than the remaining one, then the final protein will be a mixture of holo forms binding more than one ligand. Thus, this problem will introduce an error during further studies of structure or activity.

A single literature report has attempted to delipidate PPARs by loading the protein solution on the prepacked hydrophobic interaction (HIC) column at 37 °C. Since the HIC column is made of a fairly hydrophobic material, it should compete for the lipids against the proteins. To date, however, there is no report demonstrating a successful delipidation of a PPAR. In this project, we will discuss several ways to delipidate the PPARs by optimizing the conditions based on previous references.

1.10.3. **Open questions about PPAR NMR assignments**

In addition to the crystal structures presented in Section 1.4, it is possible to study the molecular structure of PPARγ using solution-state Nuclear Magnetic Resonance (NMR) spectroscopy. Since the protein functions in solution state, it is sensible to study its protein-protein and protein-ligand interactions by using solution-state NMR. So far, there are only four sets of PPAR NMR
assignments published in the Biological Magnetic Resonance Data Bank (BMRB). All of these assignments pertain to the holo-PPARγ LBD subtype bound to GW1929, rosiglitazone, MRL24 or MRL20 (BMRB: 15518, 17975, 17976 and 19777).

1.11. **Interactions between PPARs and FABPs**

Recently, many observations have been made that help to explain the mechanisms and pathways by which fatty acids as signaling molecules target and regulate lipid metabolism. First is that both PPARs and L-FABP co-localize in the nucleus\(^7^9\). L-FABP as a fatty acid-binding protein is abundant in the cytosol, but it has also been discovered in the nucleus where PPARs predominantly localize. A second observation is that some portion of L-FABP binding and transferring ligands are also agonists. For instance, the antidiabetic drug thiazolidindione, which has reported

![Figure 7](image)

Figure 7. Spectra and structure mapping published by Dr. Tony Velkov. (a) \(^1\)H-\(^{15}\)N HSQC spectrum of \(^{15}\)N apo-L-hFABP overlaid at the bottom of the spectrum of \(^{15}\)N LFABP titrated by holo-PPARα LBD(blue). (b) The highly perturbed residues (>0.6 p.p.m perturbation) titrated by holo-PPARα LBD are mapped onto the crystallographic structure of human L-FABP (PDB code: 2F73)\(^1\) and colored by red.
dissociation constants in the micro- or nanomolar range, is transferred by L-FABP\textsuperscript{80}. Finally, L-FABP expression and β-oxidation enzymes are induced by hypolipidemic drugs through PPARα\textsuperscript{80}. Only one report by Velkov et al. has appeared about the interaction between L-FABP and PPARα\textsuperscript{1} using the solution-state NMR technology (Figure 7). These investigators claimed that the LFABP\textsuperscript{1}H\textsuperscript{15}N HSQC spectrum was significantly perturbed by titrating with PPARα (Figure 7a). All of the largely perturbed residues identified in his paper have been mapped onto the structure of the LFABP (Figure 7b). However, we would expect broader HSQC peaks for the 50-kDa FABP-PPAR complex and would benefit from the use of TROSY-HSQC strategies (REF). The data obtained so far should be viewed with caution. Thus, in sum, the mapped structure of the perturbation is inconclusive and need to be studied further more.

Despite a broad range of evidence that supports the possible interaction between L-FABP and PPARα, there was no consensus until it was reported that L-FABP interacts directly with PPARs\textsuperscript{5} using the methods of immunoprecipitation, Western immunoblotting, Northern-blot analysis and a mammary epithelial cell proliferation assay (Figure 8). Before that it was simply believed that FABP works as a fatty acid transporter. Aqueous diffusion of these ligands, which are not hydrophilic, can be facilitated with the help of FABPs\textsuperscript{81}. It was discovered that I-, H- and A-FABP can lead to the dissociation of fatty acids from model membranes\textsuperscript{82}, while some recent reports found that L-FABP associates with specific nuclear membrane proteins when PPAR ligands are present \textsuperscript{83}.

Direct evidence for this protein-protein interaction came from immunoprecipitation of L-FABP and PPARα\textsuperscript{5}. According to Figure 8A, either an antibody to PPARα or an antibody to L-FABP was used. Both proteins were pulled down by the antibody, suggesting that L-FABP interacts with PPARα directly in vitro. Meanwhile, experimental results showed that only the pairs (α-PPARα,
L-FABP) and (α-L-FABP, PPARα) display a positive immunoprecipitation band (Figure 8A). This proved that the binding between L-FABP and PPARα and the binding between A-FABP and PPARγ is specific.

![Figure 8](image)

**Figure 8.** Co-immunoprecipitation of L-FABP and PPARα recombinant proteins. A: L-FABP and PPARα proteins (20 μg each) were mixed, immunoprecipitated with anti-PPARα (α-PPARα) or anti-L-FABP (α-L-FABP), and examined by SDS-PAGE and Coomassie blue staining for each protein. B: L-FABP and SREBP-1a proteins (20 μg each) were mixed, immunoprecipitated with anti-SREBP-1 (α-SREBP-1) or anti-L-FABP (α-L-FABP), and examined by SDS-PAGE and Coomassie blue staining for the presence of each protein. C: SREBP-1a and PPARα proteins (20 μg each) were mixed, immunoprecipitated with anti-SREBP-1 (α-SREBP-1) or anti-PPARα (α-PPARα), and examined by SDS-PAGE and Coomassie blue staining for the presence of each protein.⁵

Another line of research showed that L-FABP and A-FABP can respectively interact with PPARα and PPARγ, with or without ligand addition⁸⁰. By further studying the interaction between these proteins, scientists provided more evidence that cooperation between FABPs and PPARs is highly selective⁸⁴. That is, PPARγ can be activated by A-FABP, while PPARβ can be enhanced in activity by K-FABP. According to the Figure 9D, the AFABP can transfer the ligand (troglitazone) to PPARγ, but the KFABP cannot. This is another evidence that the interaction between the AFABP and PPARγ is specific, although the AFABP and KFABP are both primarily expressed in adipocyte. More reports provide evidence that L-FABP directly interacts with PPARα and is involved in the
nucleo-cytoplasmic shuttling of their ligands\textsuperscript{85,86,60}, which are found within the binding pocket of L-FABP and the ligand binding domain of PPAR\textalpha. As PPAR LBD is sufficient for interacting with the FABP\textsuperscript{39,87}, most of the proposed research focuses only the interaction between the PPAR LBD and FABP.
Figure 9 A-FABP directly channels troglitazone to PPARγ-LBD, while K-FABP does not. The dependence of the rate of transfer of troglitazone (TZD) from FABP to PPARγ was examined. PPARγ-LBD was covalently labeled with a pyrene moiety. (A) The labeled protein (1 μM) was titrated with A-FABP precomplexed with troglitazone. Fluorescence ($\lambda_{ex} = 342$ nm; $\lambda_{em} = 377$ nm) decreased upon titration until a plateau was reached at saturation. (B and C) To determine the rate constants for ligand transfer from FABP to PPARβ-LBD, pyrene-labeled PPARβ-LBD was mixed with A-FABP (B) or K-FABP (C) precomplexed with troglitazone. Mixing was accomplished using a stopped-flow apparatus. Final protein concentrations for the representative traces shown were 1 μM PPARβ-LBD and 5 μM FABP-ligand complexes. Traces were analyzed by fitting to a single first-order reaction (solid line through data points) to obtain the pseudo-first-order rate constant of the reaction. (D) $t_{1/2}$ for transfer of troglitazone from A-FABP (solid circles) or K-FABP (open circles) to PPARβ-LBD as a function of the FABP/PPAR molar ratio.
Finally, more evidence has been supplied to prove and quantify this interaction between PPARα and L-FABP both in vitro and in vivo\(^5\). Schroeder et al. quantitatively analyzed this direct interaction with the method of Fluorescence Resonance Energy Transfer (FRET)\(^5\) and obtained a PPARα--L-FABP complex dissociation constant \(K_d = 156.5 \pm 18.1\) nM with the application of sensitized emission of Cy3-apo-L-FABP and Cy5-apo-PPARα (the Cy dyes are fluorescent cyanine compounds producing an intense signal that can be detected). This result not only demonstrated an important interaction but also revealed that the associated binding affinity was moderately strong.

1.12. **Open questions about PPARs interaction with FABPs**

According to previous research, it has been demonstrated that the PPARs can form complexes with FABPs in vivo. It has also been found that the PPARα LBD specifically binds L-FABP, and the PPARγ LBD specifically binds A-FABP. But it is unknown which parts are the actual interacting surfaces on both proteins pairs and whether the interaction functions to promote the ligand transfer between the PPARs and FABPs. Since PPARs are mainly located in the cell nucleus, the ligand transfer is an important problem to solve. FABPs as one kind of lipid carrier can transfer in both the cytosol and nucleus. Meanwhile, the FABPs have been shown to form a specific complex with PPARs. It is unknown but interesting that whether the interaction between these proteins is related to the ligand transfer or not. Studying the possible ligand transfer is important and helpful to understand many biological pathways e.g. the regulation of lipid metabolism. This type of study is also promising for the design of pharmaceutical drugs, in that analogs of the native ligands could activate or inhibit the corresponding ligand transcription. In sum, studies of the interaction between these two proteins and the process of the potential ligand transfer is worthwhile to aid in understanding the signaling pathways that regulate lipid metabolism.
2. MATERIALS, PROCEDURES, AND RATIONALE


2.1. Molecular cloning of the target proteins

2.1.1. Molecular cloning of the PPAR LBDs

2.1.1.1 Molecular recombination

The PPARα LBD gene sequence (cDNA) is carried on the pCMX plasmid supplied by Dr. Judith Storch’s group. In order to overexpress and obtain enough protein for further studies, the corresponding gene needs to be combined into an expression plasmid which contains a transcription promoter, ribosome binding position and affinity tag (6 x His-tag in this project). A mature Invitrogen Gateway® system was employed to accomplish the re-combination. First, the target cDNA sequence was obtained and amplified by the polymerase chain reaction (PCR) with the pCMX as the template and by using the designed primers shown below.

**Forward Primer:**

5′-GGGGACAGTTTGTACAAAAAGCAGGCTTAAGAAAAACCTTGACTTTCAAGTCGGAAACTGCGACCTCAAATCTC-3′

**Reverse Primer:**

5′-GGGGACCACTTTTGTACAAGAAGCTGGCTCTATTAGATCATGTCTGTAGATCTCT-3′

The PCR product was purified by using an Invitrogen PCR product purification kit and introduced into the expression plasmid pET-57-DEST (or pDEST17) by the Gateway® reaction kit (Figure 10). The reason we chose the pET-57-DEST plasmid is because it can link our PPARs with a fusion protein NusA at the N-terminus, so as to increase the PPAR solubility during the overexpression. The final expression clone was sent to Genewiz for sequencing to confirm our
target protein sequence. Additionally, the PPARγ LBD recombination was achieved by Dr. Cédric Bernard in an analogous way.

2.1.1.2 Transformation

The successfully recombined expression clones, including FABPs and PPARs, were transformed into two alternative competent cell lines: BL21-AI™ and BL21(DE3)pLysS by following the heat-shock procedure. Each of the cell lines has a different protein inducing system. BL21-AI™ has the tightly regulated arabinose-inducible araBAD promoter upstream of the T7 RNA polymerase gene. BL21(DE3)pLysS recruits the lacUV5 promoter which is suitable for production of protein from target genes cloned in pET vectors by induction with Isopropyl β-D-1-thiogalactopyranoside (IPTG).

Once the competent cells were transformed, the cell medium was placed on the LB agar plates with antibiotics, which depends on what plasmid was used. The plates were incubated for 15 hours. Then a single colony was picked and inoculated into LB liquid medium with the same antibiotics. This pre-culture medium was shaken at 37°C at 225 rpm for 15 hours. The harvested culture was used for a large culture (1 liter) or stored at -80°C with addition of 5% glycerol.
2.1.2. Molecular cloning of the FABPs

The FABPs (AFABP and LFABP) were recombined in the same way shown above. The only difference was that the FABP sequences were finally introduced into the pDEST17 plasmid instead of pET-57-DEST. Compared with the pET-57-DEST, the pDEST17 links the His-tag to the FABPs directly, because FABPs do not encounter a problem of solubility. More details will be presented in a later section of this thesis.

2.2. Protein expression and purification

2.2.1. Expression and purification of the FABPs

A BL21-AI-FABP pre-culture was inoculated to a large size medium at 50 times dilution so as to grow a large amount of cells producing our target protein. The Luria-Bertani medium (LB, Sigma L3022) is used for generating unlabeled proteins, while the minimal medium (0.4% $^{12}$C- or $^{13}$C-
glucose, 2 mM MgSO$_4$, 0.1 mM CaCl$_2$, 100 µg/mL ampicillin, 1x sigma MEM vitamins solution, 20 mM KH$_2$PO$_4$, 450 mM Na$_2$HPO$_4$, 8.5 mM NaCl, 1g/L $^{14}$ or $^{15}$N-NH$_4$Cl, pH 7.2) is used for producing $^{13}$C and/or $^{15}$N labeled proteins. The culture was shaken under the same conditions as the pre-culture until the OD$_{600nm}$ (absorbance) reached ~0.600. Then 2.0% L-arabinose was added to activate the araBAD operon, so that a large amount of T7 RNA polymerase would be generated to recognize our promoter and therefore start the transcription of our target FABPs. The inducer-treated culture continued to grow for another 4 hours or until the OD$_{600nm}$ reached the plateau of the sigmoid curve. Then the cell culture was centrifuged at 4000 rpm and 4°C for 30 minutes. The cell pellets were frozen at -80°C immediately or suspended for subsequent lysis. According to this protocol, ~6 grams of cells were harvested from 1 liter culture.

The 6 grams of cell pellets were suspended with 50 mL Lysis Buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM imidazole, 1 mM phenylmethane sulfonyl fluoride (PMSF), 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0). Sonication to produce a lysate was done by using a Misonix Sonicator 3000 for 45 minutes at 30 watts power. The lysate was centrifuged to recover the supernatant. Filtration of the supernatant was done with 0.20 µm Millipore filters for the subsequent HisTrap chromatography.

Affinity purification was performed on a HisTrap HP 5 mL column (GE Healthcare) which was attached to an Äkta purifier Fast protein liquid chromatography (FPLC) system (GE Healthcare). Since the nickel charged HisTrap column can specifically bind His-tagged proteins, once the lysate supernatant flows through the HisTrap column, only our protein will bind. The column was pre-equilibrated with a Binding Buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM imidazole, 1.0 mM EDTA, pH 8.0). In order to recover our sample protein, a concentration gradient of imidazole (10-
500 mM) in Elution Buffer (50 mM Tris-HCl, 150 mM NaCl, 500 mM imidazole, 1.0 mM EDTA, pH 8.0) was employed to compete with our protein and therefore elute it.

Usually, there are some contaminants remaining after the HisTrap elution due to non-specific binding. In order to further purify the samples, gel-filtration chromatography was conducted to separate the proteins by size. A 20-mL portion of the HisTrap eluate was injected into a Superdex 75 packed XK26/100 column (GE Healthcare). The FPLC was used to pump the sample and GF buffer (10 mM potassium phosphate, 150 mM potassium chloride, 0.2 g/L sodium azide, pH 7.4) at a flow rate of 0.6 mL/min and a total volume of 700 mL. Different proteins are eluted based on their sizes, with smaller proteins eluted out later than bigger ones. Any proteins with size bigger than 75 kDa are washed out at the void volume together.

In order to track the sample quality, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used. First, the samples were collected from each step of the purification. Then they were mixed with the sample buffer (100 mM Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 25% β-mercaptoethanol, 1% bromophenol blue) and boiled in a water bath for 5 minutes to fully denature the proteins. A 5 μL portion of the mixture was loaded into the SDS-PAGE gel wells and run at a voltage of 100 V. The current was stopped when the bromophenol blue leading edge migrated to the bottom of the gel. Staining of the gel was conducted in a Staining Buffer (0.1% w/v Brilliant Blue, 50% v/v Methanol, 10% Acetic acid) for 1 hour, and destaining of the gel in Destaining Buffer (25% v/v Methanol, 10% Acetic acid) was done until the protein bands appeared clearly (~ 15 hours). Since the proteins were denatured for SDS-PAGE, the protein size is the only factor that impacts the migration rate. Smaller proteins migrate faster and show up close to the bottom of the gel. By comparing their positions with the protein standard marker ladders, it is straightforward to estimate the size corresponding to each band.
After the GF purification, the protein was checked for purity by SDS-PAGE. The His-tag was no longer required, so Tobacco Etch Virus (TEV) protease was incubated with the target protein at mass ratio (1:10) to remove the His tag. Meanwhile, a 10 kDa Millipore filter was used to remove the cut linker (3.5 kDa) as soon as possible to avoid competitive binding with the TEV protease. After completing the TEV digestion, the HisTrap column was used to extract the pure FABPs, since only pure FABPs lacked the His-tag. The resulting FABPs were checked for purity by SDS-PAGE. The typical yield was 30 mg per liter of cell culture.

2.2.2. Expression and purification of the PPAR LBDs

Since PPAR proteins are not as stable as FABPs, an expression and purification procedure similar to the one described in last section was used with minor modification. The BL21(DE3)pLysS-PPARs cell line was grown at 37°C and initially inoculated into the LB medium (minimal medium) for unlabeled (labeled) protein. When the OD$_{600nm}$ absorbance reached ~0.600, the culture was chilled on an ice bath for 10 minutes. Then 1.0 mM IPTG with 0.5 mM OLA was added to activate the lac operon and meanwhile release the lac inhibitor blocking the RNA polymerase movement. The induced culture was continuously incubated at 18°C for another 20 hours or until the OD$_{600nm}$ reached the plateau of the sigmoidal curve. Then the cell culture was centrifuged at 4000 rpm and 4°C for 30 minutes. The cell pellets were frozen at -80°C immediately or suspended for subsequent lysis. According to this protocol, ~6 grams of cells were harvested from 1 liter of culture.

The cell pellets were suspended with 50 mL Lysis Buffer (0.5% Tween20, 5% Glycerol, 50 mM Tris-HCl, 150 mM NaCl, 10 mM imidazole, 1.0 mM MgSO$_4$, 5 μg/mL DNAse I, cocktail protease inhibitor (Sigma S8830), 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0). Sonication to produce a lysate was done with a Misonix Sonicator-3000 for 45 minutes at a power of 30 watts.
The lysate was centrifuged to recover the supernatant. Filtration of the supernatant was done with 0.20 μm Millipore filters for the subsequent HisTrap chromatography.

The supernatant was loaded into a HisTrap column that had been pre-equilibrated by a Binding Buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM imidazole, 1.0 mM EDTA, 5% Glycerol, 0.5% Tween20, pH 8.0). In order to recover our sample protein, a concentration gradient of imidazole (10-500 mM) in Elution Buffer (50 mM Tris-HCl, 150 mM NaCl, 500 mM imidazole, 1.0 mM EDTA, 5% Glycerol, 0.5% Tween20, pH 8.0) was employed to compete with our protein and therefore elute it out.

The purified protein from the HisTrap column was injected into the Superdex 75 packed XK26/100 column (GE Healthcare) in order to exchange the buffer from Tris-HCl to a phosphate buffer system. The phosphate buffer system is required for the subsequent TEV protease cleavage and for recording of the NMR spectrum. Meanwhile, the GF can further remove the contaminant proteins based on size. The GF buffer contains 10 mM potassium phosphate, 150 mM potassium chloride, 0.2 g/L sodium azide, pH 7.4. The elution fractions were collected and run by SDS-PAGE to verify the purity of the sample.

The purified sample was incubated with TEV protease including 0.5 mM EDTA and 0.5 mM TCEP, so as to remove the fusion protein and His-tags connected at the N-terminal of the target protein. The mixing ratio of the TEV protease and target protein was 1:10 (g/g). The mixture stayed at 4°C for two days or until the linkers were fully removed by the TEV. This result was verified by harvesting aliquots every day and running SDS-PAGE. As the digestion proceeded, the gel band corresponding to the recombined protein became thinner and eventually disappeared. Since all of the components had a His-tag except the cleaved PPARs, only the well-cleaved PPARs flowed through a HisTrap column. The other proteins (TEV protease, removed linker, uncleaved PPARs)
bound to the HisTrap column. The purified protein amount was measured by a UV spectrometer at wavelength of 280 nm and converted to protein mass based on the molar extinction coefficient (PPAR$\alpha$: 10430 M$^{-1}$cm$^{-1}$; PPAR$\gamma$: 11920 M$^{-1}$cm$^{-1}$) and molar mass (PPAR$\alpha$: 30745.9 g mol$^{-1}$; PPAR$\gamma$: 31052.1 g mol$^{-1}$) (ExPASy – ProtParam tool). The yield was ~70-100 mg per liter of culture.

2.3. Protein delipidation

2.3.1. Delipidation of FABPs

Since FABPs can bind lipids which are generated by E. coli cells, it is necessary to remove them before conducting further structural studies. The method we use is Lipidex-5000 (Sigma, H6383) competitive binding. The Lipidex-5000 resin is a lipophilic Sephadex LH–20–100 (hydroxypropyl beaded dextran) which has been substituted with long-chain (C$_{13}$–C$_{18}$) alkyl ethers. 5 grams of dry Lipidex-5000 were immersed in 5 mL GF buffer to fully activate the beads. Then 40 mL protein solution (50 mg protein) was mixed and shaken at 225 rpm and 37°C for 2 hours. The beads were removed by using a 0.22 μm filter. The treatment was repeated to fully delipidate the FABPs. For AFABP delipidation, 0.5 mM tris(2-carboxyethyl)phosphine (TCEP) reducer is recommended to prevent possible homodimer formation. The efficiency of the delipidation was verified by subsequent $^1$H-$^{15}$N HSQC NMR experiments described below.

2.3.2. Delipidation of PPARs

In order to study the PPAR ligand binding cavity and corresponding features, it is first necessary to obtain apo-PPAR samples. Generally, during expression and purification, there are some lipids
from the host cells bound to PPAR. In another words, after purification, the PPAR is actually in a holo form carrying various lipids from the expression cell line (E. coli in this project). Thus, it is necessary to remove the unknown lipids before we continue the ligand titration or other structural determinations.

Based on previous literature, there are several common ways to delipidate the target protein. One way is to partially unfold the protein by treating with a concentration gradient of urea. The advantage is that once you destroy the binding cavity, the binding ligand will be released completely. But it is tricky to refold the protein correctly. Alternatively and more commonly, one can let the protein solution flow through a hydrophobic column. Since the binding ligands are usually highly hydrophobic, the column materials will compete with the PPAR to bind the ligand. This latter procedure is much gentler to the protein; it will not lead the protein to denature or precipitate, provided that the apo form is stable. The column can also be connected conveniently to an FPLC purification system. Velkov, et al.\textsuperscript{1} employed a Hydrophobic Interaction Chromatography (HIC) column\textsuperscript{3} to remove the ligand from the PPAR binding cavity, though no validation tests were presented. Other candidates are Lipidex-1000 or Lipidex-5000, which could also remove the ligand by hydrophobic competition.

In this project, we designed another method to delipidate our PPAR because neither HIC nor Lipidex removed the bound lipids from the protein cavity. Our method first requires preparation of the apo-AFABP following the protocol of FABP delipidation described above. It has been observed that AFABP can interact with PPAR\textgamma, so the rationale was that apo-AFABP could compete with PPAR\textgamma for lipid binding. PPAR\textgamma and apo-AFABP were mixed at a molar ratio of 1:2 at room temperature. Then the mixture was separated by flowing through a Superdex75 GF column at a flow rate of 0.35 mL/min. The PPAR\textgamma fractions were pooled and re-collected. The
cycle was repeated with new apo-AFABP for twice in total. The successful isolation of apo-PPARγ was verified by detecting the remaining lipids using ESI-MS in the negative ion mode (refer to 2.5.2.).

2.4. **NMR spectroscopy**

2.4.1. **NMR sample preparation**

^{15}N labeled FABPs were exchanged with an NMR buffer (10 mM potassium phosphate, 150 mM potassium chloride, 0.2 g/L sodium azide, pH 7.4) by using Millipore Amicon Ultra-15 Centrifugal Filter Units. Then the sample solution was concentrated to approximately 200 μM AFABP for ^{1}H-^{15}N HSQC NMR experiments. 10% D_{2}O was used for the spectrometer lock signal. The final volume of the sample was 450 μL for Shigemi Ultra Precision NMR sample tubes or at least 300 μL for Shigemi Advanced NMR microtubes.

2.4.2. **[^{1}H-^{15}N] Heteronuclear Single Quantum Correlation (HSQC) NMR**

Running a ^{1}H-^{15}N HSQC NMR experiment is a simple and efficient way to check the sample quality, buffer condition e.g. pH variation by monitoring the peak positions and linewidths. After accomplishing the sequence-specific assignment of the backbone NH peaks in the spectrum, each amino acid residue can be monitored during additional titration or interaction experiments.

^{1}H-^{15}N HSQC is a one-bond heteronuclear correlation experiment. The two frequency coordinates are the chemical shifts of a ^{15}N heteronucleus and a proton that are directly scalar coupled through one bond. In the protein spectrum, this cross-peak in the two-dimensional HSQC spectrum can be assigned as a backbone amide bond, a side-chain NH\_2 of Asn or Gln residues or a side-chain NH group from Trp, His or Arg residues.
The experiments were recorded at a temperature of 20 °C by using the “AR_hsqctetgpsisp_alii” pulse sequence on a Bruker Biospin 500MHz/B-SE/TXI NMR spectrometer equipped with a 5mm TXI cryogenic probe at the New York Structural Biological Center. The free induction decay (FID) was collected using Topspin3.5pl7 software. The raw data were processed by NMRPipe, an NMR spectral processing system (Frank Delaglio, National Institutes of Health). The NMRPipe program tracked all operations, produced the correct chemical shifts after complicated maneuvers and generated an “nv” file. Then the nv file was read and visualized by using NMRViewJ (Bruce A. Johnson, One Moon Scientific). Spectral assignment and chemical shift perturbation were accomplished and summarized by NMRViewJ.

Titration is a simple and efficient way to study and localize the interacting structural regions involved in protein-ligand and protein-protein interactions. It is actually a series of $^1$H-$^{15}$N HSQC experiments with a progression of protein-ligand or protein-protein molar ratios. For the AFABP-OLA and AFABP-LOLA titrations, the experiments started with 200 μM apo-AFABP as a reference. Then aliquots of OLA or LOLA were added to obtain AFABP-ligand ratios of 1:0.1, 1:0.25, 1:0.5, 1:0.75, 1:1, 1:1.5, 1:2 and 1:3.

2.4.1. $^1$H-$^{15}$N-TROSY-HSQC of $^{15}$N-AFABP titrated by unlabeled PPARγ

In order to identify the interface on the AFABP side, the $^{15}$N labeled AFABP was titrated by the unlabeled PPARγ in the methods of $^1$H-$^{15}$N-TROSY-HSQC NMR. In the apoAFABP-apoPPARγ titration trials, 95 μM AFABP was prepared with gradient concentration of PPARγ. The molar ratios were chosen at 1:0.1, 1:0.25, 1:0.5, 1:0.75, 1:1, 1:1.5, 1:2, 1:3, 1:4 and 1:6. The data was collected in the 800 MHz magnetic field with 1.7 mm micro cryoprobe in New York Structural Biology Center (NYSBC). The data was processed and analyzed by NMRDraw and NMRViewJ software respectively. For the OLAAFABP OLAPPARγ titration, 95% protein was saturated to
holo form in order to prevent the large amount of free ligand appearing in solution and meanwhile suppress the apo peak in spectrum, since 5 μM (5% of 95 μM) apo protein is invisible to our receiver coil.

2.4.2. Multidimensional NMR Experiments

In order to identify the residue corresponding to each cross-peak in the $^1$H-$^{15}$N HSQC NMR spectrum, we employed the $^{15}$N TOCSY-HSQC and $^{15}$N NOESY-HSQC experiments\textsuperscript{89}. In $^{15}$N TOCSY-HSQC, an isotropic mixing step transfers magnetization between networks of chemically bonded $^1$H spins. Then the magnetization is transferred to neighboring bonded $^{15}$N nuclei and back to $^1$H for detection. This experiment is useful to identify the amino acid residue types. In $^{15}$N NOESY-HSQC, the magnetization is exchanged between paired hydrogens within ~ 5 Å using the nuclear Overhauser effect (NOE). Then the magnetization is transferred to bonded $^{15}$N nuclei and back to $^1$H for detection. This latter spectrum can be used to obtain structural information on any correlation through a close distance. This is an effective way to confirm the assignment of proteins in the current work, since the part of the assignment was previously accomplished relying primarily on C\textsubscript{α}/C\textsubscript{β} connectivity (CBCA(CO)NH and HNCACB experiments and side-chain $^1$H connectivity (H(CCO)NH-TOCSY and TOCSY-HSQC)\textsuperscript{31}. The 3D CBCA(CO)NH experiment is specifically designed to correlate the $^1$H and $^{15}$N amide resonances of one residue with both $^{13}$CA and $^{13}$CB resonances of its preceding residue via the intervening $^{13}$CO spin by means of the $^1$J(NH), $^1$J(N,CO), $^1$J(CA,CO) and optional $^1$J(CA,CB) coupling constants. The 3D HNCACB experiment is designed to correlate the $^1$H and $^{15}$N amide resonances with those of the intra- and interresidue $^{13}$CA and $^{13}$CB resonances by means of the $^1$J(NH), $^{1,2}$J(N,CA) and optional $^1$J(CA,CB) coupling constants. By combining the information from these two experiments, we can assign the both the residue types and position in the polypeptide sequence.
The protein samples (apo-AFABP, holo-AFABP, apo-LFABP or holo-LFABP) had a concentration as high as 500 𝜇M to conduct the 3D experiments. The FIDs were recorded using the same 500 MHz instrument as described above.

2.5. Mass spectrometry (MS)

2.5.1. Matrix-Assisted Laser Desorption/Ionization-Time of flight (MALDI-TOF)

MS is a quite efficient and accurate way to identify the exact protein and ligand components in a sample solution according to their masses. Two types of MS were used in this project. One is Matrix-Assisted Laser Desorption/Ionization-Time of flight (MALDI-TOF)\(^9\). Samples in MALDI will be proton charged and pulse laser-ionized into an electric field. Then different molecules will be separated on the basis of the mass-to-charge ratio \((m/z\) or \(m/Q\)). This is a more accurate way to identify the purity of the sample than SDS-PAGE, although the SDS-PAGE method is faster and less expensive.

Electrospray ionization is another widely used MS method\(^9\). Unlike MALDI that denatures the protein macromolecules, ESI can spray the sample while maintaining their structure. Therefore, it is useful to detect the existence of a protein-ligand or protein-protein complex. In this dissertation, it has been applied for both protein and lipid identification.

All samples were prepared at 25 𝜇M and mixed with 30 mg/mL sinapinic acid (matrix) at a ratio of 1:1 (v/v) in a solvent of 50% Acetonitrile (ACN) and 1% Fluoroacetic Acid (F.A.). All recordings were made with the assistance of Dr. Rinat Abzalimov at the CUNY Advanced Science Research Center.
2.5.2. **Electrospray ionization mass spectrometry (ESI-MS)**

Electrospray Ionization Mass Spectrometry (ESI-MS) was performed on a Bruker Maxis II ETD instrument (Bruker Biospin, Billerica, MA). Samples were exchanged with 200 mM ammonium acetate by using 10 kDa Amicon Ultra-15 filters, then concentrated to 10 μM before injecting into the mass spectrometer at 3 μL/min. Typical native ESI-MS runs used a source temperature of 150 °C, dry nitrogen gas kept at 4 L/min, and a collision cell voltage optimized to 5 V.

In order to measure the lipid (OLA) amount in the protein solution, LC-MS was accomplished by connecting an HPLC column (Agilent, Poroshell 5um, 30SB-C3, 2.1 x 75mm) ahead of the Bruker Maxis II ETD instrument. The samples were prepared by mixing in 68% methanol:isopropanol (v/v, 1:1) and 30% aqueous buffer plus 2% formic acid so as to fully unfold the protein and release the bound lipids. Then the samples were injected into the HPLC column with a 90% aqueous elution buffer and eluted by an ascending gradient methanol buffer. Since the lipids are much soluble in methanol compared to aqueous solvent, at some point of the gradient the lipids will be eluted from the column and sprayed into the MS channel. The chromatography and MS signals were recorded and analyzed by “Data analysis 4.3”. Our target molecular weight is “281” (OLA). The peak that contains the 281 molecule can be extracted and integrated to estimate the amount of OLA in the injected samples, since the OLA amount is proportional to the “281” area. In order to establish the linear equation which is used for the OLA amount estimation, the calibration curve of “281” area versus OLA concentration was plotted using OLA standards at concentrations of 1.37, 6.86 and 13.7 uM dissolved in the same buffer (68% methanol isopropanol (1:1, v/v), 30% protein solvent buffer, 2% formic acid).
2.6. Fluorescent lipid (DAUDA) competitive titration

2.6.1. Validation of ligand binding preference between AFABP and PPARγ

It is known that the fluorescent lipid analog 11-((5-dansyl)amino)undecanoic acid (DAUDA) can alter its emission wavelength as the polarity of its environment changes. For example, the emission of the DAUDA in the presence of phosphate aqueous buffer only is ~535 nm; as a lipid-binding protein target is added the peak shifts to ~460 nm which (a blue shift), and the exact wavelength depends on the hydrophobicity of the specific binding cavity. Also when the DAUDA binds to proteins, the emission signal intensity can largely increase which can be easily detected and distinguished from the free DAUDA emission.

First, 40 μM DAUDA was mixed with 8 μM of each protein, AFABP or PPARγ, respectively. The sample was irradiated at 345 nm and the emission of DAUDA when it binds each protein was scanned from 400 to 580 nm as a reference.

Second, 4 μM AFABP and 4 μM PPARγ were mixed with 40 μM DAUDA. The emission curve was observed and averaged in three repeated trials. The ligand (OLA) was added gradually and the emission curves were collected as above.

Each profile was corrected by subtracting the background signal of the DAUDA solution. The intensity versus wavelength was plotted by GraphPad Prism 7 software with the help of Dr. Francine Katz.

The 460 nm (PPARγ-DAUDA) and 520 nm (AFABP-DAUDA) wavelengths were used to compare the changes in fluorescence intensity as each ligand was added. It was expected that a more drastic drop in intensity indicates a preference for the ligand to bind the protein and displace the DAUDA.
All of the fluorescence assays were measured using a Perkin Elmer Envision 2100 Multi-Label Microplate Reader at Columbia University with the help of Dr. Francine Katz.

2.6.2. **Study of protein-ligand binding affinities by fluorescence displacement assays**

2.6.3. **Measurement of protein-DAUDA binding affinities by fluorescence assays**

The protein-ligand binding constant (K\textsubscript{d}) can also be estimated using a fluorescence displacement assay with DAUDA. The protein-ligand binding constant obtained by this method is K\textsubscript{i} (equivalent to K\textsubscript{d} in general), where the ligand is viewed as a competitor of the protein-fluorophore complex which emits the measurable signal. In order to calculate each protein-ligand binding K\textsubscript{i}, it is necessary to measure the protein-DAUDA binding K\textsubscript{d} as a constant parameter first.

Purified apoAFABP or PPAR\textsubscript{γ} was mixed with DAUDA in 50 mM potassium phosphate, 150 mM potassium chloride, pH 7.4 at 25°C, and the resulting fluorescence was measured within one minute using a SpectraMax M5 instrument (E\textsubscript{x} = 345 nm, E\textsubscript{m} = 500 nm). The protein concentration was increased by successive addition of a purified protein solution from 0 to 8 \mu M. The DAUDA was applied by successive addition from 0 to 20 \mu M. The data were collected and analyzed by Softmax software and plotted by GraphPad Prism 7.0.

2.6.4. **Fluorescence displacement assay to determine ligand-PPAR\textsubscript{γ} and ligand-AFABP binding**

The purified PPAR\textsubscript{γ} was fixed at 0.5 \mu M. The DAUDA was gradually titrated from 0 to 8 \mu M. Every ligand (sodium oleate, linoleic acid and sodium linoleate) was added to achieve concentrations from 0 to 50 \mu M. The system was dissolved in 50 mM potassium phosphate, 150 mM potassium chloride, pH 7.4. The fluorescence was measured at E\textsubscript{x} = 345 nm, E\textsubscript{m} = 480 nm. The data were collected and analyzed by Softmax software and plotted by GraphPad Prism 7.0.
The purified apoAFABP was fixed at 2.0 μM. The $E_m = 520$ nm. The other parameters were set in the same way as above.
3. RESULTS AND DISCUSSION

3.1. Sample preparation of AFABP

3.1.1. Expression and purification of AFABP

The AFABP gene was transformed into BL21-AI cells and induced by 0.2% L-arabinose at 37°C. Cells were treated in the same way as described for PPARγ LBD. The only difference is the method of TEV protease incubation. The digested linker can compete for the TEV protease with the undigested A-FABP. Thus a 10 kDa Millipore concentrator was employed to remove the linker (~5 kDa). An aliquot of the sample from each step was analyzed by SDS-PAGE to check the purity (Error! Reference source not found.). According to the SDS-PAGE result, the A-FABP is pure, yielding 40 mg per liter of unlabeled culture.

![SDS-PAGE gel](image)

Figure 11. 4-12% SDS-PAGE gel of PPARγ LBD expression and purification.

Gel of the AFABP expression and purification: 1, protein marker ladder; 2, medium culture before induction; 3, twenty hours after induction; 4, cell lysate after sonication; 5, insoluble phase of the cell lysate; 6, supernatant of the cell lysate; 7, pooled HisTrap elution fractions; 8, supernatant after adding 13C_OLA solution; 9, pooled GF fractions; 10, after 5 minutes incubation with TEV protease; 11, after 1 day incubation with TEV protease; 12, 13, sample filtration by 10 kDa cut-off Millipore filters. 14, after HisTrap removing the TEV protease and the removed linker.
3.1.2. **Delipidation of AFABP**

Previously, we employed Lipidex-1000 beads to remove the endogenous lipids from AFABP. However, even duplicate treatments with the Lipidex-1000 packed column failed to delipidate the AFABP completely, as demonstrated by the retention of extra peaks in the HSQC NMR spectrum (data not shown). We suppose that the hydrophobicity is not high enough to compete for the ligands with AFABP. In this case, we located an alternative Lipidex-5000 product, which is 50% substituted with long-chain carbon chains and more hydrophobic. About 25 g of beads were packed into an Invitrogen XK-26 column at 37 °C, through which ~40 mg of protein was eluted. An aliquot was concentrated and examined by $^1$H-$^{15}$N HSQC to assess the degree of delipidation. Unfortunately, the delipidating effect was insufficient; after treatment there was still some portion of AFABP remaining in the holo form.

We suspected that the low efficiency might come from the short retention time. Thus, we let the protein incubate with the bulk beads directly and swirled the mixture in a 50 mL Falcon tube at 37 °C for 2 hours. After treatment, the beads were removed by a 0.22 μm Amicon Filter. In order to obtain the highest efficiency of delipidation, we repeated the treatment. In the resulting $^1$H-$^{15}$N HSQC spectra (Figure 12, D and E), we could observe that the peaks were characterized to be in the apo form. Remarkably, there were no holo peaks observed even down at the lower contour level. More than 95% AFABP is in apo form by calculating the proportion of apo and holo peak intensities. Thus nearly all of the AFABP was converted to the apo form after twice swirling incubation with Lipidex-5000 beads.
Figure 12. $^1$H-$^{15}$N HSQC spectra of the $^{15}$N AFABP delipidation by Lipidex-5000 treatment. The peaks used for confirming apo or holo forms are marked by green rectangles. A, the $^{15}$N AFABP sample obtained after TEV protease cleavage but before delipidation; B, the spectrum $^{15}$N AFABP after double swirling treatment Lipidex-5000 beads; C, lower contour levels of the spectrum in B; D, delipidated $^{15}$N AFABP (black) overlaid with holo-OLA-AFABP$^2$; E, lower contour levels of the spectrum in D.
3.1.3. **Study of homodimers of AFABP**

3.1.3.1. *The dimer can be separated from the monomer by GF*

Although it has been widely assumed that FABPs are present in monomeric form at the near-physiological concentrations used for thermodynamic and NMR studies of their interactions with ligands and other proteins, non-covalently associated dimers were proposed by Gillilan et al \(^{94}\). Such associated species would not be evident in the denaturing SDS-PAGE gels of *Error! Reference source not found.* and could conceivably be missed in the 2D HSQC NMR spectra of Figure 12 due to the modest molecular weight of this protein. To establish the oligomeric state of the apo-AFABP sample, a series of protein samples at concentrations from ~10 to ~500 \(\mu\)M were analyzed by gel filtration size exclusion chromatography \(^{89}\), typically after refrigerated storage for up to 25 days. The resulting elution profiles, which are overlaid in Figure 13A, each displayed two well-separated peaks from protein species that differed in molecular size, as deduced from their respective retention volumes. Reference to calibration standards showed that the leading and lagging peaks correspond to approximate molar masses of 32 kDa (an AFABP dimer) and 16 kDa (the monomer). Each profile showed the lagging peak to be predominant; integration yielded a nearly invariant dimer proportion of 13±0.3% over this 50-fold concentration range. Moreover, GF trials with 2:1 holo-AFABP liganded to oleate, linoleate, or troglitazone (a member of the thiazolidinediones antidiabetic drug family) revealed monomer percentages: 99±1%, 95±5%, and 91±1%, respectively, for dilution series from 500 to 10 \(\mu\)M \(^{95}\). The reason of this invariant dimer proportion is not sure. The difference might occur occasionally because later it has been proved that the dimer formation is related to the dissolved oxygen in solution.
Figure 13. Elution profiles for murine adipose fatty acid-binding protein obtained from size exclusion chromatography (gel filtration with Superdex 75). A: Dilution series (462 μM; royal blue to 11.5 μM; navy blue) of twice-delipidated apo-AFABP samples stored for one month at 4°C and analyzed in triplicate, showing reproducible dimer percentages within 1% for each sample and a nearly invariant dimer percentage (13.3±0.3%) throughout the indicated 50-fold concentration range. B: Recovered leading fraction (green) and original 15.2±5.6 μM apo-protein elution shown in panel A (blue). C: Recovered lagging fraction (red) and original 231.0±0.8 μM apo-protein from the elution shown in panel A (blue). Each trace in panels B and C represents an average of three GF chromatograms.

The trend in GF-based relative populations reported above would be anomalous if non-covalent association of AFABP molecules established a monomer-dimer equilibrium, as proposed in related protein samples under different experimental conditions based on crystallographic, fluorescence, and small-angle light scattering evidence.93-94. In that type of dimer, the predominant monomer proportion should indicate weak binding affinity; then as the sample is diluted, the dimer proportion should decrease to a vanishingly small value rather than remaining constant. Conversely, if the AFABP dimer is linked by one or more covalent bonds, then it is possible to
account for the observed constancy of the dimer proportion as a function of overall protein concentration.

In order to test this latter proposal, the leading and lagging fractions from the GF column were each recovered and subjected to another round of size exclusion chromatography. In the case of a non-covalent interaction, the leading and lagging fractions should each re-establish the identical monomer-dimer equilibrium state that would be reflected in the subsequent GF elution profile. Otherwise, there should be a greater dimer proportion observed from the recovered leading fraction and a lesser proportion from the corresponding lagging fraction, respectively. Figure 13 B and C show GF profiles that illustrate the larger relative amount of dimer in the recovered leading fraction compared to the recovered lagging fraction. This trend argues strongly for an AFABP dimer that is held together (irreversibly) by covalent bond(s). The hypothesis of a leading GF fraction in which a stable dimer predominates was also confirmed using ESI-MS (Figure 14).
Figure 14. Native ESI-MS verification of the recovered leading fraction (dimer) from the GF chromatogram of apo-AFABP (Figure 13B). The 8+ charged peak at \( m/z \) 29,748 Da corresponds to the monomer of AFABP (14,874 Da); the remaining three peaks are dimeric AFABP with various charges ranging from 12+ to 14+. The light blue lines provide a reference frame to identify the peak assignment.

3.1.3.2. The AFABP dimer is linked by disulfide bond(s)

Given the common observation of disulfide bonds in proteins \(^9^6\) and the presence of two surface-accessible cysteine residues in AFABP \(^9^7\), we hypothesized that our dimer is covalently linked in this fashion. This supposition was tested by monitoring the protein mass in the presence or absence of a \( \beta \)-mercaptoethanol disulfide bond reductant. The SDS-PAGE gels of Figure 15 showed a dimer band that disappeared upon treatment with a \( \beta \)-mercaptoethanol (BME) reductant. The retention of the 29-kDa band in the absence of BME suggests that the dimer is covalently bound rather than self-associated; its disappearance in the presence of BME supports identification of the
AFABP covalent bond as a disulfide linkage. Interestingly, only monomers were observed from the initial GF conducted during purification and in the recovered lagging fraction samples isolated directly after GF elution, but the dimer proportions grew to ~13% and ~16%, respectively, during refrigerated storage for one month.

**Figure 15.** Characterization of covalently bound AFABP oligomer by 15% SDS-PAGE. Lane 1, protein marker ladder; lane 2, AFABP monomer recovered from the lagging fraction in GF chromatography; lanes 3 and 4, AFABP dimer from the leading GF fraction, omitting (lane 3) and including (lane 4) a β-mercaptoethanol reducing agent.
3.1.3.3. The AFABP dimer forms through an N-terminal cysteine residue

Either of the two cysteine residues within the AFABP protein is a candidate for dimer formation via a disulfide bond, though the N-terminal Cys-1 is expected to have a somewhat more surface-accessible location than Cys-117. In order to verify the location, the recovered AFABP monomer and dimer fractions were each examined using 2D $^1$H-$^{15}$N HSQC NMR. The overlaid spectra of Figure 16 reveal differences in chemical shift for several peaks, i.e., significant perturbations in magnetic environment for the backbone amide group in particular molecular regions. Sites for which the weighted $^1$H and $^{15}$N perturbations exceeded one standard deviation beyond the mean value (Figure 17) were mapped onto the crystal structure of the protein (PDB: 2Q9S). Figure 18 shows that the most perturbed region of AFABP is located at the N-terminus, close to the location of Cys-1. These NMR results support the formation of a disulfide bond through the cysteine residue at the N-terminus of the protein sequence.
Figure 16. 500 MHz $^1$H-$^{15}$N HSQC NMR$^{99}$ contour plot showing overlaid spectra for the recovered monomer (blue, 400 $\mu$M) and dimer (red, 200 $\mu$M) from GF chromatography of AFABP protein samples. Chemical shifts are referenced according to the guidelines of Wishart et al$^{93,100}$. Highly perturbed residues are labeled in the spectrum; a complete plot of chemical shift perturbation as a function of protein sequence appears in Figure 16.
Figure 17. Composite $^1$H-$^{15}$N NMR chemical shift perturbations vs. AFABP residue number, comparing a disulfide-linked dimer to a monomer standard. Values for unassigned residues are left blank. The numerical values are calculated as $\Delta$(dimer-monomer) = $((\delta$HN(dimer) − $\delta$HN(monomer))^2 + {[\delta$N$(dimer) − $\delta$N(monomer)] / 6.31}^2)^{1/2}$ 93,98. The orange cut-off lines at 0.11 ppm, set slightly below the mean ± one standard deviation of perturbations for each pair of protein forms, are used to identify backbone sites with structurally significant chemical shift changes. Residue numbering for this protein construct is designated as G(-2)-M(-1)-C(1)-D(2)... to maintain consistency with published crystal structures.
Figure 18. Mapping of significant chemical shift perturbations on structures depicted with PyMOL [www.pymol.org] for the AFABP dimer with respect to the corresponding protein monomer (PDB: 2Q9S)⁴. Backbone residues highlighted in red exhibit composite ¹H-¹⁵N NMR chemical shift perturbations of at least one standard deviation beyond the mean value observed for 131 sites of the polypeptide. The cysteine residues that could form disulfide bonds are highlighted in blue.

3.1.3.4. AFABP dimer formation can be blocked by excluding oxygen gas

Given that disulfide bond formation is an energy-requiring oxidative process, we hypothesized that oxygen gas dissolved in the phosphate buffer solution could effect this chemical transformation. Formation of inter- or intramolecular S-S bridges under oxidative stress conditions has been established, for instance, in a protein kinase and a protein-tyrosine phosphatase, respectively ¹⁰¹. To test our proposal, a month-long course of dimer development was monitored for freshly purified apo-AFABP samples in a standard oxygen-saturated buffer vs. a buffer infused with oxygen-scrubbed nitrogen gas. The samples were collected at 8-10 day intervals and analyzed by GF to assess their respective dimer proportions (Figure 19). During the initial week of the time
course the dimer population grew commensurately in the two samples. Subsequently, dimer growth progressed linearly in the standard buffer but was essentially halted in the oxygen-free buffer medium. After thirty-five days, the comparative dimer proportions were 19% (oxygen-saturated control) and 5.5% (oxygen-free). (The estimated value after 25 days is 11%, in reasonable agreement with the 13% reported in Figure 13.) These results support the premise that dissolved oxygen gas is the reactant responsible for formation of the disulfide bond in the AFABP dimer.

Figure 19. Proportion of dimers present as a function of time for 56.3 μM (assuming all proteins are monomers) freshly prepared AFABP samples that were stored in buffers saturated with oxygen (blue curve) or infused with oxygen-scrubbed nitrogen gas (red curve). Percentages were derived from elution profiles of Superdex 75 size exclusion gel filtration chromatography analogous to those illustrated in Figure 13. Error bars denote results from triplicate GF runs on the same sample.
3.2. Sample preparation of LFABPs

3.2.1. Expression and purification of LFABP

The LFABP was induced in two different temperatures: 37 °C and 18 °C. According to Figure 20 (lanes 3 & 9), the LFABP was successfully expressed and produced at both temperatures. However, most of the protein was found in the insoluble form (Figure 20, lanes 5 & 11) at a growth temperature of 37 °C. Thus the 18 °C choice was more suitable for this cell line to generate soluble LFABP. This production of insoluble inclusion bodies is a quite common problem for induction of proteins at 37 °C in the BL21 cell line.\textsuperscript{102}

![Figure 20. SDS-PAGE of LFABP expression at 37 °C and 18 °C. Lanes 1 and 7, protein marker ladder; lanes 2-6, samples from 37 °C growth; lanes 8-12, samples from 18 °C; lane 2, before induction at 37 °C; lane 3, 5 hours of induction at 37 °C; lane 4, cell lysate after sonication; lane 5, insoluble fraction after centrifuging the sonicating lysate; lane 6, soluble supernatant after centrifuging the sonicating lysate; lane 8, before induction at 18 °C; lane 9, 24 hours induction at 18 °C; lane 10, cell lysate after sonication; lane 11, insoluble fraction after centrifuging the cell lysate; lane 12, soluble supernatant after centrifuging the cell lysate.](image-url)
After HisTrap and gel-filtration purification as outlined above, the LFABP with a 6 x histidine tag was pure as judged by SDS-PAGE (Figure 21A, lane 2). The procedure of primary expressions and purifications the LFABP was accomplished by Dr. Cédric Bernard and Dr. Sayantani Sarkar. In order to remove the Histidine tag, a TEV cleavage protocol was carried out at 30 °C. Unfortunately, the incubation always proceeded incompletely (Figure 21B, lanes 4 and 5). Extension of the incubation time did not improve the result (Figure 21B, lanes 7, 8, 10 and 11). We suspected that the N-terminal Histidine-tag (linker), which had a molar mass of 3.5 kDa, was a reversible competitor of the TEV protease for binding with uncleaved LFABP. As the reaction proceeded, more and more of the linker was removed and released, but it could still be recognized and bound by TEV protease. That could explain why the initial TEV digestion worked well, but as time went on the reaction rate became quite slow. In order to solve this problem, we used a Amicon Ultra centrifugal 10 kDa filter. Since our protein, the TEV, and the linker are 15 kDa, 30 kDa and 3.5 kDa respectively in mass, a 10K filter should allow only the linker to flow through while the TEV and our LFABP remained in the inner tube of the filter. By removing the linker in real time, the digestion continued to almost 100% completion (Figure 21 C, lane 15). The amount of ^15N LFABP purified in this way reached 30 mg per liter of minimal medium.
Figure 21. SDS-PAGE of LFABP purification. A) LFABP after HisTrap and gel-filtration purification. B) LFABP TEV cleavage. Lane 1, protein standards; lanes 2, 4, 7, 10, 0-3 hours processing of cleavage with old batch of TEV; lanes 3, 5, 8, 11, 0-3 hours processing of cleavage with freshly prepared TEV; lanes 6-9, flow-through of 3K filters. C) LFABP TEV cleavage. Lane 1, protein standards; lanes 2, 4, 8, 10, 14, 1-4 hours processing of cleavage with old batch of TEV and using 10K filters; lanes 3, 5, 9, 11, 15, 1-4 hours processing of cleavage with freshly prepared TEV; lanes 6, 7, 12, 13, flow-through of 10K filters.
3.2.2. **Delipidation of LFABP**

Since we have future plans to study the interaction and ligand transfer between LFABP and PPARα LBD, both apo-LFABP and apo-PPARα LBD were required. When pure LFABP and PPARα LBD are obtained after TEV cleavage, it is expected that endogenous lipids and other unknown molecules remain in the binding cavity. Prior publications, including ours and those of our collaborators, used Lipidex-1000 and monitored the removal of 14C-labeled radioactive OLA until the counts reached background levels. Radioactive monitoring was also reported for delipidation with Hydrophobic Interaction Chromatography (HIC); we used 1H-15N HSQC NMR as a monitoring method for HIC delipidation of both L-FABP and PPARα LBD to avoid the use of radioactive isotopes. However, the spectra of holo-OLA-15N-LFABP before and after HIC treatment showed only modest differences (blue and green spectra). On the premise that the protein might require more incubation time to allow the OLA to be extracted, we decreased the flow rate from 1ml/min to 0.1ml/min and allowed both duplicate and overnight incubation (Figure 22, orange, magenta) in a sequential fashion. The spectra remained quite similar to those observed before HIC. We concluded that fatty-acid (OLA) binds tightly enough within the LFABP cavity to preclude competition and ligand extraction by the HIC column.

We then proceeded with an alternative delipidation route using a Lipidex-1000 column, which suffers from high costs and requirements for a stable 37 °C working temperature, slower flow rate (0.01ml/min), and time-consuming column unpacking and re-packing of the beads before each use. The resulting NMR spectra indicated some differences with respect to the initial holo-OLA-LFABP sample (Figure 23, cyan). For instance, some holo-OLA-L-FABP peaks disappeared (Figure 23, blue box) and several new peaks appeared, as expected for apo-LFABP (Figure 23, Figure 24, blue box). These changes suggest that our sample is moving towards the apo form, but
the delipidation was viewed as incomplete. A second Lipidex treatment produced a very similar number and magnitude of peak alterations, i.e., very little further progress toward obtaining the protein in apo form. Both spectra from the Lipidex-treated samples displayed broader peaks than the holo form (Figure 24, blue circle) and were missing several peaks that had been established previously in apo-LFABP (Figure 24, green circle). Based on these observations, we can conclude that our protein started to be delipidated but could no longer progress.

Given the similarity between the current delipidation protocol and our prior successful procedures\textsuperscript{103}, we considered the possibility of improper and lengthy storage of the Lipidex beads. Re-purchase of the same beads from same vendor, including a check regarding manufacturing changes, yielded the same disappointing results (spectra not shown). Attempts were made to regenerate the beads with either methanol or petroleum ether to solubilize the highly hydrophobic fatty acids using a mobile phase (flowing solvent) that is much more hydrophobic than Lipidex. Nevertheless, we were not able to improve the delipidation and reproduce the published spectrum obtained by He, et al.\textsuperscript{28} Ultimately, this delipidation challenge was met by group members May Poh Lai and Francine Katz, who built on Cédric Bernard’s work to develop a butanol extraction delipidation protocol for LFABP that was validated spectroscopically and by re-binding of the OLA ligand to recover the holo-LFABP.
Figure 22 $^1$H-$^{15}$N HSQC spectrum of holo-OLA-$^{15}$N-LFABP (blue) overlaid with HIC delipidated-LFABP (first flow-through, light green; second flow-through, orange; flow-through after overnight incubation, magenta).

Figure 23 $^1$H-$^{15}$N HSQC spectrum of holo-OLA-$^{15}$N-L-FABP (blue) overlaid with one-time Lipidex-1000 flow-through (cyan) and two-time Lipidex flow-through (pink).
Figure 24. $^1$H-$^{15}$N HSQC spectrum of apo-$^{15}$N-LFABP (reference, red) from He et al$^{28}$ is overlaid with one-time holo-OLA-LFABP Lipidex-1000 flow-through (cyan) and two-time Lipidex flow-through (pink).
3.3. Sample preparation of PPARγ

3.3.1. Molecular cloning of the PPARγ LBD

The recombined PPARγ LBD DNA sequence designed and cloned by Dr. Cédric Bernard was validated by GENEWIZ sequencing. The confirmed plasmid (pET-57-DEST) carrying the PPARγ LBD DNA was successfully transformed into two cell lines, BL21-AI and BL21-pLysS, distinguished using LB agar plates with ampicillin antibiotics.

3.3.2. Expression and purification of the PPARγ LBD

The BL21-AI cell line expressed the protein well when it was cultured in LB medium. About 200 mg of recombined NusA- PPARγ LBD was produced from 1 L of culture, yielding about 100 mg of pure PPARγ LBD protein after removal of the NusA and histidine-tag. However, this cell line did not perform well during growth in minimal medium, which was required, for instance, to label the protein with 15N isotopes for NMR studies. In comparison, the BL21-pLysS cell line generated more protein when it was incubated in minimal medium. In Figure 25 lane 10, the PPARγ was shown to be pure after TEV cleavage.
The quality of the PPARγ LBD was validated by MALDI-TOF as shown in Figure 26. The predominant peak indicates that the PPARγ LBD was well purified and yield a measured molecular weight of the PPARγ LBD of 31062.178 Da compared to the calculated 31052.1 Da by ProtParam Expasy. In sum, the PPARγ LBD can be successfully expressed and purified by following the protocols described in an earlier section to yield about 100 mg of non-delipidated PPARγ LBD from 1 L of LB medium culture. Since the PPARγ LBD always binds endogenous lipid when it is produced in the BL21 cell line, it is critical to remove those lipids before we study its ligand binding.
Figure 26 MALDI-TOF of the PPARγ LBD after purification. The predominant peak mass-to-charge ratio (m/z) 31062.178 indicates the measuring molecular weight of the measuring object (PPARγ LBD). The minor peak m/z 15529.760 indicates the half molecular weight of the measuring object.
3.3.3. **Delipidation of the PPAR\(\gamma\) LBD**

The purified PPAR\(\gamma\) was incubated with apoAFABP which had been fully delipidated by Lipidex-5000 and then separated in a Supedex 75 gel-filtration column. The delipidated samples were denatured and launched to the ESI-MS to measure the released amount of OLA and thus estimate the proportion of PPAR\(\gamma\) in the apo form. The results are shown in Figure 27.

According to Figure 27, 97% of PPAR \(\gamma\) is in apo form. This indicates that the OLA can transfer from PPAR\(\gamma\) to AFABP by incubating and separating using a in the methods of gel filtration column. However, the biological expectation is that the ligand transfers from AFABP to PPAR\(\gamma\). More experiments and results will be discussed in later section 3.6.4. In addition, negative-mode ESI-MS is shown be an effective way to measure the OLA amount.

Since the ligand removal is based on GF chromatography, each cycle of purification result in loss of protein. The yield was about 60 mg per 1 L of LB medium.

![Figure 27 Histogram of OLA amount from each 3\(\mu\)M protein sample measured by ESI-MS. PPAR\(\gamma\)_non, the PPAR\(\gamma\) without any delipidating treatment; PPAR_2nd, the PPAR \(\gamma\) after twice incubated and separated with apoAFABP; PPAR_3rd, the PPARg after three times incubated and separated with apoAFABP; AFABP_1st, the AFABP incubated and separated from PPAR_non; AFABP_2nd, the AFABP incubated and separated from PPAR_1st, AFABP_3rd, the AFABP incubated and separated from PPAR_2nd.](image-url)
3.4. Sample preparation of PPARα

3.4.1. Attempted expression and purification of the PPARα LBD

The PPARα LBD cDNA sequence was validated by GENEWIZ sequencing. The confirmed plasmid (pDEST17) carrying the PPARα LBD DNA was successfully transformed into BL21-AI cells. The protein was quite well induced. But all of the material stayed in the insoluble phase (Figure 28, lane 6). The conditions were optimized at different temperatures (18°C or 37°C) and media. None of these modifications solved the protein solubility problem. To solve this problem, we connected the PPARα LBD to a fusion protein which is highly soluble to force our protein to fold and remain soluble.

Figure 28 SDS-PAGE of PPARα LBD expression in LB medium at 18°C. Lane 1, protein marker ladder; lane 2, non-induced culture aliquot; lane 3, 2 hours induced culture aliquot; lane 4, 20 hours induced culture aliquot; lane 5, the cell lysate after 20 hours induction; lane 6, the insoluble fraction after centrifugation of cell lysate; lane 7, the supernatant after centrifugation of cell lysate.
3.4.2. **Molecular cloning of the NusA_PPAR LBD**

The recombined NusA_PPARα LBD DNA sequence was validated by GENEWIZ sequencing. The confirmed plasmid (pET-57-DEST) carrying the PPARα LBD DNA was successfully transformed into two cell-lines, BL21-AI and BL21-pLysS, distinguished by LB agar plates with ampicillin antibiotics.

3.4.3. **Expression and purification of the NusA_PPARαLBD**

The NusA_PPARα LBD was successfully expressed with the addition of 1 mM OLA in LB medium by BL21-AI cell line. The OLA is intended to stabilize the structure of NusA_PPARα LBD and strengthen the binding to the HisTrap column. As a result, the NusA_PPARα LBD was overexpressed and purified in an approximate amount of 200 mg per 1 L of LB culture.

![Figure 29 SDS-PAGE of the PPARα LBD samples during each step of expression and purification. 1, protein marker ladder; 2, cell lysate after sonication; 3, insoluble fraction after centrifugation; 4 soluble supernatant after centrifugation; 5-10, the unbound fractions during the HisTrap; 11-15, the elution fractions of the HisTrap with a gradient of imidazole. The PPARα LBD is designated by a red arrow.](image-url)
The NusA_PPARα LBD was well expressed and purified, but when it was incubated with TEV protease to remove the histidine-tag, 99% of the protein irreversibly precipitated. The TEV co-precipitated as well, which might cause it to form an insoluble complex with PPARα LBD. Alternatively being explored in our laboratory include insertion of a Thrombin cleavage site, or re-designed by connecting the protein to His-tag at the C-terminal and scanning for the best expressing cell lines.

3.5. Assignment of AFABP HSQC spectrum

3.5.1. 3D TOCSY-HSQC and NOESY-HSQC

Previously, the AFABP HSQC spectrum was been partially assigned by Dr. Samar Rizk, but since the AFABP was discovered to not be fully delipidated, the assigned spectrum was actually a mixture of both apo and holo forms. Building on the previous assignment, the 3D TOCSY-HSQC and HOESY-HSQC NMR data were recorded and analyzed in NMRViewJ (Figure 30). 143 and 126 peaks were observed in apo-AFABP and holo-linoleate-AFABP spectra and 117 (90%) and 114 (88%) amide peaks were assigned.
Figure 30 Part of the 3D spectra used in protein assignment. NOESY-HSQC (white strip) and TOCSY-HSQC (light green); Intra-molecular interaction (blue line); sequential interaction (orange line).

<table>
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<td>143*</td>
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<td>21</td>
<td>126*</td>
<td>114</td>
</tr>
<tr>
<td>holo-oleate-AFABP</td>
<td>132</td>
<td>130</td>
<td>21</td>
<td>125*</td>
<td>114</td>
</tr>
</tbody>
</table>

* The total number of HN group in the main chain and side chain.

Table 2 Statistics of the assignment of apo-AFABP, holo-linoleate-AFABP and holo-oleate-AFABP.
3.6. Interaction between AFABP and PPARγ

3.6.1. Fluorescence titration to compare protein affinities for OLA

In section 3.3.3, the PPARγ was shown to be successfully delipidated by incubating with the apoAFABP. This result indicates that in vitro the ligand (OLA) prefers to bind AFABP rather than PPARγ, which is opposite to the biological direction of ligand transfer from AFABP to PPARγ. In order to further confirm this suspicion, a fluorescence titration was performed. Meanwhile, the ligand binding $K_d$ of PPARγ should be available from the competitive titration.

The fluorophore used was 11-((5-dansyl)amino)undecanoic acid (DAUDA), for which the emission peak will be altered in different environments. In Figure 30A, the DAUDA-PPARγ and DAUDA-AFABP emission peaks occur at 480 and 520 nm, respectively. Moreover, the PPARγ-bound DAUDA emission intensity was approximately 3-fold higher than the AFABP-bound DAUDA. This could be a sign that the PPARγ can bind a maximum of more than one ligand, a hypothesis that needs further study. It is known independently that a DAUDA blue-shift reflects the local hydrophobicity. So according to this result, we conclude that the PPARγ binding cavity is more hydrophobic than AFABP. Since the DAUDA can emit at different wavelengths, it is also possible to deduce which protein will bind the ligand preferentially based on the shift of the emission peak.

The apoAFABP and apoPPARγ were mixed at a molar ratio 1:1 and pre-equilibrated with an excess of DAUDA. In this case, both AFABP and PPARγ bind DAUDA and emit (Figure 31B red curve). As the ligand (OLA) was added, the peak intensity decreased and exhibited a blue shift as expected. The reason the peak decreased was because the DAUDA was replaced by the OLA. The blue shift indicates that the DAUDA-AFABP lost the signal more readily. In another words, the OLA binds AFABP preferentially to PPARγ.

This result can be interpreted in another way. Instead of comparing the entire scan of wavelengths, it was informative to examine the specific wavelengths of 460 nm and 520 nm. The intensities at these two extremes were compared at each titration increment (Figure 31C and 30D). Evidently, the intensity at 520 nm dropped more readily than the one at 460 nm. Since 520 nm is dominated by the AFABP, it may be concluded once again that the OLA binds AFABP more tightly than PPARγ.

Figure 31 Fluorescence assay of oleate titration of a AFABP-PPARγ-DAUDA mixture. A: red, 8 μM of apoAFABP mixed with 40 μM DAUDA; blue, 8 μM of apoPPARγ mixed with 40 μM DAUDA; B: titration curves as OLA is increased (from red to blue); C: intensity bar plot at 460 and 520 nm wavelengths (from B); D, normalized intensities from C.
3.6.2. *Fluorescence titration to obtain the relative protein-ligand binding affinities*

In order to obtain the relative binding affinities of the protein-ligand complexes based on the protein-DAUDA experiments, the ligand-bound percentages were calculated by using the formula shown below. The percentage values are plotted on decimal and logarithmic scales shown in Figure 32 A2&B2 and A3&B3.

\[
\text{Ligand bound} \% = \left( \frac{\text{slot intensity} - \text{initial intensity without ligand}}{\text{initial intensity without ligand}} \right) \times 100 \%
\]

By fitting each curve from Figure 32 A2&B2 to the Michaelis-Menten equation, the IC\textsubscript{50}\textsuperscript{52} is obtained and shown in Figure 33. It is observed that there is a trend that both AFABP and PPARγ binds the OLA tighter than LOLA. These fluorescence results are promising but preliminary in nature. Optimization is required in order to obtain better accuracy and reproducibility.
Figure 32 Protein-ligand titration fitting to the Michaelis-Menten equation. A1: 2 μM apoAFABP titrated with OLA (red) or LOLA (green) in the presence of 4μM DAUDA. B1: 0.5 μM PPARγ titrated with OLA (red) or LOLA (green) in the presence of 4μM DAUDA. A2 & B2: conversion of A1 & B1 fluorescence to ligand-bound percentage by being subtracted and dividing by the initial fluorescent signal without any ligand. A3 & B3: logarithm of x-axis of ligand concentration from A2 & B2.
Figure 33 IC\textsubscript{50} values by fitting the curves from Figure 32 A2 and B2 to the Michaelis-Menten equation. A: IC\textsubscript{50} values of AFABP bound LOLA (dark green) or OLA (dark red). A: IC\textsubscript{50} values of PPAR\textsubscript{γ} bound LOLA (dark green) or OLA (dark red).
3.6.3. **$^1$H-$^{15}$N-TROSY-HSQC of $^{15}$N-AFABP titrated with unlabeled PPARγ to define binding interface**

3.6.4. **NMR chemical shift perturbation due to $^{15}$N AFABPs - apoPPARγ interaction**

Results from titration of apoAFABP titrated by apoPPARγ are shown in Figure 34A. According to the spectra, most of the peak position were invariant; exceptions included the peaks in the zoomed region, which migrated as the apoPPARγ was added. It is clear that resonances are in the fast exchange regime which can be identified by progressive chemical shift changes of an averaged peak rather than relative intensity changes of two peaks. Fast exchange usually indicates that the binding between these two apo proteins is weak. The titration of apoPPARγ and holo-OLA-AFABP was studied as well (Figure 34B). The chemical exchange rate remained fast, similarly to the apo_apo titration. The chemical shift perturbation of each residue was calculated as shown below. (The spectrum of the holo-linoleate-AFABP_apo-PPARγ titration was not shown but was included in the calculation of the perturbations.)
Figure 34 $^1$H-15N TROSY-HSQC NMR of AFABP titrated by PPARγ. A: overlay of spectra of apo$^{15}$N AFABP titrated by 1-6 fold molar ratio of apoPPARγ; B: overlay of spectra from apo$^{15}$N AFABP titrated by 1-6 fold molar ratio of holo-oleate-PPARγ; C: overlay of spectra from apo$^{15}$N AFABP titrated by 1-6 fold molar ratio of holo-linoleate-PPARγ.
\[
\Delta \delta \ (ppm) = \sqrt{\Delta \delta_H^2 + \frac{1}{6.51} \Delta \delta_N^2}
\]

According to the Figure 35, most of the perturbed regions for each protein-protein pair span residues 29-35, which define the helical structure of the AFABP portal. By mapping onto the crystal structure (Figure 36), we observe that the most perturbed residues belong to the portal area. There are two particular residues, Asp-17 and Phe-57, which are perturbed significantly in spectrum A (apo_apo titration) but disappear in spectrum B (holo_apo titration). The disappearance indicates that these specific resonances are in intermediate exchange on the NMR timescale \((k_{ex} \sim \Delta \omega)\). Thus it is impossible to track the perturbations of these two residues which are substantially perturbed in the apo_apo titration. It is not certain whether these peaks will reappear in the NMR spectrum, since the titration has been not fully saturated. This non-global line broadening effect could indicate large changes in chemical (magnetic) environment at these sites, i.e., large \(\Delta \omega\) which becomes comparable to \(k_{ex}\).

There are two residues (Val-23, Asp-76) which are perturbed in the apo_apo titration but less perturbed in the holo_apo titration. They are mapped to crystal structures in Figure 36. The holo-OLA-AFABP is colored in green with the OLA ligand shown. The apo-AFABP is colored in red. These two residues are at the portal areas as well.

Finally, there are two residues (Lys-100, Arg-106) which are perturbed in the apo_apo titration but more impacted in the holo_apo titration. They are mapped to the crystal structure in both Figure 36 and Figure 37. It is gratifying to observe that Arg-106, which is critical to ligand binding,\(^{104}\) has been perturbed during the holo_apo but not the apo_apo titration. It is not clear that whether
this interaction is attributable to ligand binding. Future studies could attempt to untangle the protein-ligand binding and protein-protein interactions.

As the titration proceeds, the various peak intensities decrease in both apoapo and holoapo titrations as shown in Figure 38. This is a good sign that PPARγ binds the AFABP. This global broadening effect of the NMR signals is due to the addition of unlabeled PPARγ which binds the labeled AFABP and therefore leads to a slower tumbling rate of the complex.

It is also of interest to note that in the apoAFABPapopPARγ titration, residues T29-M35 of the portal area do not decrease in intensity as much as the average for the entire polypeptide. Comparatively, for the other two titrations, the residues in the portal area decrease more significantly than the average. Qualitatively, the least decreasing peaks tend to be located in the unstructured turns connecting the β-strands (Figure 39). Even though the PPARγ binding leads to a slow tumbling rate and broadening of the signals in the labeled AFABP protein, exceptions could occur at the termini or other highly mobile regions, for example the free coil region\textsuperscript{105}. In order to draw more definitive conclusions, the spin relaxation behavior of each residue must be monitored to investigate the broadening effects more rigorously.
Figure 35: Histogram of the chemical shift perturbation of each AFABP residue in apo and holo titrations. The unassigned residues are given arbitrary negative values. Red bar, chemical shift changes for apoAFABP_apoPPAR γ titration; Red line, the cutoff of the perturbations obtained by the mean value plus one deviation; Green bar, chemical shift perturbation of holo-oleate-AFABP_apoPPAR γ titration; Green line, the cutoff of the perturbations obtained by the mean value plus one deviation; Blue bar, chemical shift changes for holo-linoleate-AFABP_apoPPAR γ titration; Blue line, the cutoff of the perturbations obtained by the mean value plus one deviation. Red bars, chemical shift changes for apoAFABP_apoPPAR γ and holo-oleate-AFABP_apoPPAR γ titrations. Red bars, chemical shift changes for apoAFABP_apoPPAR γ and holo-linoleate-AFABP_apoPPAR γ titrations. Red bars, chemical shift changes for apoAFABP_apoPPAR γ and holo-linoleate-AFABP_apoPPAR γ titrations.
Figure 36 Structural mapping of the perturbed areas. The perturbation level is indicated by the gradient of colors. A & B: overlay of secondary structure and surface of apoAFABP perturbed by apoPPARγ (1LIB); C & D: overlay of secondary structure and surface of holo-OLA-AFABP perturbed by apoPPARγ (1LIB). The perturbed areas are colored by red in A & B and blue in C & D titrations; oleate ligand is colored in magenta. E & F, overlay of secondary structure and surface of the holo-OLA-AFABP perturbed by apoPPARγ (2Q9S).
Figure 37 Mapping the structure of the different perturbed areas to compare the apo-AFABP_aP-PPARγ (red cartoon) and holo-oleate-AFABP_aP-PPARγ (green cartoon) titrations.
Figure 38 Changes of peak intensity in AFABP_PPARγ titrations. Blue bar, the apoAFABP_aPOPPARγ peak intensity subtracted from the corresponding apoAFABP peak intensities; Red bar, the holo-oleate-AFABP_aPOPPARγ peak intensity subtracted from the holo-oleate-AFABP peak intensity; Green bar, the holo-linoleate-AFABP_aPOPPARγ peak intensity subtracted from the holo-linoleate-AFABP peak intensity.
Figure 39 Qualitative structural mapping of the intensity-decreasing areas. Regions of modest decrease in peak intensity are colored in gradient red; the areas where the peak intensity decrease are colored in gradient blue; the unassigned residues are colored in gray.

A: apo-AFABP (1LIB); B: holo-oleate-AFABP (1LID); C: holo-linoleate-AFABP (2Q9S).
3.6.5. **Dissociation constants \( K_d \) for \(^{15}\text{N AFABP - apoPPAR}\gamma\) interaction**

Since the formation of the protein-protein complex is accompanied by proportional changes in the chemical shift, it is possible to estimate the protein-protein dissociation constant (\( K_d \)) from the perturbed peaks. Since the resonances are in the fast exchange regime, it is possible to estimate the \( K_d \) from the chemical shift changes of the perturbed residues. The calculated perturbations are plotted in Figure 40A-C and fitted to the Michaelis-Menten equation with \( R^2 > 0.9 \) (\( R^2 \) indicates how the data points fit the curves.). According to the Figure 40A-C, all of the data points fit the curve well. All of the curves generate their own \( K_d \) and are averaged to obtain the \( K_d \) of the protein-protein complex shown in Figure 40D.

The derived \( K_d \) values are rather broadly distributed for different residues. For example, in the apoAFABP_a apoPPAR\gamma titration, the \( K_d \) ranges from 200 to 1200 \( \mu \text{M} \). Most of the data points fall in the range 250-750 \( \mu \text{M} \). There are two outliers: 993 and 1116 \( \mu \text{M} \) corresponding to G34 and V30, which are both in the portal area perturbed when apoPPAR\gamma is titrated. In the holo-OLA-AFABP_a apoPPAR\gamma titration, the \( K_d \) mainly ranges from 100 \( \mu \text{M} \) to 1 \( \text{mM} \), with one upper outlier at 1736 \( \mu \text{M} \) corresponding (V30) and three lower outliers at 100, 116 and 112 \( \mu \text{M} \) (V23, V32 and G34). In the holo-LOLA-AFABP_a apoPPAR\gamma titration, the \( K_d \) mainly ranges from 250 to 1250 \( \mu \text{M} \), with two upper outliers at 1833 and 1454 \( \mu \text{M} \) (V32 and A33). It is notable that all of these outliers pertain to residues in the most perturbed portal area of the AFABP protein.

The averaged \( K_d \)s are all in about half millimolar range, indicating that the protein-protein interactions are quite weak. The differences among these three averaged \( K_d \) are not so significant given the large variance for each titration. Although the average \( K_d \) is quite high, some specific residues V23, V32 and G34 yield small \( K_d \) values in the holo-OLA-AFABP_a apoPPAR\gamma titration. These trends suggesting modulation of the protein-protein interaction could be studied in the future.
Figure 40 Dissociation constant ($K_d$) of AFABPapoPPARγ interactions. A: residue chemical shift perturbation in apoAFABPapoPPARγ titration. B: residue chemical shift perturbation in holo-OLA-AFABPapoPPARγ titration. C: residue chemical shift perturbation in holo-LOLA-AFABPapoPPARγ titration. D: layout of $K_d$ estimated by fitting the residue perturbation to the Michaelis-Menten equation. E: comparison among three $K_d$ s estimated by perturbed V23, V32 and G34.
3.6.6. **Ligand transfer between the AFABP and PPARγ**

In section 3.3.3 we demonstrated that most PPARγ-bound oleate (>97%) can transfer from holo-oleate-PPARγ to apo-AFABP by simply mixing and separating by three successive GF column elutions. This results indicates that oleate binds to AFABP more tightly than PPARγ under our experimental conditions, although it is opposite to the biological direction. However, during the holo-AFABP-apo-PPARγ titrations it was noticed that as the apo-PPARγ was added, some apo-AFABP peaks appeared (Figure 34). The holo- and apo-proportion could be calculated based on their peak intensities, since the ligand binding is in the slow exchange rate. The results are shown in Figure 41. According to the histogram, when holo-AFABP was mixed with apo-PPARγ at molar ratio 1:1, about 3.9% or 9.1% AFABP-bound ligand (linoleate or oleate) transferred to PPARγ. As the molar ratio reached 1:4, about 10.1% or 15.7% AFABP-bound ligand (linoleate or oleate) transferred to PPARγ. Although it has been reported that the AFABP level in healthy men/women ranges 0.64~1.36 μM / 0.84~1.87 μM106, and 0.9~1.63 μM / 1.27~1.93 μM in Type 2 diabetic groups, the corresponding PPARγ has not been reported yet.

Based on the experiments of PPARγ delipidation and AFABP_PPARγ titrations, we find that the AFABP binds the ligands (oleate and linoleate) much tighter than does PPARγ. In another words, most of the ligand would bind AFABP first, which is opposite to the biological expectation. Nonetheless we also observed that about 3.9-9.1% (3.7-8.6 μM) ligand was able to transfer from AFABP (95 μM) to PPARγ (95 μM). This finding suggests that a low level of bound ligand could be present and sufficient to activate PPARγ function in gene transcription. In fact, it has been reported that the PPARγ transactivation EC₅₀ values are at about 0.01-1.0 μM range,¹⁰⁷ suggesting that PPARγ transactivation does not require a large amount of ligand to be transferred. In last paragraph, it is mentioned that in serum, the AFABP concentration is about ~1 μM. It is not known
that the AFABP concentration in nucleus. Assuming that the AFABP concentration is similar and 10% is transferred, which is about 0.1 \( \mu M \), then the ligand will fall into the PPAR\( \gamma \) transactivation EC\(_{50} \) (0.01-1.0 \( \mu M \)) range which is enough for the PPAR\( \gamma \) to initiate its biological function.

Interestingly, as discussed above, the most perturbed area is located within the portal region which also includes the NLS sequence. According to prior reports, there is an NLS sequence existing in a 3-D fold mapping to the helix-loop-helix region, which is part of the AFABP portal area. The sidechains of K21, R30 and R31 form a functional NLS by shifting their orientation to bind different ligands\(^{25} \). The non-activating ligands protrude from the portal and prevent the helical cap closure, while activating ligands favor an alternative homodimer to promote the exposure of the NLS and facilitate transfer of AFABP to the nucleus. This consideration can also explain how the PPAR\( \gamma \) obtains the activating ligand (LOLA) even though OLA has greater binding preference. Since the non-activating ligands will be blocked by the nuclear membrane, the activating ligands will become predominant in the nucleus.

In this project, only oleate and linoleate have been studied. Other ligands (neutral fatty acids or synthetic agonists such as Rosiglitazone, thiazolidinediones and Troglitazone) could also be of interest, because PPAR\( \gamma \) might specifically bind such ligands much more tightly.

AFABP is a critical lipid carrier that delivers lipids to many proteins in various organelles. It has been well established that AFABP associates with Hormone-sensitive Lipase (HSL)\(^{108} \). This research found that association to the HSL requires a bound ligand and that the interface is located at helix \( \alpha I \), which is part of the portal region. The AFABP delivers the ligand to the activated and phosphorylated HSL to inhibit its function to facilitate a feedback inhibition. Thus, study of the interaction between AFABP and HSL could inform investigation of the interaction between AFABP and PPAR\( \gamma \).
Figure 41 Bars plot showing estimation of the transferred ligand proportion by measuring the remaining holo-AFABP and apo-AFABP proportions. The mean values were calculated by averaging peak intensities of 7 residues (N=7). Left, remaining holo-AFABP peak intensity proportions at each titrating increment. Right, the newly appeared apo-AFABP peak intensity proportions at each titrating increment. Blue bars, titration of holo-oleate-AFABP by apo-PPARγ. Magenta bars, titration of holo-linoleate-AFABP by apo-PPARγ.
3.7. Conclusions

In this project, the interaction sites of AFABP with PPARγ have been studied primarily by NMR titration in the presence of different ligands. The most perturbed area is located in the portal region. This could indicate that AFABP transfers the ligand to PPARγ through interactions of that region. The corresponding PPARγ interaction sites remain undetermined, requiring future assignment of the 15N PPARγ amide NH resonances and titration by unlabeled AFABP.

By calculating the chemical shift perturbation, $K_d$ for the protein-protein complex was estimated. The binding interaction is weak (average $K_d \sim 500 \mu M$). Several residues e.g. V23, V32 and G34 behaved anomalously. For instance, in the holo-OLA-AFABP_aPOPPARγ titration, the average $K_d$ is 641 $\mu M$. The $K_d$s estimated by V23, V32 and G34 are about $\sim 100 \mu M$. It is of interest to discover that these residues are mostly perturbed and located at the portal area of the AFABP. This might be a sign that these residues are critical for the protein-protein interaction. In order to obtain more definitive information, a chemical shift perturbation study of the PPARγ interaction site and a possible NOE determination of spatial proximities would be useful.

It was surprising to discover that the oleate ligand binds the AFABP more tightly than PPARγ, since the physiological expectation is ligand transfer directly from AFABP to PPARγ in the nucleus. The previous delipidation result for PPARγ proves that AFABP binds the OLA more tightly than PPARγ. The NMR titration confirms this result. Although the fluorescence assay has not yet provided a specific protein-ligand binding constant, it is clear that the OLA preferentially binds AFABP when PPARγ is also present.

Although OLA preferentially binds the AFABP, our evidence shows that the ligand can transfer from holo-AFABP to the apoPPARγ site (10% when holo-AFABP: apoPPARγ = 1) in micromolar
level. According to previous studies, ligand present in the nanonolar range can initiate the function of the PPARγ. Thus a possible model suggests that in the nucleus, most of the PPARγ stays in the apo form; once the AFABP-bound ligand enters into the nucleus, a portion of the ligand transfers to the PPARγ. The total amount of holo-PPARγ is evidently large enough to initiate the regulating function of the related gene transcription. Validation of this proposal will require more experiments and supporting results.

The differential impact on protein-protein interaction from various ligands has been primarily studied by comparing the PPARγ-induced chemical shift perturbation without ligands and in the presence of OLA-bound and LOLA-bound AFABP. Overall, the perturbed areas are all at the portal region. Nonetheless, several residues exhibit variable perturbations depending on titration. It is inconclusive that whether the interaction is ligand independent, but there are several important residues e.g. Arg-106 which stabilizes the OLA binding is significantly perturbed in the holo-OLA-AFABP_apoPPARγ titration rather than the other two titrations. In future more ligands are should be incorporated in the protein-protein titration in order to obtain comprehensive knowledge on this interaction.

In this project, the protocols for PPARγ preparation and delipidiation have been improved. However, these protocols were not successful for PPARα preparation, although they share similar secondary structure and biological function. The main problem has been the precipitation of PPARα after tag removal. The PPARα is not quite stable and soluble without the tag and fusion protein. Different cell lines and tags are being tried by Stark Lab members. The delipidation of LFABP had not been solved when this thesis was written, but Stark Lab members are now able to delipidate the protein with the help of 1-butanol. Finally, difficulties were encountered in digestion of the TEV protease engineered between our target and fusion protein during FABP purification.
Evidently it is realized that the removed linker (histidine tag and NusA fusion protein) still competes protease with the uncleaved proteins. By using a 10 kDa Amicon filter, it was possible to remove the histidine tag and NusA fusion protein linker immediately to avoid competition with the protease for the uncleaved protein. Together, these technical advances offer an encouraging prospectus for broader investigations of FABP-PPAR interactions at the molecular level and hope for better understanding of metabolic signaling in mammalian cells.

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