

***Effect of R122L/S124A and K31L Mutations on Molecular
Structure and Function in Rat Liver Fatty Acid-Binding Protein***

By

Rima Estephan

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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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.

Date

Committee

Dr. Ruth E. Stark
Chair of Examining

Date

Dr. Lesley Davenport
Executive Officer

Dr. Fred Naider_____

Dr. Judith Storch_____

Dr. Marilyn Gunner_____

Dr. Tatyana Polenova_____

Supervisory Committee

THE CITY UNIVERSITY OF NEW YORK

Abstract

Effect of R122L/S124A and K31L Mutations on Molecular Structure and Function in Rat Liver Fatty Acid-Binding Protein

by

Rima Estephan

Mentor: Professor Ruth E. Stark

The unique binding and transfer mechanism of LFABP among proteins of the iLBP family have motivated the construction of two mutants: K31L modifies the charged residue in the first helix that is thought to interact with the first FA and to partially account for the transfer mechanism; R122L/S124A alters the binding cavity where Arg122 and Ser124 interact with the second FA. Titration and binding studies suggested that unlike K31L-LFABP, the R122L/S124A-LFABP mutant may bind just one fatty acid with a much lower affinity. Further NMR analysis of intermolecular NOE data between the ligand (oleate) and R122L/S124A-LFABP revealed that there is one oleate binding: the carboxylate end interacts with K96 and I98 near the opening of the β -barrel, and the hydrocarbon tail interacts with S39, I41, T51, and I109 in the cavity.

NMR-based chemical shift perturbation analysis revealed localized structural perturbations for the K31L mutant but more pervasive conformational changes for R122L/S124A. Nevertheless, neither of these mutations altered the transfer rate of FA to membranes. The solution secondary structures, based on chemical shift indices for $^1\text{H}_\alpha$, $^{13}\text{C}_\alpha$, and $^{13}\text{C}_\beta$ sites, indicated 10 β -strands and 2 α -helices as found for wild type-LFABP.

Interproton distance restraints derived from 3D and 4D nuclear Overhauser effect NMR experiments were used to calculate the best-fit tertiary structures of apo- and holo-R122L/S124A-LFABP. The apo-R122L/S124A-LFABP three-dimensional structures was well-defined with average pairwise RMSDs, for the 10 lowest energy structures, of 0.47 and 0.91 Å for backbone and all heavy atoms, respectively. However, the two α -helices are closer to the β -barrel in the mutant than in the wild-type protein forming a more closed portal that could explain the lower on-rate of the ligand.

The holo-R122L/S124A-LFABP preliminary three-dimensional structure was very similar (b.b. RMSD = 0.64 Å) to that of the apo- R122L/S124A-LFABP. Compared to the holo wild-type protein, one half of the β -clam (β G, β H, β I, and β J) is superimposable with that of the holo wild-type structure, but the other half of the β -clam (β A, β B, β C, β D, β E and β F) is displaced. In addition, the overall spatial relationship of the helix motif relative to the β -clam is similar to that in the holo wild-type structure. The three-dimensional structure of R122L/S124A-LFABP complexed with oleate is being further refined using both intermolecular NOE measurements and protein restraints.

DEDICATION

I would like to dedicate this dissertation to my parents, Edouard and Hanan Estephan, my siblings, Racha, Amir, and Rita and also to my husband, Joseph Alhaddad and my lovely children Charbel and Kaitlyn, who encouraged me and never stopped believing in my pursuit of this work. I give special thanks to my mother, Hanan Estephan, whom without her help and support I would have never been able to pursue my dream of being a scientist. And to my loving husband, Joseph Alhaddad, whose continuous support provided me with the motivation I needed to get through graduate school.

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LIST OF ABBREVIATIONS

Å	Angstrom
ADIFAB	Acrylodan labeled Intestinal Fatty Acid Binding Protein
AFABP	Adipose fatty acid-binding protein
AOFA	<i>n</i> -(9-anthroyloxy)fatty acids
12AO	12-(9-anthroyloxy)oleic acid
Apo	Non bound form of the protein
ARIA	Ambiguous Restraints for Iterative Assignment
BFABP	Brain fatty acid-binding protein
BSA	Bovine serum albumin
°C	Degree Celsius
CD	Circular dichroism
CL	Cardiolipin
CNS	Crystallography and NMR System
CRABP II	Cellular retinoic acid-binding protein type II
CRBP II	Cellular retinol binding protein type II
CSI	Chemical shift index
KDa	Kilo-dalton
D ₂ O	Deuterium oxide
Δδ	Chemical shift difference
DYANA	DYnamics Algorithm for Nmr Applications
EDTA	Ethylenediaminetetracetic acid

EFABP	Epidermal fatty acid-binding protein
EPC	Egg phosphatidylcholine
FA	Fatty acid
FFA	Free fatty acid
FABPs	Fatty acid-binding proteins
FRET	Fluorescence resonance energy transfer
[³ H]oleate	Tritium-labeled oleic acid
H ₃ PO ₄	Phosphoric acid
HFABP	Heart fatty acid-binding protein
Holo	Bound form of the protein
HSQC	Heteronuclear single quantum correlation
Hz	Hertz
IFABP	Intestinal fatty acid-binding protein
iLBPs	Intracellular lipid-binding proteins
ILBP	Ileal lipid-binding protein
IPTG	Isopropyl-β-D-thiogalactopyranoside
³ J _{HNα} / J _{NH-CHα}	NH-CHα coupling constants
K _d	Dissociation constant
K _p	Partition coefficient
KCL	Potassium chloride
LB	Luria Broth media
LCFA	Long-chain fatty acid
LFABP	Liver fatty acid-binding protein

MCCE	Multi-Conformational Continuum Electrostatics
MHz	Megahertz
NaCl	Sodium chloride
NaN ₃	Sodium azide
NBD-PC	<i>N</i> -(7-nitrobenz-2-oxa-1,3-diazol-4-yl) egg phosphatidylcholine
¹⁵ NH ₄ Cl	¹⁵ N-labeled ammonium chloride
NMR	Nuclear magnetic resonance spectroscopy
NOE	Nuclear Overhauser effect
NOESY	Nuclear Overhauser enhanced spectroscopy
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
ppm	Parts per million
PS	Phosphatidylserine
[θ]	Mean residue ellipticity
RMSD	Root-mean-square deviation
rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SUVs	Small unilamellar vesicles
TALOS	Torsion Angle Likelihood Obtained from Shift and sequence similarity
TBS	Tris-buffered saline
TOCSY	Total correlation spectroscopy

UV

Ultraviolet

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CHAPTER I

INTRODUCTION

Cellular transport of fatty acids to various organelles

In mammalian cells, long-chain fatty acids (LCFA) are important as a source of metabolic energy, yielding ~38kJ/g upon complete oxidation, more than twice that for the same weight carbohydrate or protein. They act as substrates for the synthesis of membrane lipids (phospholipids) and lipid mediators, in addition serving as storage molecules for metabolic energy (triglycerides) and for sterols, e.g. cholesterol esters. Because of their hydrophobicity and insolubility in water, LCFA are stored primarily in adipocytes as lipid droplets in the form of triglycerides, which must be hydrolyzed later by the action of hormone-sensitive lipase (HSL) to release the (non-esterified) LCFA. The LCFA in the blood are bound to serum albumin for transport to the plasma membrane of other tissues. At the target cells, once LCFA translocate across the plasma membrane by passive diffusion and/or by protein-translocase-facilitated diffusion, the LCFA desorb from the cytofacial leaflet into the cytoplasm. The rate of desorption may be increased by fatty acid-binding proteins (FABPs) (*Vork et al., 1993*). In addition, the high cytosolic concentrations of FABPs (0.2-1.0 mM), coupled with their high affinity for LCFA, suggest that FABPs can solubilize LCFA from membranes and act as a cytoplasmic 'sink' (*Paulussen et al., 1990*) that enhances LCFA transfer to acceptor membranes (peroxisomes, mitochondria, and endoplasmic reticulum) by aqueous transfer as in the case of liver fatty acid-binding protein (LFABP) (Figure 1) (*Hsu et al., 1996*). By contrast, other FABPs interact directly with membranes either via ionic interactions between lysine residues and acidic phospholipids in the acceptor membranes (*Hsu et al.,*

1996; Herr *et al.*, 1995; Corsico *et al.*, 1998) or by interaction with high affinity specific membrane receptors (Burton *et al.*, 1994). The importance of FABPs in lipid metabolism has also been explored more directly by creating knockout mice models. For example, creation of LFABP null mice showed that this protein is important in LCFA flux into the cytoplasm, as lipogenesis was decreased in the LFABP null mice (Martin *et al.*, 2003). Moreover, heart fatty acid-binding protein (HFABP) null mice showed severe defects in LCFA cellular transport and beta-oxidation in cardiac myocytes (Binas *et al.*, 1999; Schaap *et al.*, 1999). Various pathophysiological states are associated with marked alteration in cellular lipid homeostasis. For example, during ischaemia and perfusion there is an accumulation of fatty acids in the heart and there is an increased fatty acid catabolism in skeletal muscle during diabetes (Glatz and Van der Vusse, 1996). Therefore, FABPs are important protecting agents for the cell against the undesirable and potentially lethal effects of LCFA and their metabolites.

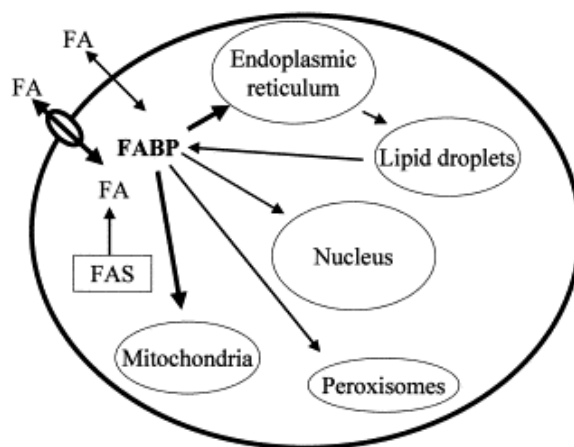


Figure 1.1 Intracellular transport of long-chain fatty acids. Influx and/or efflux of fatty acids across the plasma membrane take place by either protein-mediated or diffusive processes. Cytosolic transport to, or from, various intracellular organelles, including the mitochondria, endoplasmic reticulum, peroxisomes, nucleus and lipid droplets, may be mediated by the FABPs. Abbreviations: FA: fatty acid; FABP: fatty acid-binding protein; FAS: fatty acid synthetase (Storch and Thumser, 2000).

Characteristics of liver fatty acid-binding protein (LFABP)

Liver fatty acid-binding protein (LFABP) is a water-soluble 14-kDa protein that is expressed abundantly in both the epithelial cells of the small intestinal mucosa and in the hepatocytes of the liver. It belongs to a family of conserved intracellular lipid-binding proteins (iLBPs) of 126-134 amino acids that includes intestinal, ileal, heart, myelin, and adipocyte FABP as well as retinol- and retinoic acid-binding proteins showing various ligand binding affinities and specificities (*Banaszak et al., 1994; Veerkam et al., 1995; Glatz and Van der Vusse, 1996*). Sequence analysis of LFABP and other iLBPs reveals that proteins from the same species but different tissues exhibit a 22-73% sequence identity, whereas FABPs from the same tissue but different species show a 79-90% sequence identity (*Zimmerman and Veerkamp, 2000*). Sequence alignment of rat-LFABP with ILBP, CRABP II, BFABP, CRBP II, IFABP, and HFABP of the same species show higher sequence identity to ILBP (41%) than other FABPs (Figure 2). Consequently, aligning apo-LFABP with apo-ILBP showed closer structural similarity (RMSD=4.77 Å) than that of apo-LFABP with apo-IFABP (RMSD=6.35 Å) (Figure 3). In the case of apo-LFABP and apo-ILBP alignment, strong conformational alteration was only present in the helix-turn-helix and E/F turn regions. In contrast, alignment of apo-LFABP with apo-IFABP showed a more drastic overall conformational alteration (Figure 3).

LFABP binds preferentially to long-chain, unsaturated fatty acids with high affinity ($k_{d1} \sim 9\text{nM}$, $k_{d2} \sim 62\text{nM}$) (*Richieri et al., 1994*) and also binds to saturated fatty acids but with lower affinity relative to unsaturated fatty acids. It has also been reported to bind heme, bilirubin, lysophospholipids, bile salts, acyl CoA esters, bromosulphothalein, hexachlorophene, cyclopentenone prostaglandin, and a host of other hydrophobic

compounds with lower affinity (*Glatz and Van der Vusse, 1996*). In contrast to the other members of the FABP family, one mole of LFABP is capable of binding 2 moles of long-chain fatty acids (*Cistola et al., 1989*). For the larger ligands, such as heme, bile acids, and lysophospholipids where both fatty acid sites are required to bind the larger ligands, the stoichiometry is reported to be one to one (*Stewart et al., 1996; Glatz and Van der Vusse, 1996; Thumser et al., 1995*).



Figure 1.2 Sequence alignment and percent sequence identity of LFABP with other iLBP family members. Key: r, rat; LFABP, liver fatty acid-binding protein; ILBP, ileal lipid-binding protein; CRABP, cellular retinoic acid-binding protein; BFABP, brain fatty acid-binding protein; IFABP, intestinal fatty acid-binding protein; CRBP, cellular retinol-binding protein; HFABP, heart fatty acid-binding protein. Asterisks represent identity, colons represent high similarity, periods (full stops) represent low similarity, and spaces represent no similarity. Alignments and consensus sequence were derived using T-COFFEE, Version_1.41 (*Notredame et al., 2000*).

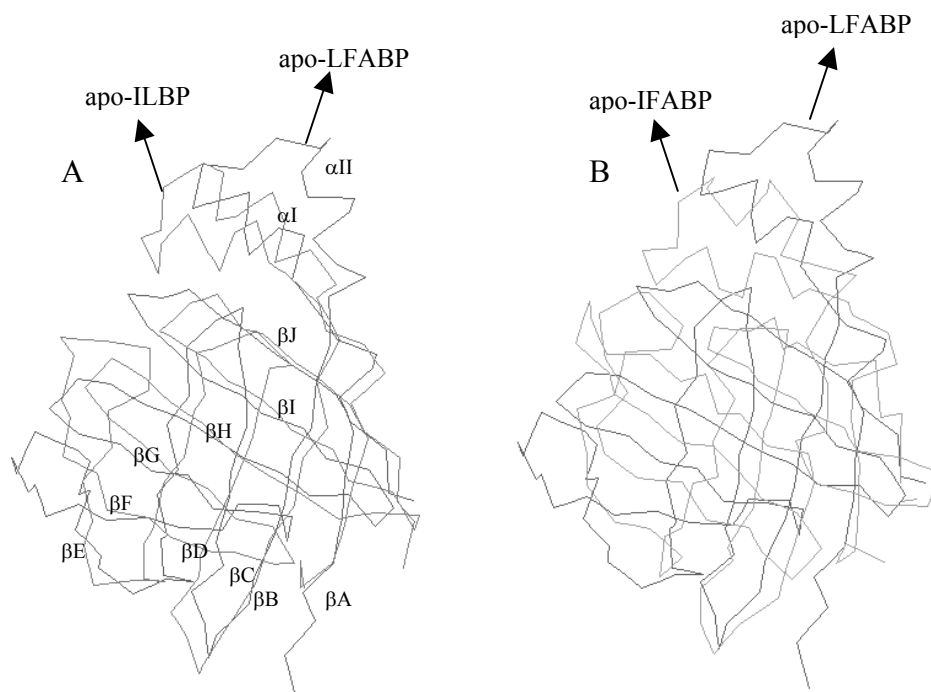


Figure 1.3 Structural alignment of apo-LFABP with other FABPs.

A: apo-LFABP with apo-ILBP. B: apo-LFABP with apo-IFABP. Key: LFABP, liver fatty acid-binding protein; ILBP, ileal lipid-binding protein; IFABP, intestinal fatty acid-binding protein. Structures from protein data bank were displayed using Swiss-PdbViewer 3.7 (Guex and Peitsch, 1997).

The three-dimensional structures have been investigated for several family members by NMR spectroscopy and X-ray crystallography (Balendiran *et al.*, 2000; Young *et al.*, 1994; Winter *et al.*, 1990; Sacchettini *et al.*, 1989; Lucke *et al.*, 1999; Zhu *et al.*, 1999). Even though these proteins display different ligand-binding affinities and specificities, they all show the same overall tertiary fold, indicating a well-adapted cavity conformation for binding small lipid molecules (Veerkamp *et al.*, 1995). The fatty acid ligand is bound inside the protein body, which consists of 10 anti-parallel β -strands, labeled βA through βJ beginning from the N-terminal, that form a β -clam-type structure enclosing an internal cavity. In addition, a short helix-turn-helix motif, linked to the main framework by the βA - αI and αII - βB polypeptide segments, closes the β -clam on one side

and is thought to participate in the trafficking of lipids (*Sacchettini et al., 1989; Hodsdon et al., 1997a; Hodsdon et al., 1997b*). In fact, small conformational changes induced by chemical modification (*Herr, et al., 1995*), mutation (*Ory et al., 1997; Herr et al., 1996*), or deletion (*Cistola et al., 1996*) in this region play a role in the protein's binding affinity and kinetics. Using an *in vitro* fluorescence resonance energy transfer (FRET) assay, Kim and Storch have shown previously that the movement of fluorescent *n*-(9-anthroyloxy)fatty acids (AOFA) from LFABP to acceptor membranes occurs via aqueous diffusion of the FA from protein to membrane. Cellular retinol binding protein type I (CRBPI), like LFABP, utilizes an aqueous diffusion mechanism to transfer its ligand to membranes (*Herr et al., 1999*). In contrast, a collisional fatty acid transfer mechanism was observed for intestinal fatty acid-binding protein (IFABP) (*Kim et al., 1992; Hsu et al., 1996; Storch et al., 2000*), adipose FABP (AFABP) (*Herr et al., 1995*), heart FABP (HFABP) (*Herr et al., 1996*), and cellular retinol binding protein type I (CRBPI) (*Herr et al., 1999*) (Figure 4).

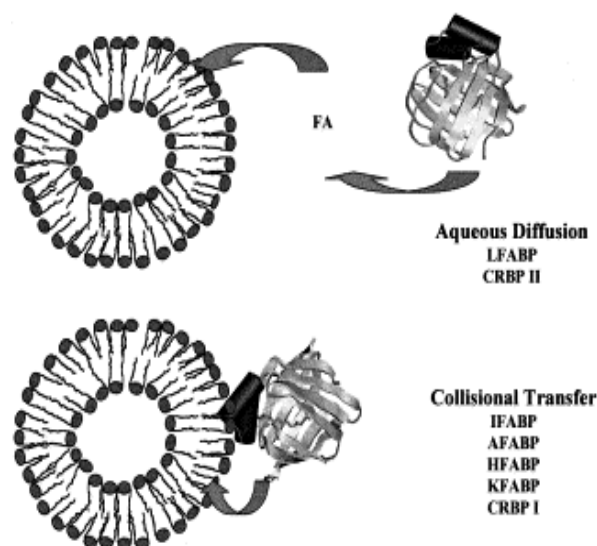


Figure 1.4. **Transfer of fatty acids from FABPs to membranes occurs by two different mechanisms:** diffusion of ligand through the aqueous phase and subsequent association with the acceptor membrane (LFABP (liver), CRBP II (cellular retinol binding protein type II)); or a collision-mediated process in which the FABP and the acceptor membrane are in direct contact (IFABP (intestinal), AFABP (adipose), HFABP (heart), KFABP (kidney), CRBP I (cellular retinol binding protein type I) (Kim *et al.*, 1992; Hsu *et al.*, 1996; Wootan *et al.*, 1993; Herr *et al.*, 1999; Herr *et al.*, 1996; Herr *et al.*, 1995; Storch *et al.*, 2000).

FABPs as early markers for cancer and /or organ damage

LFABP and other FABPs have been implicated in control of cell proliferation or cancer (Celis *et al.*, 1996). The expression of LFABP was elevated in liver during cell proliferation such as carcinogenesis or regeneration (Custer *et al.*, 1984), and transfection of LFABP into hepatoma cells increased their proliferation (Khan *et al.*, 1994). LFABP and intestinal FABP (IFABP) were elevated by 3–14-fold in prostate cancer cells compared with normal prostate cells. Adipose FABP (AFABP) and epidermal FABP (EFABP), however, were down regulated in cancer cells (Celis *et al.*, 1996). Heart FABP (HFABP) appears only in normal lactating cells and not in tumor mammary cells (Grosse

et al., 1991). Transfection of a cDNA clone of HFABP into breast cancer cells results in the loss of tumorigenicity (*Huynh et al.*, 1996).

Moreover, HFABP is an early plasma marker of acute myocardial infarction (AMI) (*Veerkamp et al.*, 1991). There are numerous studies using various FABPs as markers of specific organ damage. In view of the fact that FABPs are small in size, they can leak out of the damaged dying cells and lead to a rise of plasma and urine levels, making it possible for FABPs to be used as early markers for detecting organ damage. Consequently, understanding the structure and function of LFABP and its family FABPs provides a potentially important step toward designing therapeutic strategies against cancer, either by activating or inactivating the targeted FABP gene depending on the type and location of the cancer.

Structural analysis of LFABP in comparison to other FABPs

On the structural level, the LFABP cavity is the largest of the 10 family members, 610 Å³, as compared to 450 Å³ in AFABP and 353 Å³ in IFABP. Like other family members, 48% of this inner cavity surface consists of hydrophobic side chains, whereas 52% is associated with charged or polar residues (*Thompson et al.*, 1999). Comparing the LFABP cavity to that of other iLBPs, we can observe changes in key residues that line the surface. As one goes from the large cavity (LFABP) to the smaller cavity (IFABP) cavity, the sidechains change from small to bulky. For example, the changes are from S39 to L38, T94 to K92, and T102 to R106. The ligand molecules are bound inside a large water-filled cavity created mostly by the β -sheet structure (Figure 5 and 6). The waters are involved in hydrogen-bond networks with the interior hydrophilic residues and the carboxyl oxygen atoms of the fatty acid molecules. One fatty acid

molecule is in a U-shaped conformation and is located at the bottom of the binding cavity (Figure 5).

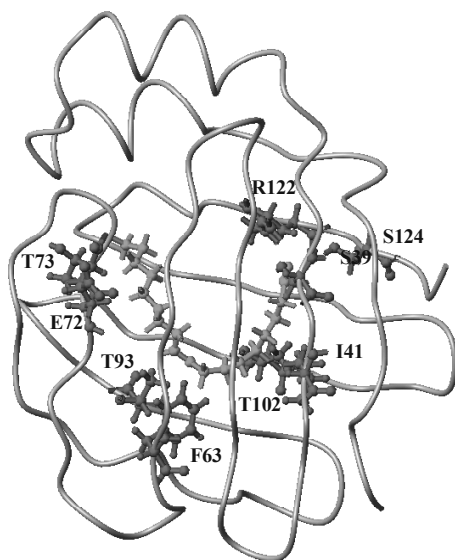


Figure 5. **Residues that interact with Oleate 1 in LFABP.**

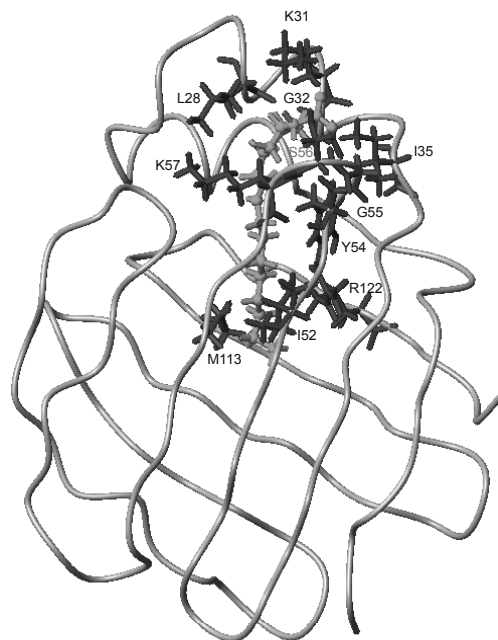


Figure 6. **Residues that interact with Oleate 2 in LFABP.**

The carboxylate of this oleate is not accessible to solvent, but it is involved in a more extensive hydrogen-bonding network with bound waters and other cavity residues in the protein than the second oleate molecule. The cavity residues involved in this first network are R122, which was previously identified by site directed mutagenesis (*Borchers et al., 1993*) and chemical modification studies (*Thumser et al., 1994; Thumser et al., 1996*), and S39 and S124, also deduced by X-ray crystallography (*Thompson et al., 1999*). Additional amino acids with atoms within 5 Å of this oleate are: I41, F63, E72, T73, T93, and T102 (*Thompson et al., 1999*). Similarly, intermolecular NOEs derived from 2D NMR spectra of ^{15}N -LFABP/ ^{13}C -oleate and $^{15}\text{N}/^{13}\text{C}$ -LFABP/oleate complexes

revealed that the first oleate interacts with residues I41, I50, F63, V83, I98, T102, I109, Y120, and S124 (*He et al., to be submitted*).

The second oleate molecule bound to LFABP is more extended and has the carboxylate moiety near the surface of the LFABP molecule (Figure 6). The cavity space occupied by the second bound oleate more closely resembles that found for AFABP. However, in AFABP, the oleate is bound with the carboxylate in the cavity interacting with Arg126 and Tyr128 (*Xu et al., 1993*). The carboxylate group of the second oleate bound to LFABP is located near residues forming the portal. The hydrogen-bonding network involves K31, Y54, and S56 (*Thompson et al., 1999*). The hydrophobic chain of the surface oleate continues down into the cavity, contacting the innermost oleate and residues in the crystal state (x-ray): L28, G32, I35, I52, Y54, G55, K57, M113, and R122 (*Thompson et al., 1999*). The location of the surface oleate deduced in solution by NMR methods is generally similar, though less contact is evident with the interior oleate. From the crystallographic and NMR data, it appears that the driving force for the first oleate to interact with the protein is electrostatic interaction. In contrast, with the second oleate the main interaction is hydrophobic. It is speculated that after the first oleate has bound, it may create a suitable hydrophobic environment for the second oleate. Therefore, it appears that the first oleate must be present before the second oleate can bind. The second site may not exist until the interior primary site is filled (*Thompson et al., 1999*).

Previous studies have shown that mutating Arg 111, one of four arginine residues that generate a positive potential that could facilitate the binding of retinoic acid (RA), to methionine in cellular retinoic acid binding protein type II (CRABP II), decreased the affinity for RA by 45 fold (*Wang et al., 1998*). This mutation also caused significant but

localized changes in the conformational and dynamic properties of CRABPII. Apo-R111M was more similar to wild-type holo-CRABPII. Another study showed that in AFABP, Ala substitutions for Arg106 and Arg126, which interact with the FA carboxylate, reduce affinities by about 100-fold (*Richieri et al., 1998*). Moreover, Thumser et al. reported that the T102Q/R122Q mutation in LFABP completely altered the structure from predominantly β -sheet to predominantly α -helix (*Thumser et al., 1996*). Binding studies with DAUDA (11-(5-dimethylamino-naphthalenesulphonyl) undecanoic acid) and dansyl-lysophospholipids showed that the T102Q/R122Q mutant did not bind any of the fluorophores due to incorrect folding of the protein (*Thumser et al., 1996*). Given the important role of positively charged residues in the protein cavity that interact with the carboxylate of the ligand, a double mutant (R122L/S124A)-LFABP was generated to study the impact of charge abolition on binding and structure.

In addition, studies that involved the portal (helix) region of the protein have shown that replacement of lysine with isoleucine in the portal region of AFABP resulted in much slower FA transfer rates to acidic membranes than those of native AFABP (*Liou et al., 2001*). In IFABP, a chemical modification by selective acylation neutralizing surface lysine residues resulted in a markedly inhibited collisional mechanism (*Corsico et al., 2005*). In addition, a more drastic change entailing the deletion of the helical domain in IFABP altered the transfer mechanism of fatty acids from a collisional transfer to an aqueous diffusion-mediated process; it diminished the capacity to interact with membranes markedly (*Corsico et al., 1998*). Furthermore, to assess whether the helix-turn-helix domain also dominates the distinctive LFABP mechanism, Corsico et al. have shown that exchanging α -helical domains between IFABP (α L β IFABP) and LFABP

($\alpha\beta$ LFABP) switched their fatty acid transfer mechanism. The α L β IFABP (helix from LFABP and β -sheet of IFABP) chimeric protein exhibits a diffusion mechanism, whereas the $\alpha\beta$ LFABP (helix from IFABP and β -sheet of LFABP) chimeric protein switches to a collision-mediated transfer mechanism (*Corsico et al., 2004*). Therefore, mutating Lys31 to Leu should reduce the positive charges on the surface of the helical domain that are important for interaction with membranes, while keeping the size of the residue constant. This mutation may reduce the transfer rate of LFABP but since the transfer mechanism is already diffusion controlled, it is not expected to change the mechanism. In addition, K31 interacts with the carboxylate of the second oleate so that mutating it to Leu might alter the binding affinity of the second oleate.

MCCE method: Electrostatic energies for interactions between LFABP and two oleates

A complementary view of the residues important in LFABP ligand recognition comes from the Multi-Conformational Continuum Electrostatics (MCCE) method; developed in Dr. Marilyn Gunner's Laboratory at CCNY (*Alexov et al., 1997; Alexov et al., 1999*). This computational protocol allowed us to calculate the ionization states of all residues throughout the LFABP protein. Electrostatic energies for self- and pair-wise interactions were calculated for LFABP complexed with two oleates. This method revealed the favorable contribution of some positively charged residues (Arg122, Lys125, Arg126) to the interaction of LFABP with oleate. It confirmed that the driving force for the first oleate to bind is mainly electrostatic interactions between the carboxylate of oleate1 and the positively charged patch (Arg122, Lys125, Arg126) at the inner surface of the cavity (*Mao et al., unpublished observations*). The second oleate is more exposed to the surface

and its carboxylate interacts most strongly with Lys31. However, since the hydrophobic chain of the surface oleate contacts the innermost oleate and some hydrophobic residues in the protein (*Thompson et al., 1999; He et al., to be submitted*), the main interaction is basically hydrophobic. The MCCE method showed that the Arg122 and Ser124 are the residues that promote the ionization of oleate 1. Analogously, Lys31 promoted the ionization of oleate 2. As a result, this method guided us in the construction of site-specific mutant LFABPs (K31L and R122L/S124A).

In brief, mutating Arg122 and S124, which interact with oleate 1, to Leu and Ala, respectively, may significantly reduce the binding affinity and may even change the binding location. The R122L/S124A LFABP mutant might bind only one oleate with the binding conformation of oleate 2 bound to wild type LFABP (tail extended in the cavity and carboxylate on the surface) (Figure 6). The rationale behind the drastic choice of substituting positively charged and polar residues with non-polar, hydrophobic residues instead of uncharged polar residues (modest change) is to completely abolish any existing electrostatic interaction with the ligand.

The goal of my thesis study is to assess whether these mutations in LFABP have affected the protein's structure and /or function. Preliminary results have shown that the R122L/S124A mutation was found to introduce the greatest perturbation in the structure and binding affinity. As a result, it is of interest to determine the solution structure of apo-R122L/S124A-LFABP and holo-R122L/S124A-LFABP, along with protein-oleate interactions in the holo protein, using multi-dimensional NMR techniques and intermolecular NOE analysis.

CHAPTER II

MATERIALS AND METHODS

Materials

The two synthetic oligonucleotide primers were obtained from Sigma-Genosys (Houston, TX). The BL21(DE3) bacterial cells and the QuikChange site-directed mutagenesis kit were purchased from STRATAGENE (La Jolla, CA). The Wizard plus Midipreps DNA Purification System was purchased from PROMEGA (Madison, WI). The ^{15}N -ammonium chloride, D- ^{13}C -glucose, isopropyl- β -D-thiogalactoside (IPTG), Luria Broth (LB), ampicillin and Lipidex-1000 were purchased from Sigma-Aldrich (St. Louis, MO). The SDS-PAGE reagents (Coomassie brilliant blue G-250, acrylamide, N,N'-methylene-bis-acrylamide, glycerol, bromophenol blue, ammonium persulfate, N,N,N',N'-tetramethylethylenediamine (TEMED), sodium dodecyl sulfate (SDS), and β -mercaptoethanol were purchased from Sigma (St. Louis, MO). The MEM vitamins solution was purchased from Invitrogen-Gibco (Carlsbad, CA). The ^3H -oleic acid was purchased from Perkin-Elmer Life sciences, Inc. (Boston, MA). The SephadexTM-G-50 medium was purchased from Amersham Biosciences (Piscataway, NJ). The DE-52 pre-swollen microgranular medium was purchased from Fisher Scientific (Atlanta, GA). The Amicon YM-10 membranes and Centricon 10 were obtained from Millipore, Inc. (Burlington, MA). The fluorescently labeled AOFA, 12-(9-anthroyloxy)oleic acid (12AO) was purchased from Molecular Probes, Inc. (Eugene, OR). Egg phosphatidylcholine (EPC), N-(7-nitro-2,1,3-benzoxadiazol-4-yl) egg phosphatidylcholine (NBD-PC), brain phosphatidylserine (PS), and bovine heart cardiolipin (CL) were obtained from Avanti Polar Lipids (Birmingham, AL). Lipids were

stored in chloroform under nitrogen at -20°C . Recombinant wild type rat LFABP plasmid was generously provided by Alan Kleinfeld (Medical Biology Institute, La Jolla, CA).

Methods

The MCCE Method

The Multi-Conformational Continuum Electrostatics (MCCE) method was developed in Dr. Marilyn Gunner's Laboratory at CCNY. The MCCE allows multiple positions of hydroxyl and water protons, alternative side chain rotamers, water positions and cofactor positions in the calculation of the pH dependence of ionization equilibria (*Alexov and Gunner 1997; Alexov and Gunner, 1999*), unlike other standard electrostatic calculations, which consider only a single structure for all heavy atoms and polar protons. Each alternate position or ionization state of a residue, cofactor, water or ligand is termed a conformer. Acidic and basic residues have ionized and neutral conformers. Neutral acids, hydroxyl residues, and waters have conformers with different proton positions. Buried waters can have their oxygens in alternate positions within a binding site. Monte Carlo sampling calculates the probability of realizing each of the initially suggested conformers in a Boltzmann distribution of microstates. Thus, the position or ionization states of particular residues, or occupancy of water sites can be determined. In order to minimize the errors, two limitations are imposed on the allowed motions. First, the backbone is kept rigid so that the motions of individual residues are not coupled together with changes in backbone position. Second, only side chains in the protein interior are allowed to move, which diminishes changes in dielectric boundaries.

Construction of K31L and R122L/S124A Mutant LFABP Genes

The QuikChange site-directed mutagenesis kit was used to achieve mutagenesis in a synthetic rat liver fatty acid-binding protein (LFABP) gene cloned into PET-11a plasmid. *pfuTurbo* DNA polymerase (provided with the kit) was used to replicate both plasmid strands with high fidelity and without displacing the mutant oligonucleotide primers. For each mutant, the basic procedure utilizes the pET-11a double-stranded DNA (dsDNA) vector containing our LFABP insert and the two synthetic oligonucleotide primers (sense and antisense) containing the desired mutation (Table 2.1). The oligonucleotide primers, each complementary to opposite strands of the vector, are extended during temperature cycling by *pfuTurbo* DNA polymerase. Incorporation of the oligonucleotide primers generates a mutated plasmid containing staggered nicks.

Table 2.1. Oligonucleotide Primers For Mutagenesis.

Mutations	Primers	Sequences 5' → 3'
Single (Lys31 → Leu)	K31L(sense)	GAC CTC ATC CAG <u>TTA</u> GGG AAG GAC ATC AAG
	K31L(antisense)	CTT GAT GTC CTT CCC <u>TAA</u> CTG GAT GAG GTC
Double (Arg122Leu & Ser124 Ala)	R122L/S124A (sense)	GAC ATC GTC TAC AAG <u>TTA</u> GTC <u>GCC</u> AAG AG
	R122L/S124A (antisense)	CT CTT <u>GGC</u> GAC <u>TAA</u> CTT GTA GAC GAT GTC

Following temperature cycling the parental DNA template is digested using *Dpn I* (provided with the kit). The *Dpn I* endonuclease is specific for methylated and hemimethylated DNA, forms characteristic in *Escherichia coli* (*E.coli*) strains and in this case the parental DNA template. The nicked DNA vector containing the desired

mutations is then transformed into XL1-Blue supercompetent cells (provided with the kit). XL1-Blue cells containing the pET-11a vector with the mutant LFABP insert were grown and plasmid DNA was isolated using Wizard plus Midipreps DNA Purification System. To verify that the mutation indeed took place, the University of Georgia Molecular Genetics Instrumentation Facilities sequenced plasmid DNA from each sample.

Expression of wild type and mutant LFABP

The pET-11a vector containing the mutant LFABP cDNA was transformed into BL21(DE3) bacteria on agar plates containing ampicillin (200 µg/ml). Individual colonies of the transformed cells were inoculated into 5mL of LB medium/ampicillin (200 µg/ml) and allowed to grow at 37 °C and 225 rpm to late log phase ($OD_{600} \sim 0.6$). The 5mL of BL21(DE3) cells containing the mutant LFABP were transferred to 2 liters of LB media and 200 µM ampicillin (for selection) and grown shaking at 225 rpm and 37 °C to late log phase ($OD_{600} \sim 0.6$). Cells were then centrifuged and resuspended in 1 liter of ^{15}N labeled Minimal Media (200 ml 5X M9 salt, 1g $^{15}\text{NH}_4\text{Cl}$, 8 ml 50 % glucose (or ^{13}C -glucose, 4g/L), 1 ml of 2M MgSO_4 , 0.2 ml of 0.5M CaCl_2 , and 10 ml MEM vitamins) / 200 µM ampicillin. After 1 hr incubation, the LFABP expression was induced with 1.0 mM IPTG. The bacterial culture was incubated at 37 °C shaking at 225 rpm and cells were harvested 3 hrs later (*Marley, et al., 2001*). A 1-mL aliquot was collected before induction and after 3 hrs induction to check the protein expression. Then protein expression was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15 %). Cells were then stored at -70 °C until proteins were to be purified.

Purification of wild type and mutant LFABP

Cells were lysed with 20 ml of T₁₀EK buffer (10mM Tris, 1mM EDTA, 100mM KCl, pH 7.7) followed by sonication and ultracentrifugation for 45 minutes at 20,000 rpm. The resulting supernatant was mixed with [³H]oleate (5 Ci/mmol) in order to monitor the fractionation of our protein. The protein was then purified at 10°C, first on a size exclusion chromatographic column (Sephadex G-50) equilibrated with KCl-phosphate buffer (0.15 M KCl, 0.05 M phosphate, and 0.02 % sodium azide, pH 7.4). Fractions of 10 ml were collected, and the protein concentration and radioactivity of each fraction were measured. Only fractions containing both protein and radioactivity were pooled and concentrated by ultrafiltration using a YM-10 membrane. The concentrate was re-chromatographed on an anion exchange chromatographic column (DE-52) equilibrated with 10 mM Tris phosphate (10mM Tris base adjusted to pH 8.3 with H₃PO₄). Fractions containing protein were pooled and applied to a Lipidex-1000 column (*Glatz, et al., 1983*) equilibrated with pH 7.4 KCl-phosphate buffer at 37°C to remove protein-bound [³H]oleate. Protein purity (>98%) was assessed by SDS-PAGE (15 %), and protein concentration was determined by the Bradford method, using BSA as a standard (*Bradford M., 1976*) (concentrations corrected by factor of 0.6; *Glatz et al., 1985*), or by UV spectroscopy at 280 nm using an extinction coefficient of 6400 M⁻¹cm⁻¹. Typical protein yields were 20-30 mg per liter of culture. The purification steps were carried out in conjunction with the laboratory of Dr. Judith Storch at Rutgers University.

Vesicle preparation

Small unilamellar vesicles (SUVs) were prepared from 90 mol % EPC and 10 mol % of the energy transfer quencher NBD-PC for the standard; additionally, 25 mol % PS

or CL, 65 mol % EPC, and 10 mol % NBD-PC to increase the negative charge of the acceptor vesicles. The lipids from chloroform stocks were mixed in a round bottom flask and dried by nitrogen evaporation followed by overnight storage under vacuum to remove residual chloroform. The lipids were hydrated in Tris-buffered saline (TBS) (40 mM Tris and 100mM NaCl, pH 8.1); except for SUVs containing CL, 1mM EDTA was added to the buffer and then the mixture was vortexed. These lipid suspensions were then incubated for 20 min, shaken slightly at 37°C, and then subjected to sonication for 45 min on ice and using N₂ gas, followed by ultracentrifugation for 45 min at 50,000 rpm (*Huang, et al., 1974*). The phospholipid concentration of the vesicles was determined by quantification of inorganic phosphate (*Gomori, 1942*).

Transfer of 12AO from LFABPs to SUVs

The transfer of 12AO (12-(9-anthroyloxy)oleic acid) from wild type and mutant LFABPs (K31L & R122L/S124A) to acceptor membranes was monitored using a fluorescence resonance energy transfer (FRET) assay (*Storch, et al., 1990*). 10 μ M LFABP was incubated with 1 μ M 12AO for 10 min at room temperature. Equal volumes of the LFABP-12AO complex were mixed (mixing time = 0.01 sec) with 300-1200 μ M acceptor vesicles (SUVs) using a temperature-controlled (25°C) DX-17MV stopped-flow spectrofluorometer (Applied Photophysics Limited, Staines, UK) at Rutgers University. Excitation was set at 383 nm, and emission was monitored at 450 nm. Since the SUVs contained NBD-PC quencher, the transfer of 12AO from LFABP to acceptor membranes was monitored by the time-dependent decrease of 12AO fluorescence intensity over 200 sec. Data were collected in photocounting mode and were analyzed using software provided with the instrument (*Storch, et al., 1990*). All experimental transfer curves were

fit by a single exponential function to obtain the transfer rate constant. For each experimental condition, at least six replicates were made. Controls to ensure elimination of photobleaching were performed prior to each experiment (*Hsu and Storch, 1996*). The standard assay buffer was TBS (40 mM Tris and 100mM NaCl, pH 8.1) except for SUVs containing CL for which 1mM EDTA was added to the buffer.

ADIFAB binding assay

ADIFAB (Acrylodan labeled Intestinal Fatty Acid Binding Protein) is a fluorescent molecule that provides the basis for an assay of the aqueous concentration of free (unbound) fatty acids. For ADIFAB, excitation was set at 390 nm and emission was monitored at both 505 nm and 432 nm. In the absence of fatty acids (FA), the acrylodan is located in the binding pocket and exhibits a fluorescence emission maximum in the blue at 432 nm. In contrast, in the presence of FA, the acrylodan is displaced from the binding pocket, the emission shifts to the green with a peak at 505 nm. Thus the ratio of fluorescence (R) at 505 nm and 432 nm is indicative of the amount of free fatty acid present. ADIFAB can be used to determine FA binding constants to any unlabeled protein, in this case LFABP, by monitoring free fatty acid levels as a solution of ADIFAB and native protein is titrated with fatty acid (*Richieri et al., 1994*). Free fatty acid concentration can be determined by:

$$[\text{FFA}] = K_d Q (R - R_0) / (R_{\text{max}} - R) \quad (\text{Eq.1})$$

Where Q and R_{max} are predetermined values for ADIFAB. Values of Q and R_{max} for the SLM 8000C fluorometer at Rutgers University were found to be 19.5 and 11.5, respectively (*Richieri et al., 1992*). R_0 is the fluorescence ratio (505/432) of a solution containing ADIFAB (0.2 μ M), LFABP (2 μ M) and measuring buffer (20 mM HEPES, 140

nM NaCl, 5 mM KCl, 1 mM Na₂HPO₄, at pH 7.4). Then the solution is titrated with small aliquots of known FA concentration (0.25μM - 5.0μM) and the R value (I₅₀₅/I₄₃₂) is measured after each addition. The background intensity (buffer alone) is subtracted from each measurement. After each titration 5 minutes are allowed for equilibration before measuring R. For each R measured, the amount of fatty acid bound to the protein is calculated using Eq.(2):

$$[FA]_{\text{bound}} = [FA]_{\text{total}} - [FFA] - [ADIFAB]_{\text{bound}} \quad (\text{Eq.2}),$$

where $[FA]_{\text{total}}$ is the total fatty acid concentration in the solution after each addition, $[FFA]$ is the free fatty acid concentration and $[ADIFAB]_{\text{bound}}$ is the concentration of fatty acid bound to ADIFAB, where it can be calculated as:

$$[ADIFAB]_{\text{bound}} = [ADIFAB]_{\text{total}} Q(R - R_0)/(R_{\text{max}} - R + Q(R - R_0)) \quad (\text{Eq.3})$$

In the case of single binding site, the titration data can be analyzed by the Scatchard method as:

$$([FA]_{\text{bound}}/[Protein]_{\text{total}})/[FFA] = (-1/K_d * [FA]_{\text{bound}})/([Protein]_{\text{total}} + n/K_d) \quad (\text{Eq.4}),$$

where $[Protein]_{\text{total}}$ is the concentration of binding protein added, n is the number of fatty acid binding sites, and K_d is the binding constant of the fatty acid to the protein. Plotting the data as $([FA]_{\text{bound}}/[Protein]_{\text{total}})/[FFA]$ vs. $[FA]_{\text{bound}}/[Protein]_{\text{total}}$ yields a straight line with a slope equal to $-1/K_d$ and a x-axis intercept equal to n . For multiple binding sites of different affinities, as in the case for LFABP, the Scatchard plot is nonlinear. Thus $([FA]_{\text{bound}}/[Protein]_{\text{total}})/[FFA]$ was fitted to Eq.5:

$$([FA]_{\text{bound}}/[Protein]_{\text{total}})/[FFA] = n_1/(K_d^{(1)} + [FFA]) + n_2/(K_d^{(2)} + [FFA]) \quad (\text{Eq.5})$$

Relative partition coefficients

The partition coefficients (K_p) for 12AO (12-(9-anthroyloxy)oleic acid) partitioning between LFABPs (wild type and mutants) and EPC vesicles containing NBD-PC were determined by measuring the decrease in 12AO fluorescence upon titration of 12AO-containing LFABPs with EPC vesicles. A 2 mL solution containing 5 μ M protein and 0.5 μ M 12AO was prepared in TBS buffer (40 mM Tris, 100 mM NaCl, pH 8.1), and allowed to equilibrate at room temperature. Successive volumes of NBD-PC were then added to provide a total concentration of 100-1000 μ M acceptor vesicles (EPC), or a LFABP: EPC ratio of 1: 20 – 1: 200. Excitation was set at 383 nm, and emission was monitored at 430-460 nm. The titration data were analyzed by the Scatchard method as:

$$1/\Delta F = (K_p) (1/\Delta F_{\max}) ([EPC]/[LFABP]) + 1/\Delta F_{\max} \quad (\text{Eq.6}),$$

where ΔF is the difference between the initial fluorescence of 12AO in LFABPs and the 12AO fluorescence at a given LFABP: EPC ratio, measured as the area under the curve from 430 to 450 nm, and $\Delta F_{\max}$ is the maximum difference in 12AO fluorescence. A plot of $1/\Delta F$ versus $(1/\Delta F_{\max}) ([EPC]/[LFABP])$ has a slope of K_p (*Massey et al., 1997; Storch, 1990*). The inner filter effect was corrected at both the excitation and emission wavelengths of 383 and 450 nm, respectively, using the equation:

$$F_{\text{icor}} = (F_i - B)10^{0.5b(A_{\lambda_{\text{ex}}} + A_{\lambda_{\text{em}}})} \quad (\text{Eq.7}),$$

where F_{icor} is the corrected value of the fluorescence intensity, F_i is the experimentally measured fluorescence intensity, B is the background fluorescence intensity, b is the path length in centimeters, and $A_{\lambda_{\text{ex}}}$ and $A_{\lambda_{\text{em}}}$ are the absorbances of the sample at the excitation and emission wavelengths, respectively (*Lehto and Sharom, 2002*).

Circular Dichroism Spectroscopy

The circular dichroism (CD) spectra of wild type and mutant LFABPs at a concentration of $\sim 25 \mu\text{M}$ in phosphate buffer (10 mM sodium phosphate, 5 μM EDTA, 0.02% sodium azide) were recorded on an AVIV model 62-DS CD instrument (AVIV Associates, Lakewood, NJ), which is interfaced with a computer used for all mathematical calculations. A Quartz cuvette with path length of 1 mm equipped with a thermostated cell holder was used for all spectral studies. All spectra were acquired as the average of 4 scans between 260 and 190 nm, obtained at intervals of 1 nm with 1-second integration times at each wavelength. The bandwidth for each measurement was set to 2 nm. CD spectra are often reported in terms of molar ellipticity, $[\theta]$, ($\text{deg}\cdot\text{cm}^2/\text{dmol}$). For macromolecules such as proteins the mean residue molar ellipticity $[\theta]$ is:

$$[\theta] = \theta_{\text{obs}} / (10 \times C_r \times l) \quad (\text{Eq.8}),$$

where θ_{obs} is the ellipticity observed in millidegrees, l is the cell path length in cm, and C_r is the mean residue molar concentration. $C_r = (n \times 1000 \times c_g) / M_r$, where n is the number of amino acid residues, c_g is the protein sample concentration in g/ml, and M_r is the molecular weight of the protein (g/mol) (*Yang et al., 1986*). Prior to calculation of the final ellipticity, all spectra were corrected by subtracting the background (sample buffer) spectrum.

NMR Spectroscopy

All NMR experiments were conducted at 10°C , on either a four-channel Varian ^{UNITY}INOVA 600 MHz spectrometer at the College of Staten Island (Varian NMR Inc., Palo Alto, CA) or on a four-channel Bruker *AVANCE*TM 800 MHz spectrometer at the New York Structural Biology Center (Bruker BioSpin Corp., Billerica, MA), each

equipped with a 5-mm $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ triple-resonance probe, z-axis pulsed field gradients, and a cryoprobe for the Bruker spectrometer. The ^1H carrier frequency was placed on resonance with the H_2O signal (4.91 ppm); gradients were used for solvent suppression and all multi-dimensional experiments used the enhanced-sensitivity pulsed-field gradient method (*Kay et al., 1994; Palmer et al., 1991*). All NMR spectra were processed using the suite of programs provided with NMRPipe/NMRDraw software (*Delaglio et al., 1995*). The frequency domain spectra acquired at 600 MHz were recalibrated in ^1H , ^{15}N , and ^{13}C dimensions for consistency with the 800 MHz data to account for small inconsistencies in chemical shifts (in ppm). The transformed data sets were reduced to include only the regions of interest and analyzed using the NMRView program (*Johnson and Blevins, 1994*).

Sample preparation

Protein samples obtained from the lipidex purification step (described above) were subjected to Centricon-10 ultrafiltration (Millipore) in order to reduce the volume and achieve the desired concentration. The concentration of $^{13}\text{C}/^{15}\text{N}$ -LFABP mutants ranged from 500 to 800 μM in KCl-phosphate buffer (0.15 M KCl, 0.05 M phosphate, and 0.02 % NaN_3 , pH 7.4); samples were prepared in 95% H_2O /5% D_2O for ^{15}N -edited experiments (^{15}N -HSQC, NOESY-HSQC, TOCSY-HSQC, H(CCO)NH-TOCSY, C(CO)NH-TOCSY, CN4DNOESY) and 99% D_2O (the protein was lyophilized and then dissolved in 99% D_2O buffer) for ^{13}C -edited experiments (HCCH-TOCSY, C3DNOESY, C4DNOESY, ^{13}C -HSQC). Holo-R122L/S124A LFABP samples were made from $^{15}\text{N}/^{13}\text{C}$ -labeled protein with unlabeled oleic acid or ^{15}N -labeled protein with ^{13}C -labeled oleic acid (Isotec, Miamisburg, OH) for the study of intermolecular protein-ligand

interactions. A stoichiometry of 1:3 (protein:ligand) was used for all holo-LFABP samples (mutant and wild type).

Molecular images were generated using the visualization program MOLMOL. MOLMOL is a molecular graphics program for display, analysis, and manipulation of three-dimensional structures of biological macromolecules (*Koradi et al., 1996*).

NMR Experiments:

1. Two-dimensional ^1H - ^{15}N -heteronuclear single-quantum correlation (2D ^1H - ^{15}N -HSQC)

The ^1H - ^{15}N HSQC (Heteronuclear Single Quantum Coherence) experiment is a two-dimensional experiment that employs heteronuclear magnetization transfer using an INEPT (Insensitive Nuclei Enhanced by Polarization Transfer) scheme (Figure 2.1). It provides chemical shift and correlation information on the heteronucleus ^{15}N and a proton that is directly scalar coupled through one bond. Each residue (except proline) in the protein is represented by a cross-peak at (ω_1, ω_2) , where ω_1 and ω_2 are the amide ^{15}N and ^1H shifts, respectively (*Reid, 1997*). This experiment establishes correlations linking each ^{15}N nucleus with its one-bond coupled proton spin. The chemical shifts of ^1H and ^{15}N of each amide of the protein backbone and in the side-chain are presented as a contour map, serving as a fingerprint for the protein structure. Because there is only one amide proton per amino acid, each HSQC signal represents one single amino acid, except that each side-chain NH_2 group will have two crosspeaks in the HSQC spectrum as well.

In order to determine the mutant protein sites with the greatest structural perturbations compared to wild-type LFABP, the chemical shift difference in Hz was calculated for the combination of amide proton (HN) and ^{15}N resonances ($\Delta\delta = \sqrt{[(\Delta\text{HN})^2 + (\Delta\text{N})^2]}$). Then a plot of $\Delta\delta$ vs. residue number was generated. Residues with perturbations greater than one standard deviation beyond the mean $\Delta\delta$ indicate that the mutation has perturbed these residues significantly. The perturbation location on the three-dimensional structure was displayed using the MOLMOL program (Koradi *et al.*, 1996).

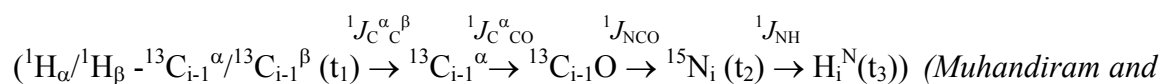
Protein-ligand interactions

Titration studies were conducted to evaluate whether the R122L/S124A and K31L mutations caused any changes in the binding stoichiometry or ligand location. An aliquot of a 100 mM stock solution of ^{13}C -labeled sodium oleate in NaOH was added to ~ 500 μM of ^{15}N -labeled protein at a total volume of 500 μl to achieve ratios of 1:0.4, 1:0.8, 1:1.2, 1:1.6, and 1:2. At each titration step a ^{13}C -HSQC (correlations each ^{13}C nucleus with its one-bond coupled proton spin) was acquired for the oleate. Then each spectrum was compared with its corresponding oleate spectrum in the wild type LFABP-oleate complexes.

2. Multidimensional NMR experiments:

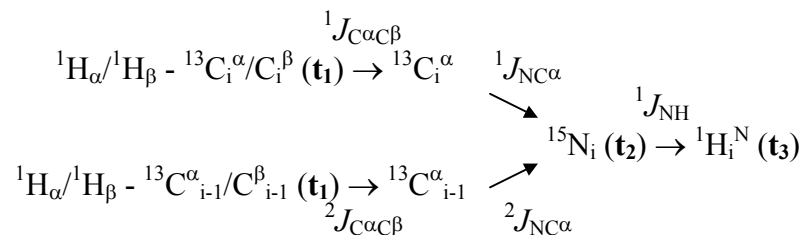
Backbone resonance assignments

Since the R122L/S124A-LFABP mutant, unlike the K31L-LFABP mutant, showed pervasive differences in the chemical shifts compared to wild type, which is an indication that the structure has been altered, resonance assignments cannot be done based on visual comparison with wild type LFABP HSQC spectrum. Therefore, CBCA(CO)NH (Grzesiek and Bax, 1992) and HNCACB (Kay *et al.*, 1994) experiments were performed to assign sequential backbone $^{13}\text{C}^\alpha$ and $^{13}\text{C}^\beta$ resonances. The CBCA(CO)NH experiment correlates the amide proton (HN) and the ^{15}N resonances of one residue with the $^{13}\text{C}^\alpha$ and $^{13}\text{C}^\beta$ resonances of the preceding residue. It relies on magnetization transfer via the $^1J_{\text{C}\alpha\text{C}\beta}$ coupling, followed by magnetization relay from $^{13}\text{C}^\alpha$ via the carbonyl to the ^{15}N of the following residue (Figure 2.2a)



Kay, 1994). In contrast, the HNCACB experiment correlates the HN and ^{15}N resonances

with those of the intraresidue $^{13}\text{C}^\alpha$ and $^{13}\text{C}^\beta$ nuclei using magnetization transfer via the $^1J_{\text{NC}\alpha}$ coupling. The magnetization transfer pathway for HNCACB is illustrated as follows and in Figure 2.2b:



Thus, since each amide nitrogen is coupled to its own $^{13}\text{C}^\alpha$ ($^1J_{\text{NC}\alpha} \sim 12$ Hz) more strongly than to the $^{13}\text{C}^\alpha$ preceding it ($^2J_{\text{NC}\alpha} \sim 7$ Hz) (Muhandiram and Kay, 1994; Wittekind and Mueller, 1993), the strip for each HN-N contains intense peaks for its own $^{13}\text{C}^\alpha$ and $^{13}\text{C}^\beta$ resonances and weaker peaks for the $^{13}\text{C}^\alpha$ and $^{13}\text{C}^\beta$ resonances of the previous residue. The way that magnetization is transferred between $^{13}\text{C}^\alpha$ and $^{13}\text{C}^\beta$ causes the correlations for these carbons to have opposite signs; this is codified by the use of black and green peaks. Aligning the weak peaks in one strip with the strong peaks from another confirms that the corresponding HN-N pairs are adjacent in the polypeptide sequence.

First, we start the assignment by looking for a unique pair of $^{13}\text{C}^\alpha$ and $^{13}\text{C}^\beta$ chemical shifts that belong to a distinctive amino acid, for example Ser ($^{13}\text{C}^\alpha \sim 56$ ppm, $^{13}\text{C}^\beta \sim 65$ ppm), Thr ($^{13}\text{C}^\alpha \sim 61$ ppm, $^{13}\text{C}^\beta \sim 70$ ppm), Gly ($^{13}\text{C}^\alpha \sim 45$ ppm), and Ala ($^{13}\text{C}^\alpha \sim 50$ ppm, $^{13}\text{C}^\beta \sim 20$ ppm). Then we assign sequence pairs that have these unique amino acids such as Ser⁴Gly⁵, Gly⁵⁵Ser⁵⁶, Thr⁷⁵Gly⁷⁶, Ala⁸¹Val⁸², and Thr⁹³Thr⁹⁴. The connectivities to other residues from these segments are extended by correlating C_i^α , C_i^β , C_{i-1}^α , and C_{i-1}^β frequencies from CBCA(CO)NH and HNCACB experiments. In some

cases a blockage is encountered due to an overlap of chemical shifts, a very weak HN signal due to intermediate chemical exchange rates, or proline residue (no NH resonance). Under these circumstances, it is necessary to choose another distinctive sequence pair and proceed again. Finally, by combining the results of CBCA(CO)NH with HNCACB, sequential assignments are obtained and now HN and ^{15}N resonances in the ^1H - ^{15}N HSQC spectrum can be identified.

Side-chain resonance assignments

Aliphatic side chain assignments (^1H and ^{13}C) were derived from C(CO)NH-TOCSY (Montelione *et al.*, 1992), H(CCO)NH-TOCSY (Logan *et al.*, 1993), and HCCH-TOCSY (Kay *et al.*, 1993) experiments. These experiments achieve total correlation of the side-chain by isotopic mixing of ^{13}C spins through $^1J_{\text{cc}}$ coupling, therefore are more efficient than ^{15}N -TOCSY-HSQC. C(CO)NH-TOCSY correlates all aliphatic carbons in the spin system of residue (i-1) with the HN and ^{15}N on residue (i) (Figure 2.2c). The amide proton (HN) is in the first dimension, all the carbon chemical shifts correlated through TOCSY to the amide HN are displayed in the second dimension, and the amide ^{15}N is in the third dimension. H(CCO)NH-TOCSY correlates all aliphatic protons on residue (i-1) with HN and ^{15}N on residue (i) (Figure 2.2d). HCCH-TOCSY is a three-dimensional ^{13}C - ^{13}C correlated experiment used to obtain complete side-chain ($^1\text{H}_i^\alpha$ - $^1\text{H}_i^\beta$ - $^1\text{H}_i^\gamma$...) resonance assignments in each residue in the proteins (Bax *et al.*, 1990) (Figure 2.2e). The transfer of magnetization along the carbon side-chain follows the path of:

$^1\text{H}_i(t_1) \xrightarrow{J_{\text{CH}}} ^{13}\text{C}_i(t_2) \xrightarrow{J_{\text{CC}}} ^{13}\text{C}_j \xrightarrow{J_{\text{CH}}} ^1\text{H}_j(t_3)$ (Cavanagh *et al.*, 1996), where $^1\text{H}_{i(j)}$ and $^{13}\text{C}_{i(j)}$ are one-bond coupled and $^{13}\text{C}_i$ and $^{13}\text{C}_j$ are part of the same side-chain spin network. Thus the experiment correlates $^1\text{H}_i$ and $^{13}\text{C}_i$ with $^1\text{H}_j$ and $^{13}\text{C}_j$ of the same side-chain spin

network. New assignments can be made for the residues with very weak NH resonances using this experiment, and side chain proton assignments derived initially from a ^{15}N -edited TOCSY-HSQC and NOESY-HSQC spectra can be confirmed.

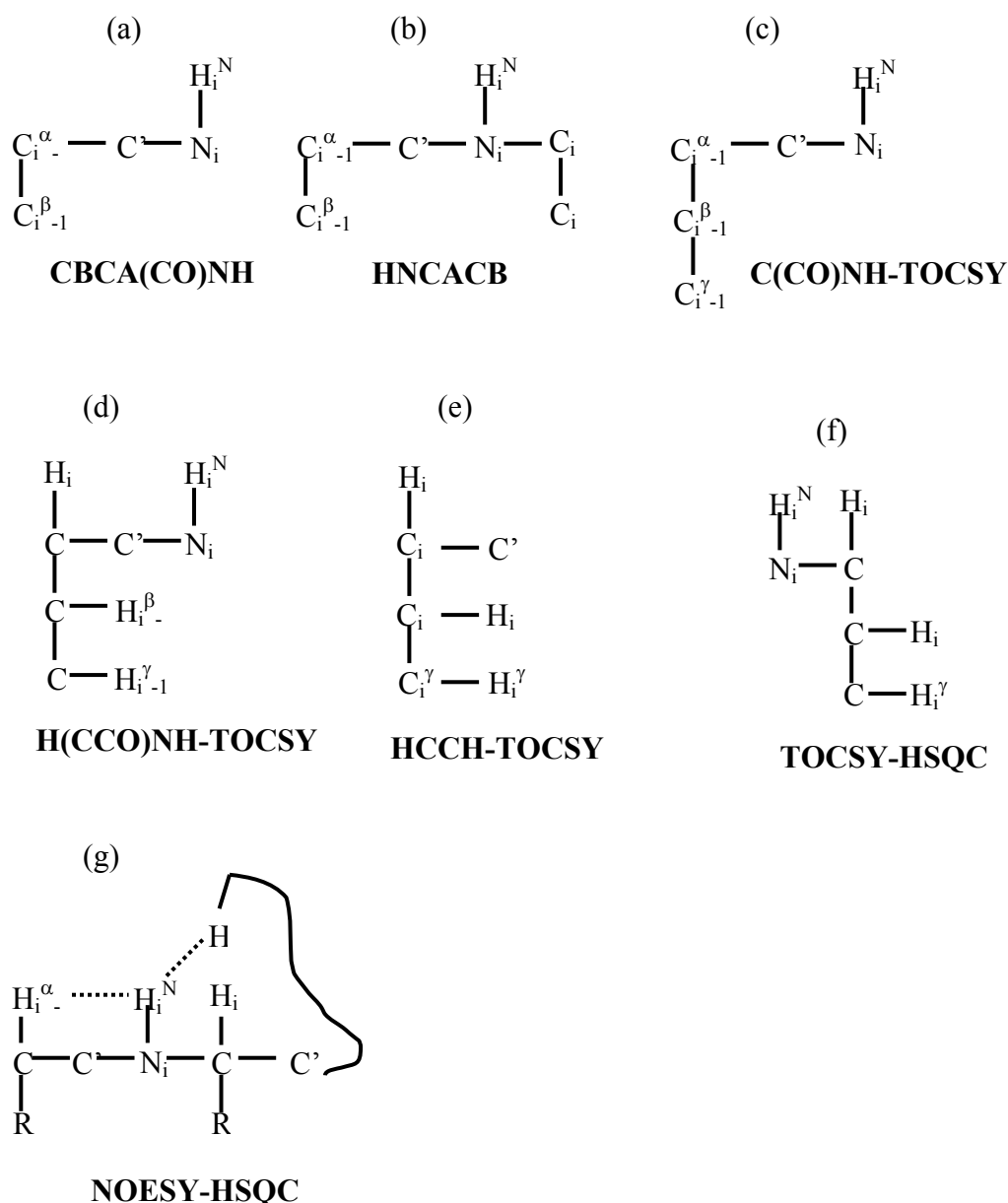


Figure 2.2. **Schematic representation of seven 3D experiments used for protein resonance assignments.** The nuclear spins of the i th amino acid residue are designated H_i^N for the amide proton spin, N_i for the amide ^{15}N spin, H_i^κ and C_i^κ for aliphatic proton and ^{13}C spins ($\kappa = \alpha, \beta, \gamma$, etc.), and C_i' for the carbonyl ^{13}C spin (*He, 2000; PhD thesis*).

Chemical Shift Indices (CSI)

Once all assignments have been made, the chemical shift indices for H^α , C^α , and C^β were calculated using the Chemical Shift Index (CSI) program (*Wishart et al., 1992*), to determine the secondary structure of a protein. The chemical shift ranges of H^α , C^α , and C^β have been established for random coil structures. If the observed shift deviates beyond the range, an index of 1 (beta sheet) is assigned. But if the shift deviates negatively beyond the range, an index of -1 (helix) is assigned. A shift within the range is assigned a value "0" (coil) (*Wishart et al., 1992*). Then a consensus secondary structure for each region of the protein is calculated from the three types of shifts. In general, a consensus estimate of secondary structure has a predictive accuracy in excess of 92%.

3D/4D-NOESY assignments

Since none of the backbone or side-chain resonance assignments can give any information about the specific orientation of the amino acid residues with respect to each other, three- and four-dimensional NMR experiments such as NOESY3DC (NOE interaction between C-H and any other proximal proton), NOESY3DN (NOE interaction between N-H and any other proximal proton), NOESY4DCC (NOE interaction between C-H and C-H), and NOESY4DCN (NOE interaction between C-H and N-H) (*Reid, 1997; Wang et al., 1998*), were performed to obtain constraints for the three-dimensional structure in aqueous solution.

In general, the NOESY spectrum provides very important data about distance restraints, derived from nuclear Overhauser effects (NOEs) between hydrogen atoms separated by less than 5 Å, for 3D structure calculation. Molecules that undergo Brownian motion in a liquid experience dipole-dipole interactions, resulting in the

transfer of magnetization between spins which gives rise to NOEs. NOEs are quantified by determining the volume of the corresponding cross-peak in the NOESY spectrum, where the volume is correlated with distance (r) according to r^{-6} .

3D and 4D heteronuclear-edited NOESY spectra contain similar information to 2D homonuclear NOESY spectra. However, by separating each ^1H - ^1H NOE cross-peak into a third dimension (heteronucleus attached to one proton) and fourth dimension (heteronucleus directly attached to the other proton), ambiguities due to limited resolution and ^1H resonance degeneracies are minimized (*Cavanagh et al., 1996*).

Intermolecular NOE assignment

Intermolecular NOEs are the most useful NMR parameter for the analysis of protein-ligand complexes. They can give us the location of the ligand, its orientation, and the kind of interaction present between the protein and its ligand (oleate). Protein-ligand interactions can be studied either by transferred NOE experiments (if the exchange rate is fast: small ligand, weak binding) (*Clare, G.M. and Gronenborn, A.M., 1982; Campbell and Sykes, 1991*) or by $^{13}\text{C}/^{15}\text{N}$ -filtered NOE experiments (if the exchange rate is slow: high affinity binding) (*Zwahlen et al., 1997; Ikura and Bax, 1992*). In our case, slow-exchange conditions exist (*He et al., to be submitted*) and therefore isotope-filtered techniques were used. This technique is especially useful in eliminating ambiguities between intra- and intermolecular NOEs by distinguishing ligand signals from protein signals. At the beginning of NOE mixing, only protons without labels remain due to the use of an isotope filter. After the NOE spin transfer, ^{13}C -editing is used to detect protons with ^{13}C -labels.

Two 3D NOESY experiments from ^{13}C -purged protons to ^{13}C -edited protons were acquired concomitantly to provide information about the intermolecular NOEs (interactions) between the R122L/S124A-LFABP mutant and oleate. In these experiments all ^1H s with ^{13}C -labels are rejected before evolution in t_1 . After NOE mixing, the destination protons are edited with ^{13}C -HSQC to detect their ^{13}C and ^1H resonances in t_2 and t_3 , respectively (Zwahlen *et al.*, 1997). One experiment uses the $^{15}\text{N}/^{13}\text{C}$ -labeled protein with unlabeled oleate to detect NOEs between the unlabeled oleate protons and the protein protons that are ^{13}C -labeled. The other experiment uses ^{15}N -labeled protein (R122L/S124A-LFABP), which does not have ^{13}C -labels, with ^{13}C -labeled oleic acid (Isotec, Miamisburg, OH) to detect NOEs from protein protons to oleate protons.

Structure Calculations

The ARIA (Ambiguous Restraints for Iterative Assignment) software package version 2.0 was used for automated structure determination from distance restraints derived from NOESY spectra. Distance restraints for structure calculations were derived from cross-peak volumes ($D = (\alpha^{-1}V)^{-1/6}$) of 3D/4D ^{15}N -edited NOESY-HSQC and 3D/4D ^{13}C -edited NOESY-HSQC data sets. All NOE mixing times were 150 ms. The program uses CNS (Crystallography and NMR System) software employing a simulated annealing strategy to calculate structures. The input for ARIA/CNS included the chemical shift list, partially assigned peak lists from 3D/4D ^{15}N -edited NOESY, and 3D/4D ^{13}C -edited NOESY, hydrogen bond restraints from 3D ^{15}N -edited NOESY, dihedral angle restraints derived from the program TALOS (Cornilescu *et al.* 1999) that predicts phi and psi angles by comparing secondary shifts and amino acid sequence of triplet of residues in the target protein relative to that of proteins of known structure in a database, and

$^3J_{\text{H}^{\text{N}}\text{H}^{\alpha}}$ coupling constants derived from HNHA experiment (Cavanagh *et al.*, 1996) and converted into backbone torsion angle (ϕ) restraints (Karplus, 1959). The frequency window tolerance for assigning NOEs was ± 0.04 ppm for the protons and ± 0.5 ppm for nitrogen and carbon shifts.

HNHA experiment

The magnitude of the homonuclear three-bond J-coupling constant ($^3J_{\text{H}^{\text{N}}\text{H}^{\alpha}}$) derived from the HNHA experiment relies on a quantitative analysis of the diagonal to cross-peak intensity ratio (Cavanagh *et al.*, 1996) using the relationship

$$\frac{S_{\text{cross}}}{S_{\text{diagonal}}} = \frac{-\sin^2(2\pi ^3J_{\text{H}^{\text{N}}\text{H}^{\alpha}} \delta_2)}{\cos^2(2\pi ^3J_{\text{H}^{\text{N}}\text{H}^{\alpha}} \delta_2)} = -\tan^2(2\pi ^3J_{\text{H}^{\text{N}}\text{H}^{\alpha}} \delta_2) \quad (\text{Eq.9})$$

Where δ_2 is a time delay and $^3J_{\text{H}^{\text{N}}\text{H}^{\alpha}}$ is the coupling constant. In turn, the $^3J_{\text{H}^{\text{N}}\text{H}^{\alpha}}$ coupling constant provides valuable information about the backbone dihedral angle ϕ in peptides and proteins (Kay and Bax, 1990). The magnitudes of the $^3J_{\text{H}^{\text{N}}\text{H}^{\alpha}}$ coupling constants are correlated with the torsion angle ϕ by the Karplus equation (Karplus, 1959):

$$J(\phi) = A \cos^2(\phi) - B \cos(\phi) + C \quad (\text{Eq.10}),$$

where A, B, and C are empirically derived parameters found from structures with known values of ϕ .

First, NOE peaks were picked and assigned with the program NMRVIEW (Johnson and Blevins, 1994). Eight iterative cycles of ARIA assignment and CNS structure calculation were performed. Structures were refined using the default simulated annealing protocol (anneal) in the program ARIA 2.0 (except that $\text{vtol} = 0.2$ was used

instead of 0.1 for the last three iterations). In the last iteration, 20 calculated structures with the lowest energies were used to analyze restraint violations and assign additional NOE cross-peaks. After several rounds of calculation, it was possible to delete consistently violated restraints derived from NOESY cross-peaks that were judged likely to be wrongly assigned or overlapped. The 20 structures with lowest NOE energies were retained and validated by the program PROCHECK (*Laskowski et al., 1993; Morris et al., 1992*). Structures were visualized using the program MOLMOL (*Koradi et al. 1996*).

CHAPTER III

RESULTS AND DISCUSSION

Electrostatic interactions of LFABP complexed with oleate (MCCE modeling)

In order to understand the function of this intracellular lipid binding protein, it is important to develop a fundamental understanding of its molecular structure. One approach uses the Multi-Conformational Continuum Electrostatics (MCCE) method, developed in Dr. Marilyn Gunner's Laboratory at CCNY. The MCCE method allows multiple positions of hydroxyl and water protons, alternative side-chain rotamers, water positions and cofactor positions for calculation of the pH dependence of ionization equilibria in proteins (*Alexov and Gunner, 1997; Alexov and Gunner, 1999*), unlike other standard electrostatic calculations that consider only a single structure for all heavy atoms and polar protons.

The MCCE method was used to calculate the ionization states of all residues of the LFABP protein, given coordinates from the x-ray structure of holo-LFABP complexed with two oleates (*Thompson et al., 1999*). Electrostatic and non-electrostatic energies for self- and pair-wise interactions were calculated for doubly ligated LFABP. Pairwise interactions with OLA128 (Table 3.1) and OLA129 (Table 3.2) were chosen as representative results. Tables 3.1 and 3.2 represent the pH dependence of ionization equilibria between each residue in the protein and each oleate. Energies are given in ΔpK units, the energy required to change a residue's pK_a by 1 pH unit ($1\Delta pK = 2.303 k_bT = 1.36 \text{ kcal/mol}$). As detailed below, this calculation gives us detailed information about the nature of the interaction between the ligands and the residues in the binding cavity. MCCE calculations, in conjunction with functional assays and x-ray structures, can guide

us in the construction of site-specific mutant LFABPs that test structure-function relationships.

It is postulated that the driving force for the first oleate (OLA129) to bind is mainly electrostatic interaction between its carboxylate and the positively charged residues (Arg122, Lys125, Arg126) at the inner surface of the protein cavity. The second oleate (OLA128) is more exposed to the surface, and its carboxylate interacts most strongly with Lys31. The main interaction is basically hydrophobic. The x-ray data (*Thompson, 1997*) show that the tail of OLA128 interacts with OLA129 and some hydrophobic residues in the LFABP cavity. Table 3.2 shows that Arg122 with the large favorable contribution of its positive charge ($-11.9 \Delta pK$) has compensated for the large desolvation energy of OLA129 ($10.3 \Delta pK$, not shown) that disfavors ionization. In addition, Ser124 contributes (-0.9 to $-1.7 \Delta pK$) more towards ionization of OLA129 than Lys125 ($-0.1 \Delta pK$) and Arg126 ($-0.6 \Delta pK$). Thus, Arg122 and Ser124 were viewed as the most important residues involved in the interaction with OLA129, suggesting that mutating these residues should have a great impact on the binding properties of the protein. As for the pairwise interactions of OLA128, Lys31 showed the largest contribution to compensate for the unfavorable desolvation energy of OLA 128 ($3.1 \Delta pK$, not shown). As a result, we chose Lys31 to be mutated to Leu in order to test the importance of this residue to electrostatic protein-ligand interactions.

Table 3.1 Pairwise interactions to OLA128 (“oleate2”) (ionized form)

pH	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0	11.0	12.0
LY+ 31 (ΔpK)	-2.9	-2.9	-2.9	-2.9	-2.9	-2.9	-2.9	-2.9	-2.9	-2.9	-2.9	-2.9
TY- 54 (ΔpK)	-0.2	-0.2	-0.2	-0.2	-0.2	-0.2	-0.2	-0.2	-0.2	-0.2	-0.2	-0.2
SER 56 (ΔpK)	0.4	0.4	0.1	-0.4	-0.9	-1.0	-1.0	-1.0	-1.0	-1.0	-1.0	-1.0
LY+ 57 (ΔpK)	-0.6	-0.6	-0.6	-0.6	-0.6	-0.6	-0.6	-0.6	-0.6	-0.5	-0.4	-0.2
AR+ 122 (ΔpK)	-0.4	-0.4	-0.4	-0.4	-0.4	-0.4	-0.4	-0.4	-0.4	-0.4	-0.4	-0.4

* Only residues that interact with the carboxylate of OLA2 (X-ray structure) and those that correspond to the highest ionization energy are shown. 1 ΔpK = 1.36 kcal/mol

Table 3.2 Pairwise interactions to OLA129 (“oleate1”) (ionized form)

pH	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0	11.0	12.0
SER 39 (ΔpK)	0.4	0.2	0.3	0.4	0.5	-0.3	-1.6	-2.5	-2.6	-2.6	-2.6	-2.6
THR 102 (ΔpK)	-0.6	-0.6	0.6	-0.6	-0.6	-0.6	-0.7	-0.7	-0.7	-0.6	-0.6	-0.6
AR+ 122 (ΔpK)	-11.9	-11.9	-11.9	-11.9	-11.9	-11.9	-11.9	-11.9	-11.9	-11.9	-11.9	-11.9
SER 124 (ΔpK)	-0.9	-0.9	-1.0	-1.0	-1.1	-1.3	-1.5	-1.7	-1.8	-1.7	-1.7	-1.7
LY+ 125 (ΔpK)	-0.1	-0.1	-0.1	-0.1	-0.1	-0.1	-0.1	-0.1	-0.1	-0.1	-0.1	0.0
AR+ 126 (ΔpK)	-0.6	-0.6	-0.6	-0.6	-0.6	-0.6	-0.6	-0.6	-0.6	-0.6	-0.6	-0.6

* Only residues that interact with the carboxylate of OLA129 (X-ray structure) and those that correspond to the highest ionization energy are shown. 1 ΔpK = 1.36 kcal/mol

Construction, expression and purification of LFABP wild-type and mutants

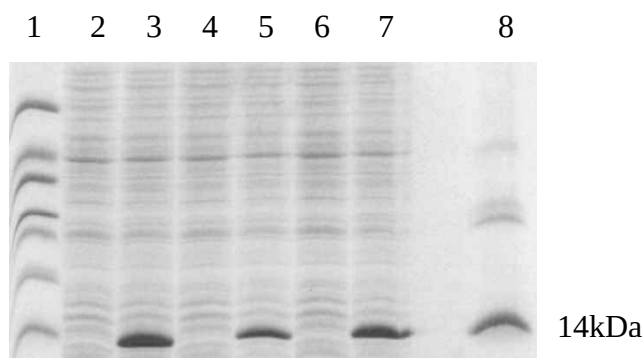
Both structural data (X-ray and NMR) (*Thompson et al., 1999; He et al., to be submitted*) and MCCE calculations implicate residues at positions 31, 122, and 124 as important contributors to ligand interactions. Therefore, mutating these positively charged and polar residues to hydrophobic residues of similar size should completely abolish any interaction of these residues with the ligands and establish the importance of electrostatic interactions in the binding process. LFABP mutants (K31L & R122L/S124A) were successfully generated and identified via sequence analysis of the LFABP mutant plasmid DNA (Table 3.3).

After the proteins were expressed in BL21(DE3) cells and before purification, protein overexpression was assessed by SDS-PAGE (Figure 3.1) in order to verify the presence of the engineered proteins. SDS-PAGE showed that both mutants (K31L & R122L/S124A in lanes 3 and 5, respectively express the same level as the wild-type (lane 7) after induction with 1 mM IPTG for three hours. Since expression of the mutants was successful, our working hypothesis was that the introduction of these mutations has no adverse effect on protein folding.

Table 3.3 Partial sequences of the wild-type and mutant L-FABPs

Wild-type	27 28 29 30 31 32 33 34 35 36 5' GAC CTC ATC CAG AAA GGG AAG GAC ATC AAG 3' <i>D L I Q K G K D I K</i>	a.a residue # cDNA sequence amino acid sequence
Single Mutant (K31L)	27 28 29 30 31 32 33 34 35 36 5' GAC CTC ATC CAG TTA GGG AAG GAC ATC AAG 3' <i>D L I Q L G K D I K</i>	a.a residue # cDNA sequence amino acid sequence
Wild-type	117 118 119 120 121 122 123 124 125 126 5' GAC ATC GTC TAC AAG AGA GTC AGC AAG AGA 3' <i>D I V Y K R V S K R</i>	a.a residue # cDNA sequence amino acid sequence
Double Mutant (R122L/S124A)	117 118 119 120 121 122 123 124 125 126 5' GAC ATC GTC TAC AAG TTA GTC GCC AAG AGA 3' <i>D I V Y K L V A K R</i>	a.a residue # cDNA sequence amino acid sequence

* Mutations in the DNA and amino acid sequences are shown in bold.

**Figure 3.1 LFABP (¹⁵N-labeled) overexpression in BL21(DE3)**

Lane 1 is the mixed marker, lanes 2 & 3 are the R122L/S124A mutant at 0 hr & 3 hrs induction respectively, lanes 4 & 5 are the K31L mutant at 0 hr & 3 hrs induction respectively, lanes 6 & 7 are the wild-type at 0 hr & 3 hrs induction respectively, lane 8 is the 14 kDa marker.

Subsequent to expression, cells were lysed; the soluble protein was fractionated and purified on a Sephadex G-50 gel filtration column (Figure 3.2; lane 4) followed by anion exchange (DE-52) chromatography (Figure 3.2; lanes 5-7), and finally delipidation using a Lipidex-1000 column. As judged by SDS-PAGE, protein purity was estimated to be > 95%; protein concentration was monitored by the method of Bradford (*Bradford, 1976*) using bovine serum albumin (BSA) as a standard.

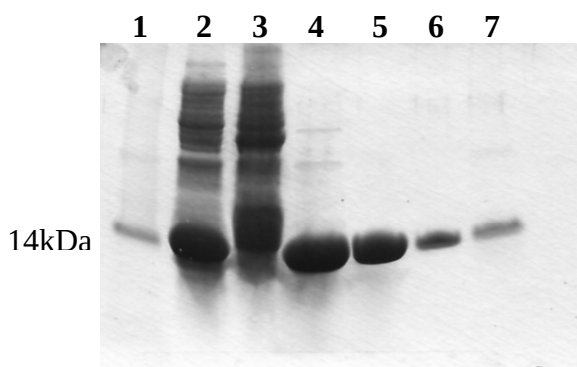


Figure 3.2 R122L/S124A-LFABP purification

Lane 1: 14kDa marker; lane 2: supernatant after cell lysis; lane 3: pellet after cell lysis; lane 4: purified protein after G-50 size exclusion column; lanes 5, 6, & 7: protein fractions from DE-52 ionic exchange column.

Transfer of 12AO from LFABPs to SUVs

Direct interactions of FABP with phospholipid vesicles may be affected by FABP and/or vesicle concentration, as well as the characteristics of the phospholipid headgroup and the nature of interactions between FABP and vesicles (*Kim and Storch, 1992; Hsu and Storch, 1996; Wootan et al., 1993*). In order to differentiate between diffusion- and collision-based mechanisms of FA transfer, the vesicle concentration and phospholipid

headgroup composition were varied systematically. When AOFA transfer from FABP to acceptor vesicles containing 25% anionic phospholipids is compared with neutral vesicles, a diffusion-mediated mechanism is implicated if no change in transfer rate is observed. However, a collisional-mediated mechanism is indicated if an increase in the transfer rate is observed. In addition, the diffusional mechanism is independent of acceptor vesicle concentration, but for the collisional mechanism the rate of transfer should increase with increasing acceptor concentration.

Transfer of 12-(9-anthroyloxy)oleic acid (12AO) from R122L/S124A-LFABP and K31L-LFABP (using wild-type LFABP as a control) was monitored with increasing concentrations of neutral EPC vesicles (Figure 3.3). Both mutants, like the wild-type protein, exhibited essentially no increase in transfer rate with increasing acceptor vesicle concentration, suggesting that the transfer process remains diffusion-mediated.

Additionally, the rate of 12AO transfer was not enhanced by increasing the negative charge on the phospholipid headgroup of the acceptor vesicles (Figure 3.4), confirming that the transfer mechanism of the mutant LFABPs remains diffusional as for the wild-type protein. It may be surprising that neither mutation (R122L/S124A and K31L) affected the transfer mechanism of the LFABP. The R122L/S124A mutation was not expected to alter the transfer mechanism, since such changes have been observed previously with mutations only in the helix region with IFABP (*Corsico et al., 1998; Corsico et al., 2005*). On the other hand, the K31L mutation is in the helix, but prior reports have only included mutations that change the mechanism from a rapid collision-mediated process to a slower diffusion-controlled mechanism. In retrospect, it is unlikely that changing from a polar Lys to a nonpolar Leu would favor the collisional FA transfer.

Thus fatty acid transfer data from LFABP and two mutants are consistent with an aqueous diffusion-mediated process. The rationale is that LFABP may act as a cytosolic buffer for fatty acids, maintaining the unbound fatty acid concentration in the cytosol rather than being involved in the uptake and transfer of fatty acid to subcellular membranes (*Storch and Bass, 1990; Kim and Storch, 1992; Hsu and Storch, 1996*).

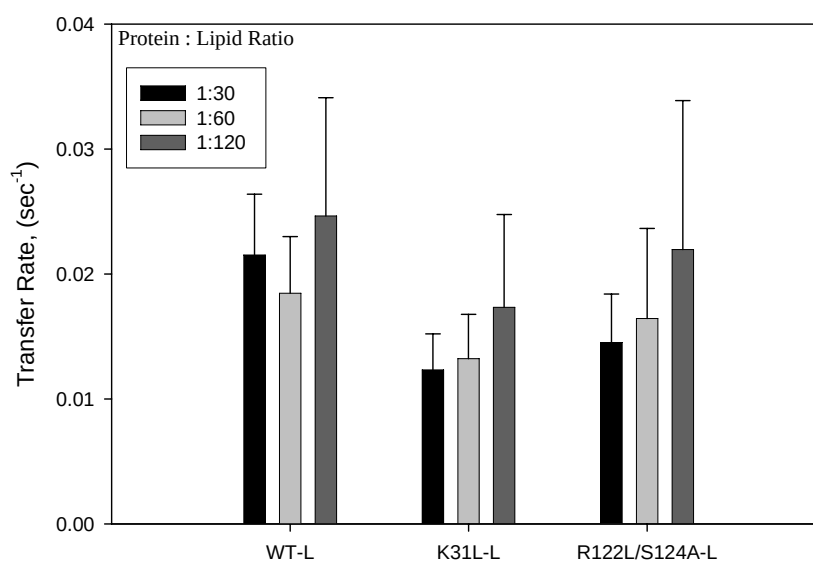


Figure 3.3 Effect of acceptor (EPC) concentration on the rate of 12AO transfer from wt-LFABP and mutants. Transfer of 1 μ M 12AO (18:1) from 10 μ M wt-LFABP, K31L-LFABP and R122L/S124A-LFABP to 300 μ M, 600 μ M, and 1200 μ M EPC vesicles was measured at 25 °C, as described under “Materials and Methods”.

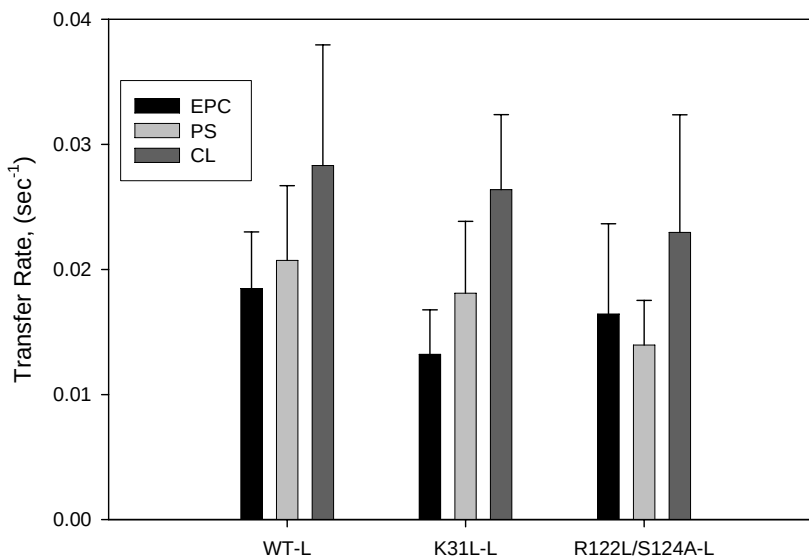


Figure 3.4 Effect of increasing negative charge of acceptor vesicles on the rate of 12AO transfer from wt-LFABP and mutants. Transfer of 1 μ M 12AO (18:1) from 10 μ M wt-LFABP, K31L-LFABP and R122L/S124A-LFABP to 600 μ M EPC, PS, and CL vesicles was measured at 25 °C, as described under “Materials and Methods”.

Binding affinity of oleate to mutant LFABPs

The binding affinity of mutant LFABPs for fatty acids was assessed by the fluorescent probe ADIFAB (acrylodated intestinal fatty acid-binding protein). This probe allows for the examination of binding characteristics of native fatty acids rather than requiring fluorescently labeled compounds. The emission spectrum of the acrylodan fluorophore in ADIFAB undergoes a red shift when ADIFAB is bound to fatty acid, and therefore this probe can be used to determine the concentration of unbound fatty acid (FFA) in a solution. In addition, the sensitivity of the probe is such that it allows for the determination of FFA levels without perturbing the equilibrium between bound and free ligand (*Richieri et al., 1994*). Given the known K_d of ADIFAB for a particular ligand,

equilibrium binding properties were determined for the unknown mutant LFABP (see Materials and Methods). Comparison of the absolute K_d values from the wild-type and mutant LFABP (Table 3.4) gave us clear insights into the effect of K31L and R122L/S124A mutations on binding affinity.

As expected, the first binding site exhibited a 10-fold decrease in affinity for the R122L/S124A mutant ($K_{d1} = 452 \mu\text{M}$): this mutation is in the cavity and, as discussed previously, Arg122 and Ser124 are important for electrostatic interactions with the ligand. They provide a favorable environment for the ligand, so that if these interactions are abolished then oleate might not bind in that position or may bind only weakly. Conversely, the K31L mutation was not significant enough to change the binding affinity to the first fatty acid (oleate) that binds: the binding constant for the K31L mutant ($K_{d1} = 0.052 \mu\text{M}$) was close to that of the wild-type protein ($K_{d1} = 0.043 \mu\text{M}$). Lys 31 interacts with the carboxylate of the second oleate on the surface of the protein, and therefore it was not expected to alter the binding affinity of the first ligand. K_{d2} was large ($>2\mu\text{M}$) but poorly determined for all LFABPs.

This assay can also provide information on the number of binding sites on the protein. The first binding site (n_1) had a stoichiometry close to 1 for both mutants, similar to wild-type LFABP. However, since the binding affinity for the second site was poorly determined, the stoichiometry for the second binding site (n_2) was not well determined either. Thus the second binding site could not be evaluated unambiguously using this assay. It may be that this assay was not sensitive enough to give an accurate binding constant for the second site. As a result, we could not determine whether any of the mutations caused a change in the affinity for binding the second oleate. Nevertheless,

we know from NMR data (see below) that the K31L mutant, like wild-type LFABP, is still binding two oleates in its cavity. By contrast, the R122L/S124A mutant is likely to bind only one oleate. These results can provide a framework with which to better understand the biological function of LFABP and the FA-LFABP interaction.

Table 3.4 The Binding Affinity of oleate to LFABP Mutants Compared to wt-LFABP (using ADIFAB binding assay)

protein	Binding constants (μM)		Stoichiometry (number of binding sites)	
	K_{d1}	K_{d2}	n_1	n_2
wt-LFABP	0.043 ± 0.032	high	1.0 ± 0.53	Not well determined
K31L-LFABP	0.052 ± 0.037	high	1.0 ± 0.53	Not well determined
R122L/S124A-LFABP	0.452 ± 0.305	high	1.15 ± 0.29	Not well determined
wt-LFABP (Richieri <i>et al.</i> , 1994)	0.009 ± 0.002	0.062 ± 0.025	1.0 ± 0.1	1.0 ± 0.1

Partitioning of 12AO between mutant LFABPs and phospholipid membranes.

The relative partition coefficient (K_p) is a molar ratio that represents the preferential distribution of fatty acids between FABPs and membranes. This assay is measured by the fluorescence that remains at equilibrium after mixing the 12AO-LFABP complex with NBD-PC vesicles, and is due solely to 12AO bound to LFABP. To confirm the binding affinity of oleate to mutant LFABPs, the relative distribution of 12AO between mutant LFABPs and phospholipid membranes (EPC vesicles) was calculated as described under “Materials and Methods”. The values for the partition coefficient of 12AO with wild-type LFABP in the present experiment (Table 3.5) are slightly lower than values reported by Storch and Bass (LFABP/EPC ratio of 51:1) (Storch and Bass, 1990), possibly because

they were determined differently. Our calculations followed Thumser and Storch (2000), but whereas they measured the partition of 12AO from membranes to LFABP, we measured the reverse process.

In the present study, the relative partitioning of 12AO between LFABP mutants and membranes was conducted as a complementary measurement of the binding affinity of oleate to the LFABP mutants. Whereas the ADIFAB assay looks at the preference of oleate for protein versus aqueous environments, this latter study assesses the competition between protein and membranes for fatty acid. The partitioning of 12AO between K31L-LFABP and EPC vesicles was similar to wild-type protein (Table 3.5), in agreement with the preceding binding data (Table 3.4) and confirming the constancy of the equilibrium binding affinity. By contrast, the partitioning of 12AO between R122L/S124A-LFABP and EPC vesicles (26:1) was substantially lower than for 12AO and K31L-LFABP (42:1) or wt-LFABP (42:1) (Table 3.5), reflecting the lower equilibrium binding affinity of R122L/S124A-LFABP. These results support our preceding finding from the binding assay using ADIFAB, in which oleate showed a significantly weaker binding to the R122L/S124A-LFABP mutant. The values in Table 3.5 represent the means of three separate determinations with variation < 10%.

Table 3.5 Coefficients for the partitioning (K_p) of 12AO between wt-LFABP and Mutants, and EPC vesicles

Protein (donor)	Partition coefficient, K_p (LFABP : EPC_{acceptor}, mol:mol)
Wt-LFABP	42:1
K31L-LFABP	42:1
R122L/S124A-LFABP	26:1

Overall secondary structure determination by Circular Dichroism Spectroscopy (CD)

Circular dichroism (CD) spectroscopy is a form of light absorption spectroscopy that measures the difference between the extinction coefficients for right- and left-circularly polarized light passing through a chiral molecule (Woody, 1995). When plane-polarized light passes through a circularly dichroic medium, differential absorption of the two circular components converts the plane-polarized light into elliptically-polarized light. CD spectroscopy is a quick but reliable technique used to gain information about the secondary structure of proteins in solution. Each different type of secondary structure displays a distinctive CD spectrum. For example, an α -helix conformation produces a CD spectrum with a positive peak ~ 190 - 195 nm, with a magnitude of about 60,000 to 80,000 $\text{deg}\cdot\text{cm}^2/\text{dmol}$ ($\pi \rightarrow \pi^*$ transition), and two negative peaks at 208 nm ($\pi \rightarrow \pi^*$ transition) and 222 nm ($n \rightarrow \pi^*$ transition), respectively, with magnitudes of $-36,000 \pm 3000$ $\text{deg}\cdot\text{cm}^2/\text{dmol}$. The CD spectrum of a β -sheet has one positive peak ~ 195 - 200 nm ($\pi \rightarrow \pi^*$ transition), magnitude 30,000 to 50,000 $\text{deg}\cdot\text{cm}^2/\text{dmol}$, and the $n \rightarrow \pi^*$ transition at about 215 to 220 nm (single negative peak), magnitude $-10,000$ to $-20,000$ $\text{deg}\cdot\text{cm}^2/\text{dmol}$ (Johnson, 1988; Fasman, 1996; Chen *et al.*, 1974).

The CD spectra of the mutant LFABPs were compared to those of the wild-type. It has been shown previously by Thumser *et al.* that a T102Q/R122Q mutation in LFABP alters its structural integrity by changing from predominantly β -sheet to α -helix, that is incorrect protein topology with respect to the wild-type protein (Thumser *et al.*, 1996). Therefore, one might expect that mutating Arg122 to Leu and Ser124 to Ala at the same time would have a similar effect on the structure.

LFABP has a predominantly β -sheet structure characterized by a CD spectrum with a positive peak ~ 195 - 200 nm and a negative peak ~ 215 - 220 nm (Figure 3.5). Likewise the R122L/S124A and K31L LFABP mutants have maintained their overall β -sheet structure (Figure 3.5). The variation in ellipticity between the mutants and the wild-type LFABPs may be due to the slight variation in the protein concentration. Thus in contrast to T102Q/R122Q, the similar CD data for all three LFABPs verify that no overall folding modifications occur in our mutant LFABP structures. These common overall features are also confirmed by detailed analysis of the respective NMR spectra (see below).

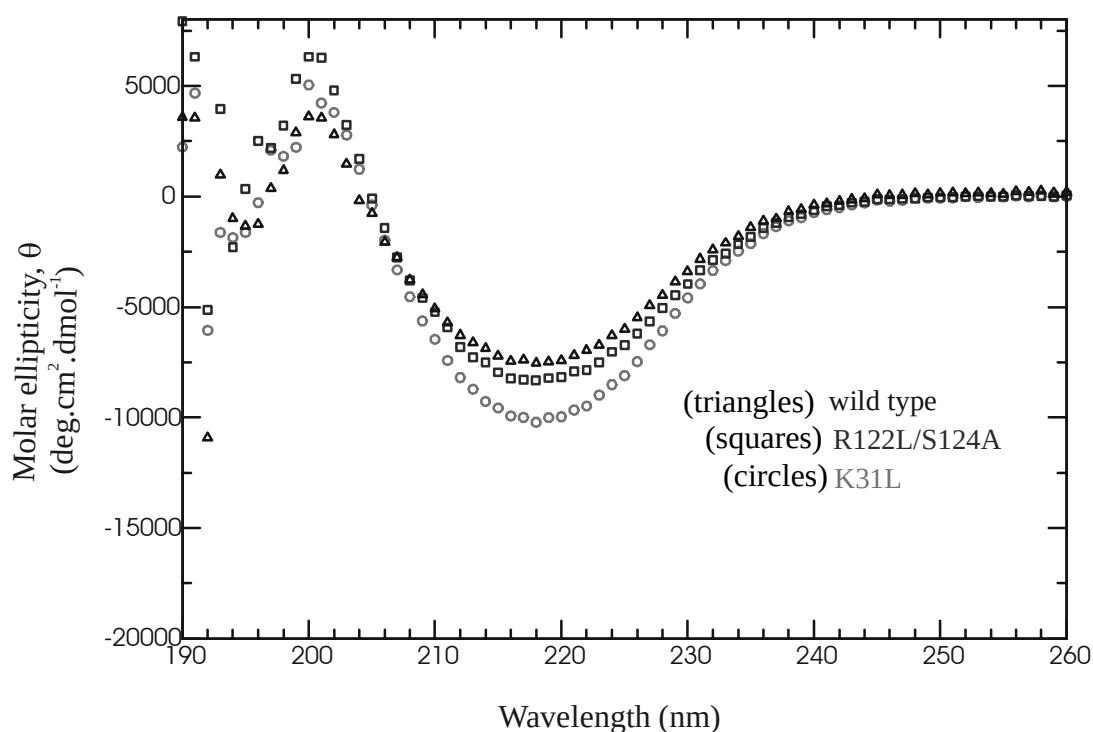


Figure 3.5 CD spectrum of wild-type LFABP, K31L-LFABP and R122L/S124A-LFABP. Spectra were measured in phosphate buffer (10 mM sodium phosphate, 5 μ M EDTA, 0.02% sodium azide, pH 7.0) at 25 °C.

NMR Spectroscopy:

Two-dimensional ^1H - ^{15}N -heteronuclear single-quantum correlation (2D ^1H - ^{15}N -HSQC)

2D ^1H - ^{15}N HSQC experiments are a simple and effective way to check whether the sample conformation has changed due to ligand binding, mutation, pH variation, or denaturation. The chemical shifts of wild-type LFABP have already been assigned previously (Wang *et al.*, 1998). Therefore, the ^1H - ^{15}N HSQC chemical shift dispersions of the LFABP mutants were analyzed and compared to that of the wild-type LFABP by using the NMRVIEW program to check the degree of similarity and location of differences. If both spectra are superimposable, which indicates that they have similar backbone amide peak positions, then the mutant's secondary structure can be determined through analogy to the wild-type protein. The spectra of the K31L-LFABP mutant (Apo; Figure 3.6 A & Holo; Figure 3.6 B) were superimposable with those of the wild-type protein except for the seven residues surrounding the mutation site, which suggests that they have similar backbone amide peak positions and thus very similar structure. Thus backbone amide proton and nitrogen chemical shifts were assigned based on analogy with wt-LFABP. In contrast, the spectrum of the R122L/S124A-LFABP mutant (Apo; Figure 3.6 C & Holo; Figure 3.6 D) showed considerable differences in the chemical shifts compared to wild-type protein. As a result, we deduced that the R122L/S124A mutant's structure has been altered, and many resonances could not be assigned based on the analogy with the wild-type protein. Therefore, other experiments (e.g. CBCA(CO)NH, HNCACB, C(CO)NH-TOCSY, H(CCO)NH-TOCSY, HCCH-TOCSY, TOCSY-HSQC, NOESY-HSQC) were performed to assign the resonances.

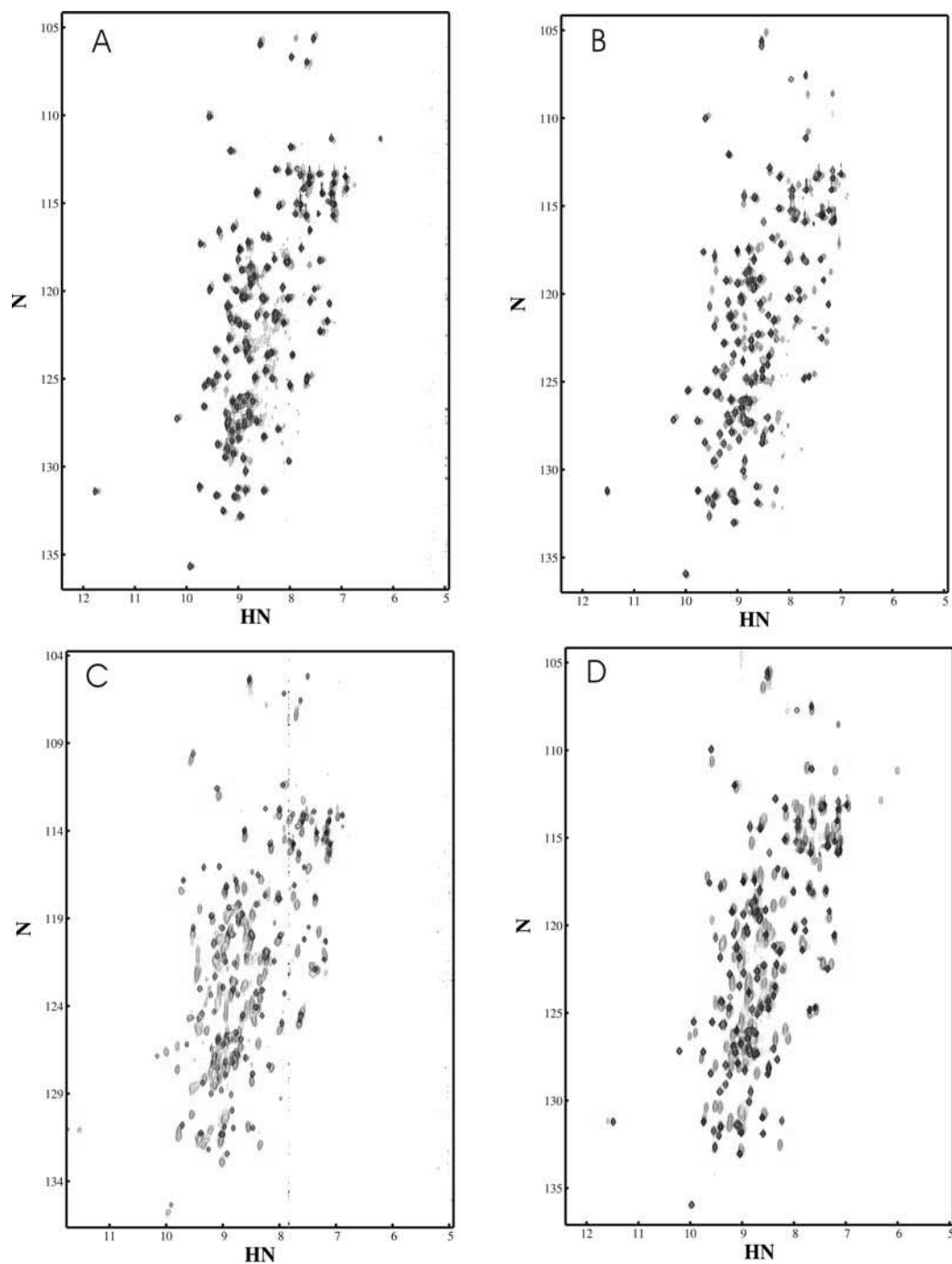


Figure 3.6 2D ^1H - ^{15}N HSQC spectra of mutant LFABPs superimposed with wild-type LFABP. (A) Apo K31L-LFABP (gray) superimposed with wt-LFABP (black). (B) Holo K31L-LFABP (gray) with wt-LFABP (black). (C) Apo R122L/S124A-LFABP (gray) with wt-LFABP (black). (D) Holo R122L/S124A-LFABP (gray) with wt-LFABP (black). Spectra were measured in KCl-phosphate buffer (0.15 M KCl, 0.05 M phosphate, and 0.02 % NaN_3 , pH 7.4) at 10 °C.

In addition, this experiment provides an important test of the feasibility of an NMR structure determination since the magnetization transfer steps associated with this experiment are present, i.e. INEPT and reverse-INEPT steps, in many more complex triple resonance pulse schemes and excellent spectra at this stage indicate that the sample is suitable for further structural studies. Furthermore, comparing the 2-D ^1H - ^{15}N HSQC spectra of ^{15}N -labeled target protein in the absence and presence of ligand provides information about protein–ligand interactions (Figure 3.6). First, the chemical shifts of the spectrum move if ligand binds, and second, the chemical shift perturbation represents a change of chemical environment, which is the results of ligand-binding, directly or indirectly.

Chemical Shift Perturbation Mapping

In order to determine the mutant protein sites that experience the greatest structural perturbation with respect to wt-LFABP, the absolute sum of chemical shift difference (in Hz) was calculated for both HN proton and ^{15}N resonances for the following pairs of proteins: apo-K31L-LFABP and apo-wt-LFABP (Figure 3.7); apo-R122L/S124A-LFABP and apo-wt-LFABP (Figure 3.8); holo-K31L-LFABP and holo-wt-LFABP (Figure 3.9); holo-R122L/S124A-LFABP and holo-wt-LFABP (Figure 3.10). The quantity $\Delta\delta$ ($\Delta\delta = [(599.944 (\text{NH}_{\text{WT}} - \text{NH}_{\text{M}}))^2 + (60.80 (\text{N}_{\text{WT}} - \text{N}_{\text{M}}))^2]^{1/2}$) was plotted as the function of the amino acid residue number. Residues with perturbation greater than one standard deviation beyond the mean were: A21, L28, Q30, K31, G32, I35, K36, H60, N61 for apo-K31L-LFABP (Figure 3.7) and Q12, V38, T53, V58, H60, V92, T102, I109, K121, V123, A124, K125 for apo-R122L/S124A-LFABP (Figure 3.8). For the holo-K31L-LFABP (Figure 3.9), the residues that experienced the greatest perturbation

were: F15, E16, K20, L28, Q30, L31, I35, V38, K58, H60, E62, M74, K78, R126, in contrast to holo- R122L/S124A-LFABP (Figure 3.10) where the perturbation was observed solely for the β -sheet residues: V9, V38, V39, M74, V83, V123, A124. Regions with the greatest perturbation are depicted using the NMR structure for the apo-LFABP using the MOLMOL program. The solution structure model is color coded in order to illustrate the degree of chemical shift difference between each LFABP mutant and wild-type. Those residues with a perturbation greater than one standard deviation beyond the mean are colored light gray. Residues with chemical shift differences below the calculated threshold value are colored dark gray. Using a 84 Hz cutoff, which corresponds to a 0.1 ppm perturbation in ^1H and a 1 ppm perturbation in ^{15}N , we show that the analysis of the chemical shift perturbation of the apo-K31L mutant as compared with the wild-type protein (Figure 3.7) indicates a localized region of perturbation in the area of mutation, located on the α -II helix but leaving the β -sheet region almost unaffected. On the other hand, the apo-R122L/S124A mutant revealed a more pervasive structural perturbation encompassing the β -sheet region (Figure 3.8), suggesting a change in its three-dimensional structure.

For the holo-K31L-LFABP (Figure 3.9) the average $\Delta\delta$ perturbation was almost double (41 Hz) in magnitude of apo-K31L-LFABP (27 Hz), which means this mutation produces a greater deviation in the holo-protein structure. Although the average chemical shift perturbation was comparable for apo- and holo-R122L/S124A-LFABP (113 and 117 Hz, Figures 3.8 and 3.10), the perturbations were distributed less pervasively in the holo form. Particularly interesting is the large perturbation around M74. Previous X-ray study has demonstrated that M74 methyl lies near the methyl group of the first bound oleate. It

is straightforward to understand the substantial perturbation around the mutation sites (R122 and S124). A large difference is also noticed around the loop area between α II-helix and β B strand, which is known to be part of the lipid portal for the FABP family of proteins.

In order to assess the impact of ligand binding on wild-type protein as compared with each of the mutants, the values of chemical shift perturbation ($\Delta\delta$) for holo K31L-LFABP (Figure 3.11) and holo R122L/S124A-LFABP (Figure 3.12) were plotted with respect to their apo forms. A striking feature of Figure 3.12 is that the binding of oleate to apo R122L/S124A-LFABP produces substantially less overall chemical shift perturbation than binding to apo K31L-LFABP (Figure 3.11), which is similar to the wild-type protein (data not shown). Moreover, the locations of chemical shift perturbation effects should inform us about ligand-protein interactions. Thus, Figure 3.12 suggests that the depth of the interaction of the fatty acid with the R122L/S124A-LFABP is 'shallower' than that in K31L-LFABP or wt-LFABP. In contrast, ligand binding to K31L-LFABP, as for the wild-type protein, shows more interaction with residues that span the inner cavity. These trends also underscore the distinctive pattern of oleate binding to the R122L/S124-LFABP mutant as compared with the wild-type and the K31L-LFABP proteins, though this analysis does not confirm definitively whether one or two ligands is binding to the double mutant.

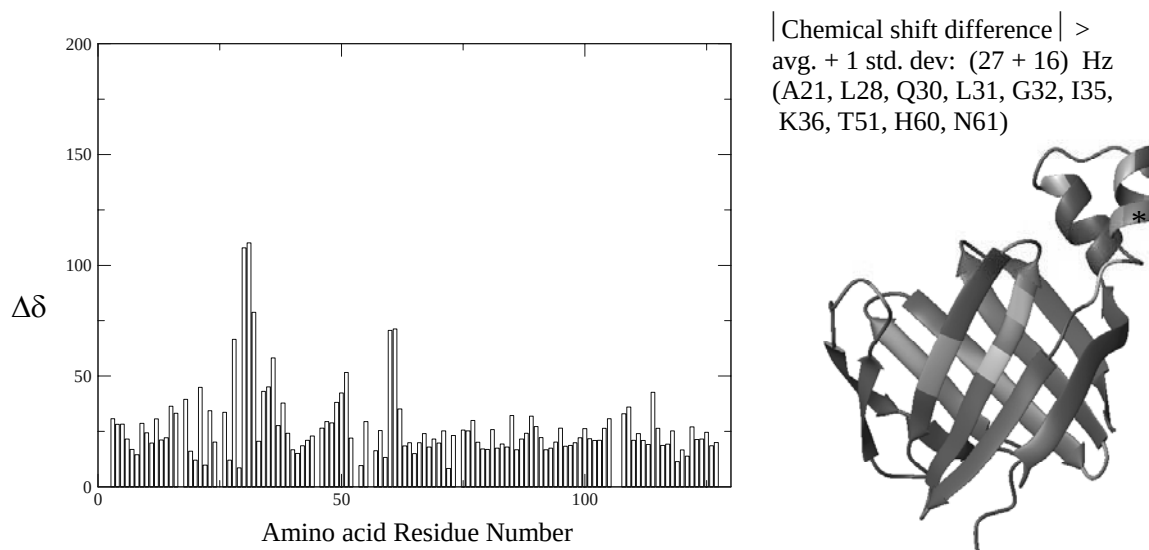


Figure 3.7 Chemical shift perturbation mapping of Apo K31L-LFABP with respect to Apo wt-LFABP. $\Delta\delta = [(599.944 (\text{NH}_{\text{WT}} - \text{NH}_{\text{SM}}))^2 + (60.80 (\text{N}_{\text{WT}} - \text{N}_{\text{SM}}))^2]^{1/2}$ Mutation site is marked with an asterisk.

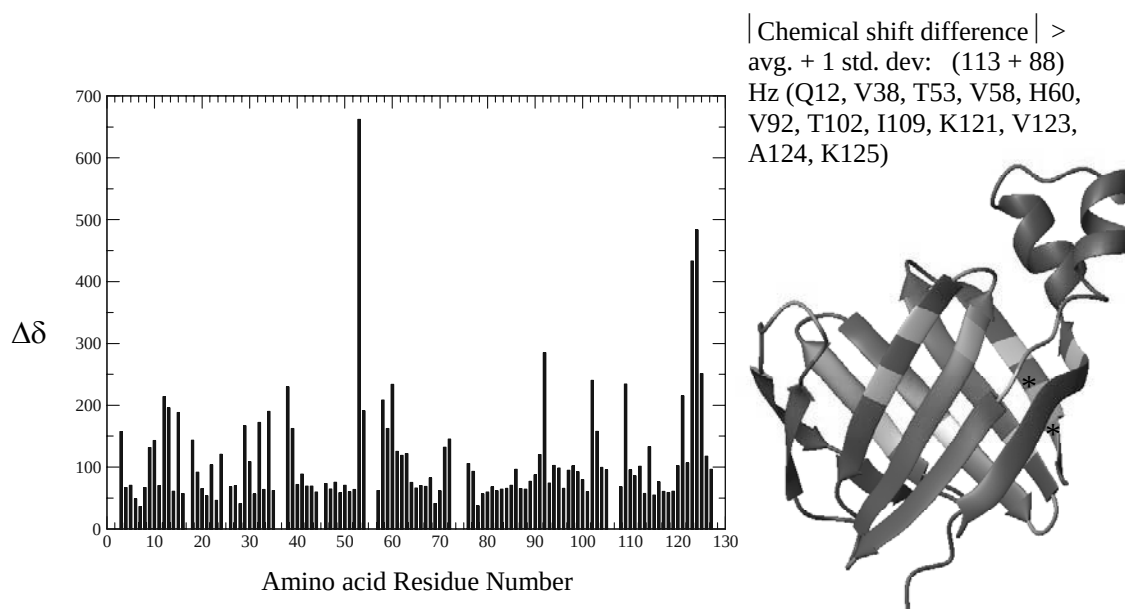


Figure 3.8 Chemical shift perturbation mapping of Apo R122L/S124A-LFABP with respect to Apo wt-LFABP.

$\Delta\delta = [(599.944 (\text{NH}_{\text{WT}} - \text{NH}_{\text{DM}}))^2 + (60.80 (\text{N}_{\text{WT}} - \text{N}_{\text{DM}}))^2]^{1/2}$ Mutation sites are marked with an asterisk.

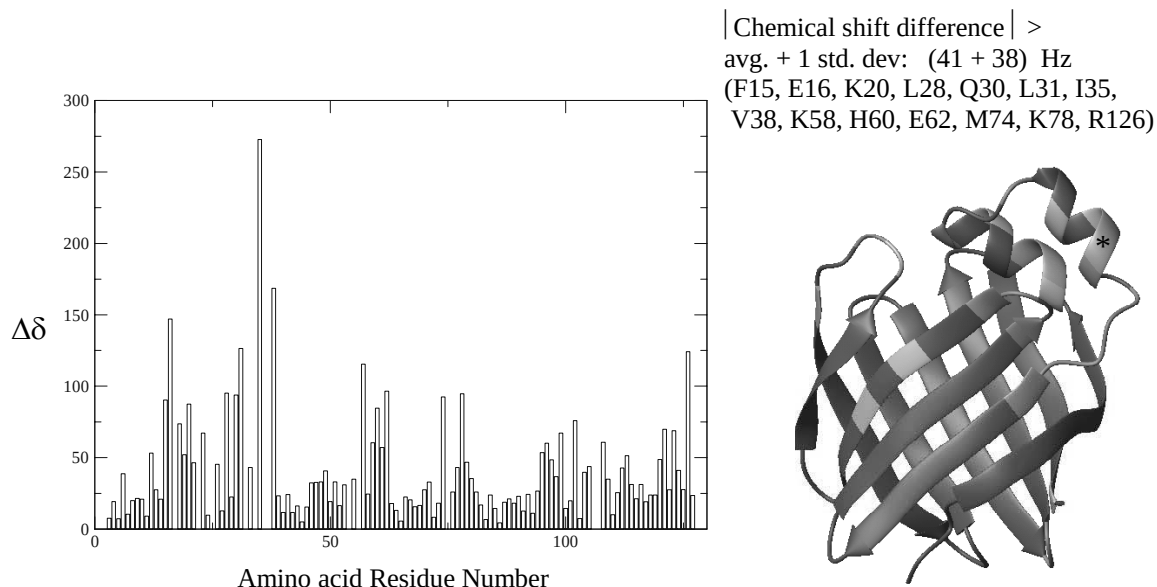


Figure 3.9 Chemical shift perturbation mapping of Holo K31L-LFABP with respect to Holo wt-LFABP. $\Delta\delta = [(599.944 (\text{NH}_{\text{WT}} - \text{NH}_{\text{SM}}))^2 + (60.80 (\text{N}_{\text{WT}} - \text{N}_{\text{SM}}))^2]^{1/2}$
Mutation site is marked with an asterisk.

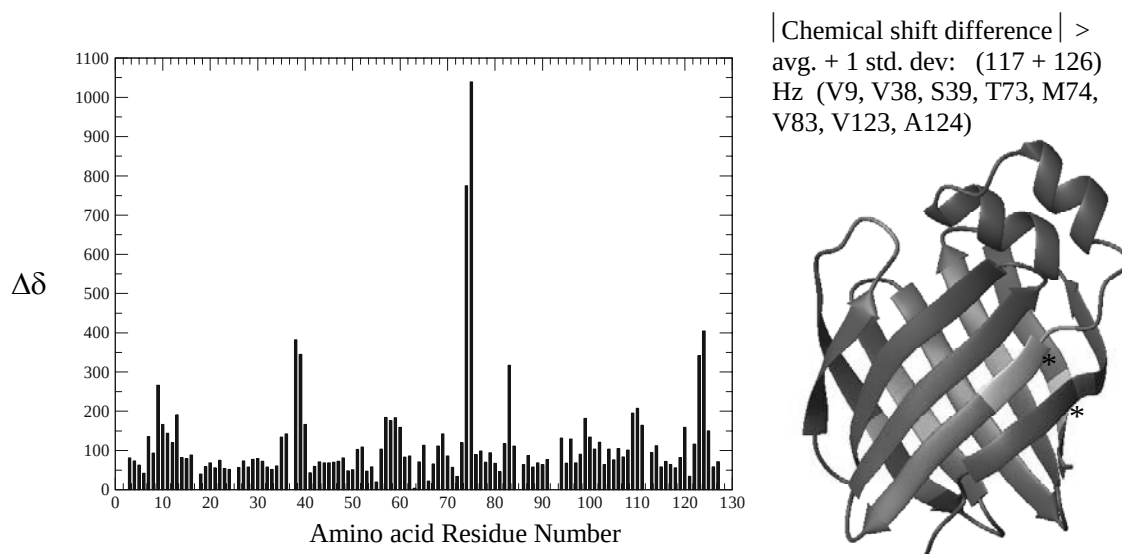


Figure 3.10 Chemical shift perturbation mapping of Holo R122L/S124A-LFABP with respect to Holo wt-LFABP.

$$\Delta\delta = [(599.944 (\text{NH}_{\text{WT}} - \text{NH}_{\text{DM}}))^2 + (60.80 (\text{N}_{\text{WT}} - \text{N}_{\text{DM}}))^2]^{1/2}$$

Mutation sites are marked with an asterisk.

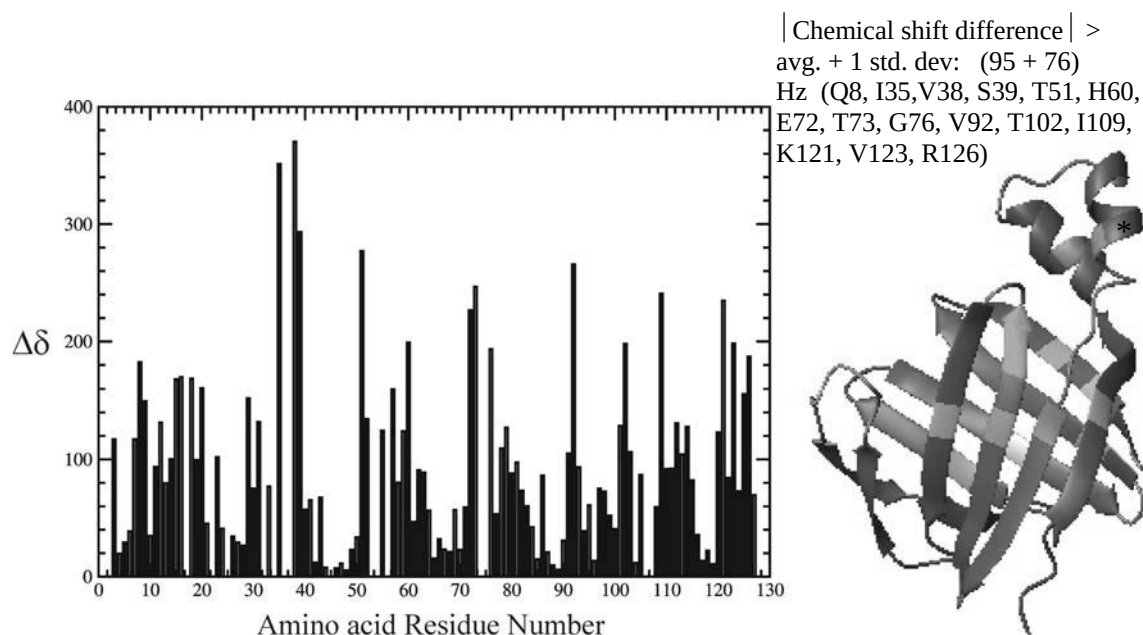


Figure 3.11 Chemical shift perturbation mapping of holo K31L-LFABP with respect to apo K31L-LFABP.

$$\Delta\delta = [(599.944 (\text{NH}_{\text{SMapo}} - \text{NH}_{\text{SMholo}}))^2 + (60.80 (\text{N}_{\text{SMapo}} - \text{N}_{\text{SMholo}}))^2]^{1/2}$$

Mutation site is marked with an asterisk.

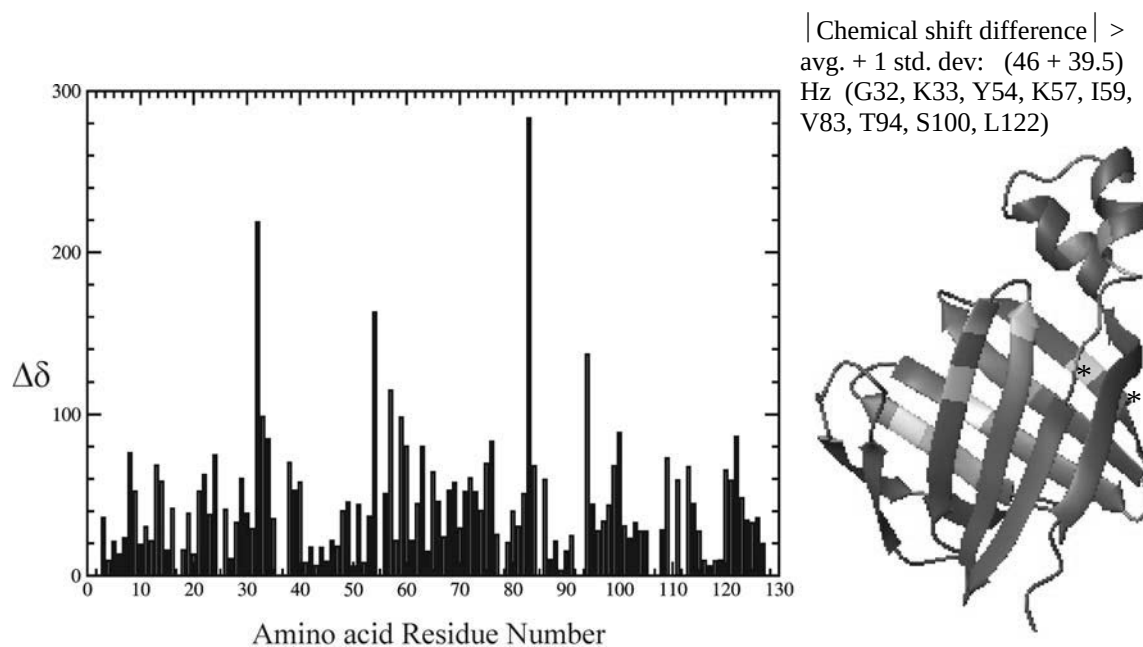


Figure 3.12 Chemical shift perturbation mapping of holo R122L/S124A-LFABP with respect to apo R122L/S124A-LFABP.

$$\Delta\delta = [(599.944 (\text{NH}_{\text{DMapo}} - \text{NH}_{\text{DMholo}}))^2 + (60.80 (\text{N}_{\text{DMapo}} - \text{N}_{\text{DMholo}}))^2]^{1/2}$$

Mutation sites are marked with an asterisk.

Protein-ligand interactions

The next logical question is whether these mutants bind two oleates (as for the wild-type protein) or one oleate. R122L/S124A-LFABP and K31L-LFABP mutants were titrated with [U- ^{13}C]-oleate to obtain holo-protein samples with molar ratios of 1:0.4, 1:0.8, 1:1.2, 1:1.6, and 1:2, respectively. The fatty acid environments at each titration step were monitored through the behavior of α - and ω -carbons observed in the 2D ^1H - ^{13}C HSQC spectra. The α -CH₂ and ω -CH₃ resonances were examined and compared with those of the wild-type protein. The oleate complexed with the R122L/S124A mutant displayed a single ω -CH₃ signal (Figure 3.13) (as well as a weak signal corresponding to excess oleate) and also one set of α -CH₂ signals (Figure 3.14), compared with two distinct sets of ω -CH₃ (Figure 3.13) and α -CH₂ (Figure 3.14) resonances for K31L-LFABP and wild-type LFABP.

The α -CH₂ resonance at (40.85, 2.15) for the oleate complexed with the R122L/S124A mutant lies close to the α -CH₂ resonance (40.8 ppm, 2.20 ppm) of the second binding site in wild-type LFABP. By contrast, although the ω -CH₃ resonance (15.75, 0.59) of oleate with the R122L/S124A mutant occurs at a distinctive position, it lies closer to that of the first binding site (16.9, 0.7) than the second binding site (17.0, 0.85) of wild-type LFABP. Furthermore, the α -CH₂ of the first oleate bound to the K31L- and wild-type protein shows two peaks corresponding to the two prochiral protons. Since the α -CH₂ of the first oleate likely binds to R122 in both wt- and K31L-LFABP, the two prochiral protons will be tethered by R122, restricting their motion and thus producing different chemical shifts for each. In this regard, the α -CH₂ of this first bound oleate in K31L-LFABP may be more constrained in space, judging from the observation that the

spectrum showed more distinct resonance positions (1.6 ppm and 1.75 ppm) for the two prochiral protons than in the wild-type protein. On the other hand, the α -CH₂ of the oleate bound to R122L/S124A-LFABP may not be tethered because of the replacement of R122 by Leu. Therefore, the two prochiral protons could rotate freely and rapidly to produce just one peak representing the average of the two magnetic environments.

These observations suggest two possibilities: (1) that a single molecule of FA binds to R122L/S124A LFABP at a site near the surface, with the carboxylate in a similar environment to that of the second binding site in wild-type LFABP and the ω -carbon tail extending into the inner cavity; or (2) both FA molecules are bound but they are undergoing rapid exchange to yield an average resonance. To distinguish between these possibilities, a binding assay was performed (Table 3.4 above), but the results are inconclusive. Alternatively, the analysis of intermolecular NOE crosspeaks between R122L/S124A LFABP and oleate (below) might shed some light on such interactions. This procedure could provide important information regarding the orientation and location of oleate in the protein cavity.

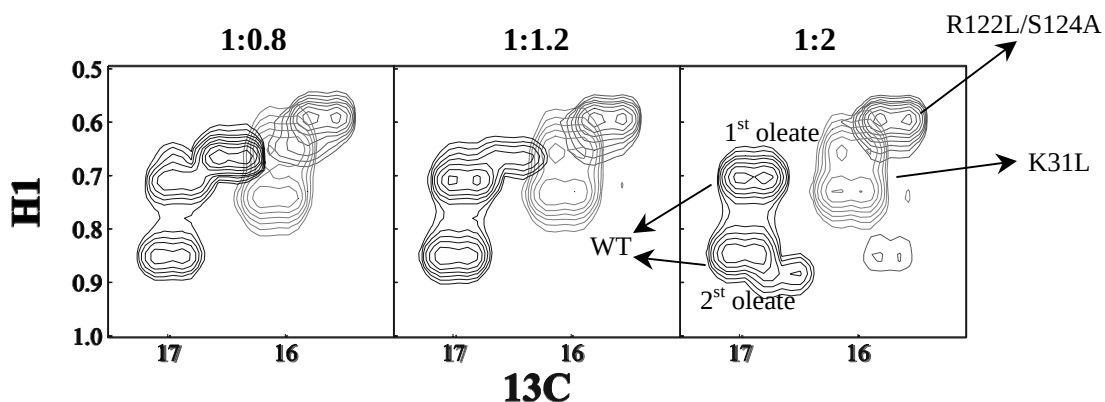


Figure 3.13 ω -carbons of a 2D ^1H - ^{13}C HSQC of $[\text{U-}^{13}\text{C}]$ -oleate complexed with ^{15}N -LFABP (wild-type, R122L/S124A and K31L mutants). Oleate

structure : ω 17 16 m 11 10 9 8 n β α
 $\text{CH}_3\text{CH}_2\text{CH}_2(\text{CH}_2)_4\text{CH}_2\text{CH}=\text{CHCH}_2(\text{CH}_2)_4\text{CH}_2\text{CH}_2\text{COOH}$

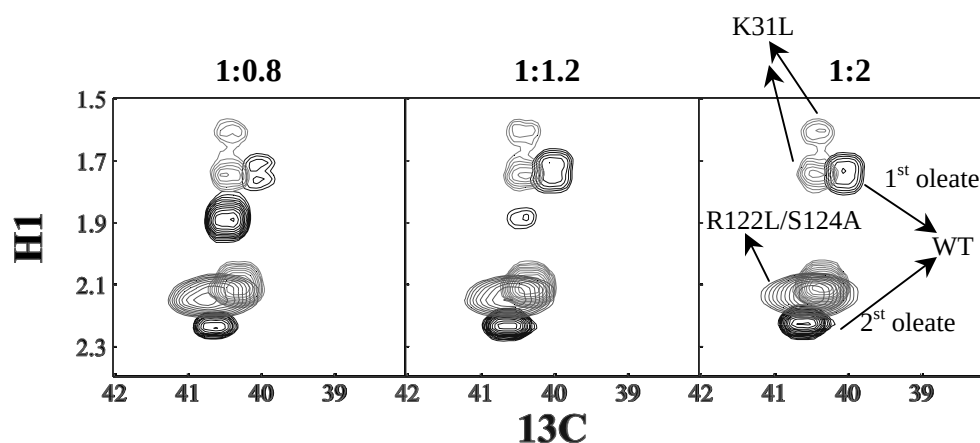


Figure 3.14 α -carbons of a 2D ^1H - ^{13}C HSQC of $[\text{U-}^{13}\text{C}]$ -oleate complexed with ^{15}N -LFABP (wild-type, R122L/S124A and K31L mutants).

Backbone and side-chain resonance assignments

The sequential backbone C^α and C^β resonance assignments of the R122L/S124A-LFABP mutant were obtained by a combination of CBCA(CO)NH, HNCACB and ^1H - ^{15}N HSQC experiments (see “Materials and Methods” chapter). The intra- and inter-residual C^α and C^β resonances were correlated to backbone amide ^1H and ^{15}N obtained from crosspeaks in the ^1H - ^{15}N HSQC spectrum. In particular, the CBCA(CO)NH experiment correlates the amide HN and the ^{15}N resonances of one residue with the $^{13}\text{C}^\alpha$ and $^{13}\text{C}^\beta$ resonances of the preceding residue (Figure 3.15 A, C, and E), whereas, the HNCACB experiment correlates the HN and ^{15}N resonances with those of the intraresidue $^{13}\text{C}^\alpha$ and $^{13}\text{C}^\beta$ nuclei (intense peaks) as well as those of the preceding residue (weaker intensity peaks) (Figure 3.15 B, D, and F). The way that magnetization is transferred between $^{13}\text{C}^\alpha$ and $^{13}\text{C}^\beta$ causes the correlations for C^α and C^β carbons in HNCACB spectra to have opposite signs ($^{13}\text{C}^\beta$ is negative and $^{13}\text{C}^\alpha$ is positive); this is coded by the use of black and gray peaks. Thus, CBCA(CO)NH and HNCACB data are

used alternately to make the connectivity of corresponding C_i^α , C_i^β , C_{i-1}^α , and C_{i-1}^β frequencies illustrated by the example shown in Figure 3.15. Strip F in Figure 3.15 displays the $^{13}\text{C}^\alpha$ and $^{13}\text{C}^\beta$ resonances of lysine 84 (intense peaks) and that of the preceding residue, valine 83 (weaker peaks) which is confirmed by looking at its corresponding CBCA(CO)NH strip (E). Aligning the weak peaks in one strip (for example, E) with the strong peaks from another (for example, D) confirms that the corresponding HN-N pairs are adjacent in the polypeptide sequence. Finally, by combining the results of CBCA(CO)NH with HNCACB, sequential assignments are obtained and now HN and ^{15}N resonances in the ^1H - ^{15}N HSQC spectrum can be identified.

Once the backbone nuclei had been identified, the resonance assignments were extended to resonances of peripheral side-chain nuclei in each spin system. Aliphatic side-chain (^1H and ^{13}C) assignments were derived from C(CO)NH-TOCSY, H(CCO)NH-TOCSY, and HCCH-TOCSY. C(CO)NH-TOCSY and H(CCO)NH-TOCSY correlates the HN and ^{15}N of one residue with all aliphatic carbons and protons in the spin system of preceding residue, respectively. $^{13}\text{C}^\alpha$ and $^{13}\text{C}^\beta$ resonances from C(CO)NH-TOCSY were confirmed using the HNCACB experiment. Alternatively, the ^1H assignments were obtained from HCCH-TOCSY, which correlates $^1\text{H}_i$ and $^{13}\text{C}_i$ with $^1\text{H}_j$ and $^{13}\text{C}_j$ of the same side-chain spin network (see “Materials and Methods” chapter), producing ^1H - ^1H planes edited by ^{13}C in the third dimension.

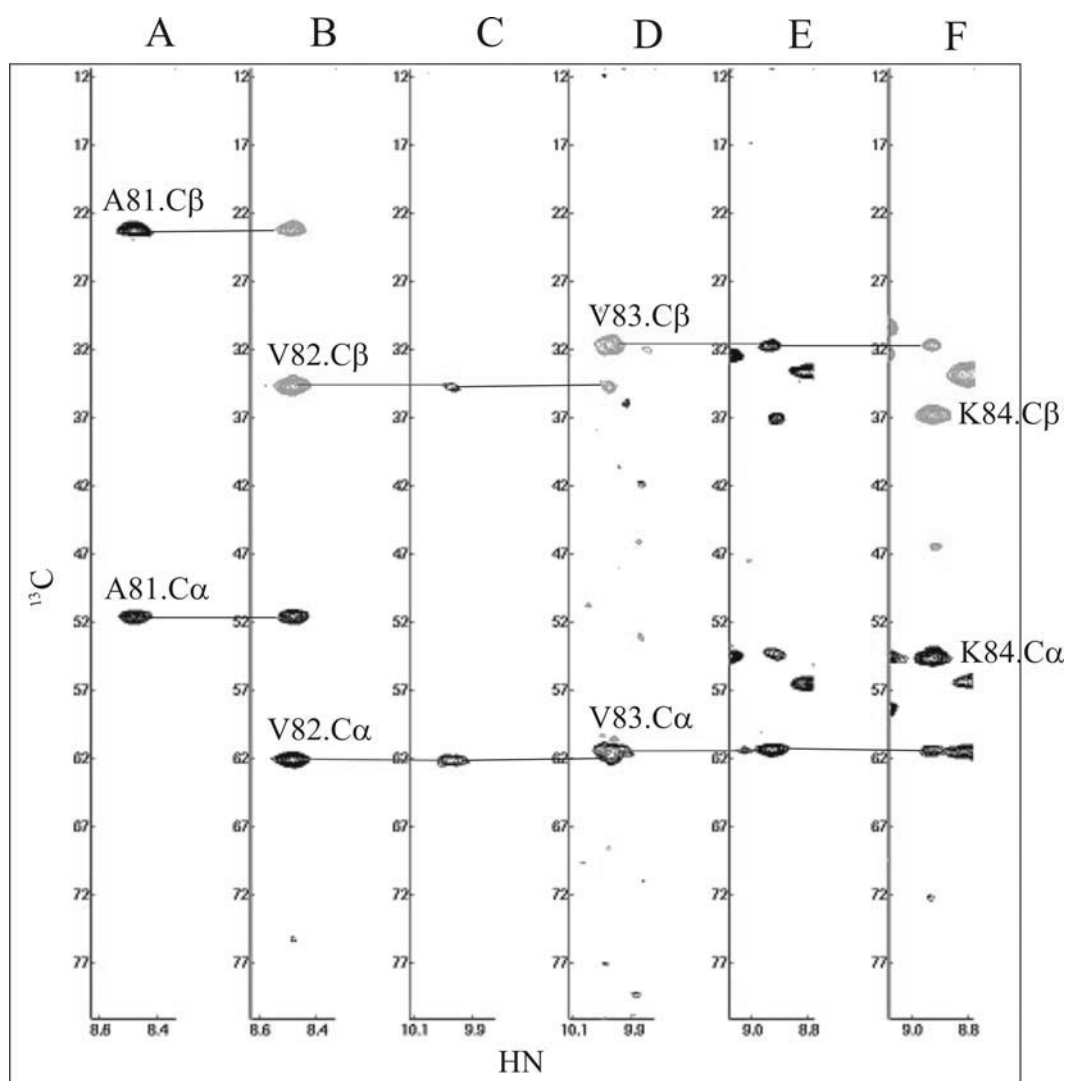


Figure 3.15 NMRview display of six strips of ^1H - ^{13}C planes from 3D CBCA(CO)NH (A, C, and E) and HNCACB (B, D, and F) experiments. Positive peaks ($^{13}\text{C}^\alpha$) are black and negative peaks ($^{13}\text{C}^\beta$) are gray.

The analysis of the complete K80 spin system with HCCH-TOCSY is illustrated in Figure 3.16. Each panel represents a ^{13}C slice where the ^{13}C resonances were obtained from the C(CO)NH-TOCSY. The ^1H that is directly attached to the ^{13}C is drawn in the Y-axis. Magnetization is transferred from a side-chain (or backbone) proton to the directly

attached carbon atom, by ^1J coupling to the neighboring carbon atoms and finally to their attached protons. These TOCSY-correlated protons are presented in the X-axis. Therefore, the HCCH-TOCSY experiment looks in principle the same as ^{13}C -HSQC-TOCSY. For example, panel A correlates the ^1H - $^{13}\text{C}_\epsilon$ with all other neighboring ^1H - $^{13}\text{C}_{\alpha/\beta/\gamma/\delta/\epsilon}$, whereas panel E correlates the ^1H - $^{13}\text{C}_\alpha$ with all other neighboring ^1H - $^{13}\text{C}_{\alpha/\beta/\gamma/\delta/\epsilon}$. Following this pattern, the sequential side-chain ($^1\text{H}_i^\alpha$ - $^1\text{H}_i^\beta$ - $^1\text{H}_i^\gamma$...) resonance assignments in the R122L/S124A-LFABP protein were obtained.

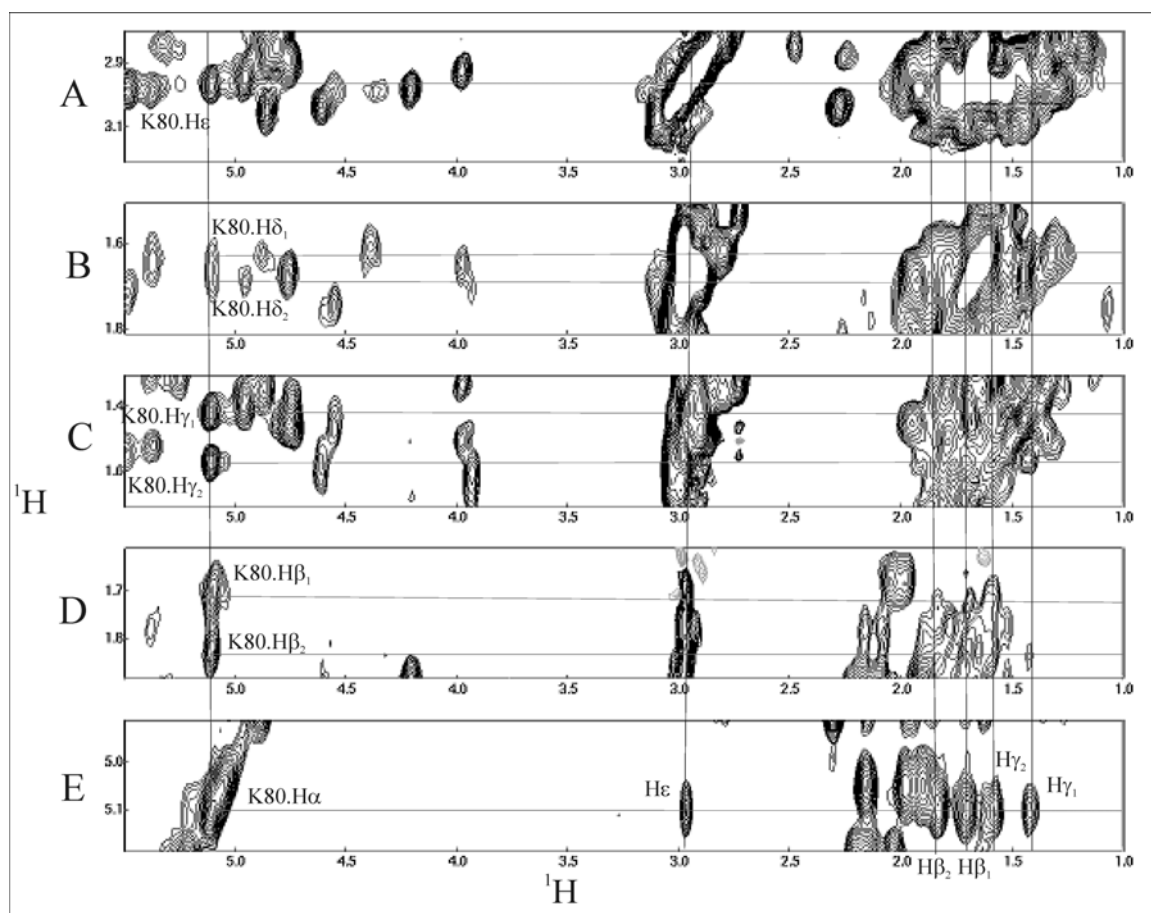


Figure 3.16 NMRview display of five strips of ^1H - ^1H planes of lysine 80 of apo R122L/S124A-LFABP from 3D HCCH-TOCSY experiment. Panels A, B, C, D, and E represent respectively the ^1H - $^{13}\text{C}\epsilon$, ^1H - $^{13}\text{C}\delta$, ^1H - $^{13}\text{C}\gamma$, ^1H - $^{13}\text{C}\beta$, and ^1H - $^{13}\text{C}\alpha$ connectivities to neighboring ^1H - $^{13}\text{C}\alpha/\beta/\gamma/\delta/\epsilon$. The y-axis displays the proton of the directly attached carbon atom (z-axis, third dimension) and the x-axis is the protons attached to the neighboring carbon atoms.

The sequential resonance assignments for the apo and the holo forms of R122L/S124A-LFABP at pH 7.0 and 10 °C are summarized in Tables 3.6 and 3.7.

Table 3.6 ^1H , ^{15}N and ^{13}C chemical shifts for apo R122L/S124A-LFABP at pH 7.0 and 10 °C.

Spin	^{15}N	^1HN	$^1\text{H}^\alpha$	$^{13}\text{C}^\alpha$	$^1\text{H}^\beta$	$^{13}\text{C}^\beta$	Other ^1H	Other ^{13}C	NH ₂ & NH (sc)
Asn ²			4.821	52.207	2.835 2.550	40.52			δ 115.634 7.662,7.159
Phe ³	131.072	11.530	4.340	57.242	3.059 2.327	38.679			
Ser ⁴	117.287	8.742	4.432	61.455	4.143 3.993	64.639			
Gly ⁵	111.996	9.078	4.020 3.897	45.297					
Lys ⁶	122.520	8.347	5.309	55.368	1.658	34.857	γ 1.365,1.181 ϵ 2.846,2.844	γ 26.044	
Tyr ⁷	121.399	9.148	5.310	55.986	2.552 2.425	42.353			
Gln ⁸	123.895	9.305	5.390	53.958	2.068 1.917	33.188	γ 2.425,2.225	γ 34.005	ϵ 113.242 7.514,6.970
Val ⁹	130.324	8.921	3.715	66.525	2.236	33.236	γ^1 1.019; γ^2 1.133	γ^1 21.298; γ^2 23.443	
Gln ¹⁰	126.610	10.002	4.550	55.980	2.180 1.962	32.126	γ 2.421	γ 34.520	ϵ 114.403 7.360,7.220
Ser ¹¹	113.093	7.999	4.768	57.676	3.916 3.850	65.425			
Gln ¹²	117.299	8.625	5.480	55.391	2.271	34.114	γ 2.503,2.385		ϵ 112.130 7.578,6.214
Glu ¹³	122.086	9.459	4.879	55.498	2.146 1.961	34.035	γ 2.304	γ 36.400	
Asn ¹⁴	118.461	9.405	4.520	54.945	3.750 3.230	36.452			δ 114.521 7.906,7.169
Phe ¹⁵	119.760	8.572	3.628	62.718	3.020 2.844	40.687			
Glu ¹⁶	117.407	9.739	3.813	63.312	2.205	26.847	γ 2.339	γ 37.716	
Pro ¹⁷			4.258	65.637	2.335 1.780	30.936	γ 2.117; δ 3.723,3.660	γ 28.050; δ 49.929	
Phe ¹⁸	119.803	7.304	3.921	62.532	3.006 2.839	40.467			
Met ¹⁹	116.766	8.318	4.071	56.612	1.628 1.364	31.364	γ 1.771, 1.515	γ 32.426	
Lys ²⁰	121.386	8.336	3.975	59.642	1.787	32.414	γ 1.502,1.340 δ 1.643; ϵ 2.925	γ 25.589; δ 29.144; ϵ 41.998	
Ala ²¹	125.038	7.649	4.035	54.833	1.329	17.972			
Met ²²	116.223	7.492	4.215	55.325	1.924 1.871	32.701	γ 2.211,2.087	γ 33.000	
Gly ²³	107.405	7.709	4.116 3.675	45.383					
Leu ²⁴	124.291	7.591	4.435	54.009	1.381 1.227	41.648	γ 1.659; δ^1 0.834; δ^2 0.771	γ 28.324; δ^1 25.589; δ^2 24.769	
Pro ²⁵			4.470	62.861	2.442 2.092	32.870	γ 2.143; δ 4.018,3.524	γ 28.050; δ 51.023	
Glu ²⁶	123.324	8.851	3.873	60.148	2.105 2.047	29.959	γ 2.350	γ 36.255	
Asp ²⁷	117.501	8.961	4.376	57.166	2.665 2.662	40.035			
Leu ²⁸	121.150	7.190	4.207	57.358	1.808 1.602	41.952	γ 1.716; δ^1 0.992; δ^2 0.913	γ 27.777; δ^1 25.316; δ^2 23.948	

Table 3.6 -----Continued.

Spin	¹⁵ N	¹ HN	¹ H ^α	¹³ C ^α	¹ H ^β	¹³ C ^β	Other ¹ H	Other ¹³ C	NH ₂ & NH (sc)
Ile ²⁹	122.152	7.824	3.549	66.481	2.019	37.998	γ ¹ 1.692,0.393; γ ² 0.942; δ 0.662	γ ¹ 29.691; γ ² 18.205; δ 13.704	ε 115.169 7.820,7.123
Gln ³⁰	117.579	8.211	3.974	58.887	2.179	28.169	γ 2.528	γ 33.567	
Lys ³¹	118.210	7.683	4.205	58.955	1.909	33.256	γ 1.673,1.528; δ 1.668; ε 2.983	γ 25.852; δ 29.405; ε 42.033	
Gly ³²	106.823	8.231	4.151 3.945	46.629					
Lys ³³	120.932	7.680	3.317	59.047	1.849 1.500	32.379	γ 1.535,1.427; δ 1.712,1.444; ε 3.100,3.057	γ 24.026; δ 29.789; ε 41.998	
Asp ³⁴	117.727	7.755	4.810	54.572	2.850 2.478	42.107			
Ile ³⁵	121.960	7.330	3.849	62.122	1.753	38.460	γ ¹ 1.046,1.368 γ ² 0.422; δ 0.672	γ ¹ 27.854; γ ² 17.314; δ 13.603	
Lys ³⁶	130.144	8.906	4.382	54.474	1.861	31.096	γ 1.479,1.314; δ 1.612; ε 2.987	γ 24.674; δ 29.144; ε 42.345	
Gly ³⁷			4.208 4.000	45.473					
Val ³⁸	121.328	8.504	5.095	61.128	1.980	36.046	γ ¹ 0.960; γ ² 0.987	γ ¹ 21.487; γ ² 21.845	
Ser ³⁹	120.860	9.443	5.587	56.140	3.630 3.581	67.291			
Glu ⁴⁰	126.081	9.353	5.300	55.586	2.159 1.907	32.667	γ 2.329,2.161	γ 36.857	
Ile ⁴¹	127.616	9.799	5.152	60.579	2.252	40.952	γ ¹ 1.792,0.992 γ ² 0.810; δ 0.895	γ ¹ 28.569; γ ² 18.011; δ 13.587	
Val ⁴²	131.518	9.394	4.225	63.261	2.082	33.341	γ ¹ 0.950; γ ² 0.992	γ ¹ 20.940; γ ² 20.414	
His ⁴³	132.912	9.015	5.530	53.024	3.346 2.509	34.606			
Glu ⁴⁴	131.751	9.037	4.372	54.894	1.908 1.845	31.799	γ 2.060	γ 36.184	
Gly ⁴⁵	118.562	8.752	3.972	47.261					
Lys ⁴⁶	126.448	8.981	4.612	56.551	2.281 1.862	33.709	γ 1.627,1.548; δ 1.784; ε 3.033	γ 25.656; δ 29.438; ε 42.160	
Lys ⁴⁷	121.353	8.221	4.755	56.325	1.944 1.674	33.646	γ 1.451,1.327; δ 1.670; ε 2.896	γ 25.084; δ 28.867; ε 41.724	
Val ⁴⁸	125.814	8.765	4.548	61.433	0.516	33.803	γ ¹ 0.390; γ ² 0.595	γ ¹ 22.238; γ ² 21.899	
Lys ⁴⁹	126.337	8.688	4.742	55.607	1.849 1.637	34.689	γ 1.492,1.371; δ 1.690,1.634; ε 2.893	γ 25.589; δ 29.698; ε 41.915	
Leu ⁵⁰	127.960	9.062	5.366	53.597	1.773 1.201	46.495	γ 1.524; δ ¹ 0.897	γ 25.553; δ ¹ 24.474	
Thr ⁵¹	128.720	9.528	5.221	62.506	4.070	70.223	γ 1.119	γ 22.854	
Ile ⁵²	131.071	9.218	4.995	60.400	1.703	41.213	γ ¹ 1.572,0.971	γ ¹ 28.030	
Thr ⁵³	125.420	9.287	5.151	62.592	3.970	70.372	γ 1.124		

Table 3.6 -----Continued.

Spin	¹⁵ N	¹ HN	¹ H ^α	¹³ C ^α	¹ H ^β	¹³ C ^β	Other ¹ H	Other ¹³ C	NH ₂ & NH (sc)
Tyr ⁵⁴	131.652	9.286	4.577	57.394	3.052 2.649	41.164			
Gly ⁵⁵			4.226 3.599	47.731					
Ser ⁵⁶	125.209	9.442	4.405	58.787	4.058 3.902	64.148			
Lys ⁵⁷	125.348	7.986	4.553	55.582	1.990	33.861	γ 1.460; δ 1.747; ε 2.986	γ 24.989; δ 28.627; ε 42.545	
Val ⁵⁸	126.155	8.368	4.995	61.443	1.871	34.008	γ ¹ 0.651; γ ² 0.904	γ ¹ 21.093; γ ² 21.487	
Ile ⁵⁹	127.807	9.223	4.532	59.676	1.741	40.761	γ ¹ 1.392, 1.251 γ ² 0.765	γ ¹ 27.936; γ ² 18.084	
His ⁶⁰	131.721	9.427	5.270	56.259	3.124 2.980	33.198			
Asn ⁶¹	122.890	8.638	5.221	53.114	2.588 2.507	45.221			
Glu ⁶²	121.512	9.099	5.213	55.482	2.038	32.923	γ 2.204, 2.151	γ 36.366	
Phe ⁶³	119.458	8.580	5.098	56.225	3.278 3.259	41.439			
Thr ⁶⁴	118.657	8.872	5.100	61.646	3.954	70.440	γ 1.225	γ 21.213	
Leu ⁶⁵	131.114	9.773	4.475	54.198	1.918 1.672	42.209	γ 0.996; δ ¹ 0.920; δ ² 0.957	γ 26.498; δ ² 23.738	
Gly ⁶⁶	109.949	9.564	4.426 3.463	45.763					
Glu ⁶⁷	120.310	8.022	4.824	54.416	2.194 1.946	32.403	γ 2.233, 2.164	γ 36.016	
Glu ⁶⁸	125.953	9.082	4.469	58.311	2.041 1.980	30.398	γ 2.180	γ 37.855	
Cys ⁶⁹	125.282	9.442	5.191	56.341	3.475 3.125	32.639			
Glu ⁷⁰	120.508	8.519	5.060	55.746	1.905	32.243	γ 2.149, 1.985	γ 38.090	
Leu ⁷¹	126.848	9.074	4.632	53.162	1.182 0.849	44.682	γ 1.380; δ ¹ 0.243; δ ² 0.469	γ 26.323; δ ¹ 25.115; δ ² 23.414	
Glu ⁷²	126.342	9.803	4.794	55.973	1.953	31.108	γ 2.419, 2.064	γ 36.802	
Thr ⁷³	113.203	8.082	4.373	61.198	4.551	70.540	γ 1.196	γ 22.185	
Met ⁷⁴	127.342	9.142		56.176		32.200			
Thr ⁷⁵	115.856	8.390		61.362		69.246			
Gly ⁷⁶	111.359	7.875	4.377	45.234					
Glu ⁷⁷	121.791	7.422	4.219	56.281	1.826	30.867	γ 2.256, 2.117	γ 36.305	
Lys ⁷⁸	125.431	8.707	5.361	54.963	1.524	33.426	γ 1.524, 1.278; δ 1.633; ε 2.987	γ 25.316; δ 29.114; ε 41.998	
Val ⁷⁹	120.177	9.074	4.678	58.998	2.137	35.145	γ ¹ 0.653; γ ² 0.875	γ ¹ 19.586; γ ² 21.786	
Lys ⁸⁰	123.896	8.485	5.101	55.772	1.827 1.703	32.958	γ 1.581, 1.417; δ 1.684, 1.612; ε 2.965	γ 25.330; δ 29.046; ε 41.965	
Ala ⁸¹	127.597	8.978	4.860	51.565	1.226	23.146			
Val ⁸²	118.782	8.479	4.269	62.033	1.930	34.694	γ ¹ 0.723; γ ² 0.870	γ ¹ 20.779; γ ² 20.926	
Val ⁸³	135.780	9.971	4.552	61.466	1.594	31.703	γ ¹ 0.595; γ ² -0.100	γ ¹ 18.240; γ ² 21.899	

Table 3.6 -----Continued.

Spin	¹⁵ N	¹ HN	¹ H ^α	¹³ C ^α	¹ H ^β	¹³ C ^β	Other ¹ H	Other ¹³ C	NH ₂ & NH (sc)
Lys ⁸⁴	126.024	8.930	4.951	54.606	1.791	36.786	γ 1.386; δ 1.687; ε 2.945	γ 25.396; δ 29.556; ε 41.987	
Met ⁸⁵	120.202	8.919	5.461	54.295	2.397 2.041	31.811	γ 2.843,2.637	γ 32.225	
Glu ⁸⁶	127.328	8.779	4.583	56.251	1.892 1.782	32.446	γ 2.120	γ 36.824	
Gly ⁸⁷	114.331	8.616	4.213 3.807	45.738					
Asp ⁸⁸	119.147	8.667	4.768	55.344	2.735	41.868			
Asn ⁸⁹	115.048	8.147	5.198	53.261	3.804 2.817	40.024			δ 113.484 7.434,7.134
Lys ⁹⁰	117.907	7.368	6.017	54.808	2.254 1.526	36.214	γ 1.502,1.181; δ 1.588,1.181; ε 2.865	γ 25.338; δ 29.493; ε 41.701	
Met ⁹¹	120.737	8.991	5.285	55.107	1.713 1.607	36.148	γ 2.446,2.108	γ 34.053	
Val ⁹²	122.280	9.462	5.327	60.241	2.001	35.772	γ ¹ 0.951	γ ¹ 21.402	
Thr ⁹³	120.127	8.906	4.865	61.654	4.053	69.715	γ 0.956		
Thr ⁹⁴	117.710	8.015	5.534	59.887	4.041	71.770	γ 1.099	γ 20.341	
Phe ⁹⁵	122.084	8.257	4.855	56.403	3.275 2.755	40.066			
Lys ⁹⁶	119.849	9.536	3.926	56.699	2.145 1.866	29.946	γ 1.703,1.591; δ 1.797,1.716; ε 3.020	γ 25.295; δ 28.621; ε 41.353	
Gly ⁹⁷	105.468	8.519	3.996 3.520	45.530					
Ile ⁹⁸	124.103	8.372	4.380	60.568	1.546	39.501	γ ¹ 1.609; γ ² 0.871; δ 0.741	γ ¹ 28.871; γ ² 14.806; δ 18.489	
Lys ⁹⁹	127.532	8.145	4.868	55.587	1.853 1.708	34.295	γ 1.353,1.257; δ 1.629; ε 2.879	γ 25.438; δ 29.324; ε 41.716	
Ser ¹⁰⁰	123.064	9.050	5.465	56.907	3.770 3.244	64.957			
Val ¹⁰¹	130.490	8.981	4.655	62.103	2.027	34.810	γ ¹ 0.842; γ ² 0.955	γ ¹ 21.213; γ ² 20.930	
Thr ¹⁰²	130.204	9.555	4.783	61.946	3.813	70.030	γ 0.683	γ 23.833	
Glu ¹⁰³	127.394	9.179	5.069	54.857	1.673	32.773	γ 2.040,1.990	γ 37.082	
Phe ¹⁰⁴	126.880	8.799	4.499	57.649	2.710 2.349	40.489			
Asn ¹⁰⁵	124.868	8.439	5.041	52.664	2.810 2.582	40.310			δ 114.087 7.611,7.158
Gly ¹⁰⁶	112.851	8.670		44.680					
Asp ¹⁰⁷			4.850	55.025	3.058 2.885	41.666			
Thr ¹⁰⁸	113.339	7.817	5.331	61.444	4.103	72.633	γ 1.172	γ 21.471	
Ile ¹⁰⁹	124.541	8.946	4.716	59.946	0.996	42.050	γ ¹ 0.894,0.189 γ ² 0.172; δ -0.237	γ ¹ 28.031; γ ² 17.876; δ 13.009	
Thr ¹¹⁰	121.427	8.762	4.912	61.142	3.835	70.779	γ 0.989	γ 21.760	
Asn ¹¹¹	128.541	9.461	5.761	52.615	2.767 1.774	44.443			
Thr ¹¹²	124.208	9.120	5.158	61.642	3.965	70.575	γ 1.124	γ 21.166	

Table 3.6 -----Continued.

Spin	¹⁵ N	¹ HN	¹ H ^α	¹³ C ^α	¹ H ^β	¹³ C ^β	Other ¹ H	Other ¹³ C	NH ₂ & NH (sc)
Met ¹¹³	128.147	9.322	5.509	54.754	1.792 1.527	36.534	γ 2.497,2.266	γ 34.067	
Thr ¹¹⁴	119.031	8.919	5.325	61.761	3.984	71.021	γ 1.170	γ 21.213	
Leu ¹¹⁵	131.345	9.006	4.690	54.298	1.703 1.253	45.136	γ 1.355; δ ¹ 0.483; δ ² 0.695	γ 27.743; δ ¹ 26.011; δ ² 22.870	
Gly ¹¹⁶	119.118	9.181	3.953 3.624	47.543					
Asp ¹¹⁷	127.197	8.761	4.631	54.323	2.770 2.694	41.182			
Ile ¹¹⁸	123.549	8.343	4.105	61.861	2.232	39.012	γ ¹ 1.723,1.089; γ ² 1.008; δ 0.798	γ ¹ 27.392; γ ² 18.181; δ 13.902	
Val ¹¹⁹	128.290	8.485	4.796	61.682	1.997	32.776	γ ¹ 0.745; γ ² 0.892	γ ¹ 21.744; γ ² 20.636	
Tyr ¹²⁰	131.928	9.335	5.445	54.441	2.660 1.978	39.896			
Lys ¹²¹	131.958	8.347	5.246	54.428	1.679 1.517	37.055	γ 1.308,1.131; δ 1.530,1.653; ε 2.726	γ 25.418; δ 29.483; ε 41.752	
Leu ¹²²	125.570	8.924	5.133	54.177	1.701 1.563	46.410	γ 1.645; δ ¹ 0.790, δ ² 0.797 γ ² 0.880	γ 28.970; δ ¹ 27.061 δ ² 25.772 γ ² 21.025	
Val ¹²³	124.102	8.579	4.920	61.567	2.044	34.251			
Ala ¹²⁴	131.463	9.783	5.296	50.145	1.069	22.622			
Lys ¹²⁵	121.254	8.574	5.477	54.116	1.925 1.788	36.705	γ 1.568,1.515; δ 1.712; ε 2.985	γ 24.863; δ 29.152; ε 41.998	
Arg ¹²⁶	127.599	8.909	4.386	57.410	1.765	31.695	γ 1.587,1.452; δ 3.412,3.132	γ 27.169; δ 43.639	
Ile ¹²⁷	130.913	8.550	4.243	63.014	1.905	40.623	γ ¹ 1.239,0.840; γ ² 0.943; δ 0.828	γ ¹ 27.141; γ ² 18.762; δ 14.518	

Table 3.7 ^1H , ^{15}N and ^{13}C chemical shifts for a complex of R122L/S124A-LFABP with oleate (1:2) at pH 7.0 and 10 °C.

Spin	^{15}N	^1HN	$^1\text{H}^\alpha$	$^{13}\text{C}^\alpha$	$^1\text{H}^\beta$	$^{13}\text{C}^\beta$	Other ^1H	Other ^{13}C
Asn ²			4.812	52.150	2.809 2.543	40.566		
Phe ³	131.167	11.589	4.321	56.697	3.034 2.311	38.836		
Ser ⁴	117.248	8.757	4.417	61.301	4.124 3.972	64.571		
Gly ⁵	112.080	9.112	3.977 3.882	45.312				
Lys ⁶	122.656	8.364	5.277	55.402	1.652 1.652	34.794	γ 1.315,1.113; δ 1.610; ϵ 2.831	γ 25.655; δ 29.797; ϵ 42.163
Tyr ⁷	121.783	9.147	5.292	55.975	2.533 2.415	42.134		
Gln ⁸	123.184	9.201	5.380	53.859	1.914	33.213	γ 2.398,2.202	γ 34.018
Val ⁹	130.885	8.987	3.682	66.469	1.979	33.449	γ^1 0.993; γ^2 1.119	γ^1 21.643; γ^2 23.848
Gln ¹⁰	126.319	10.014	4.545	55.992	2.153 1.940	31.922	γ 2.404,2.404	γ 34.598
Ser ¹¹	113.204	7.950	4.750	57.517	3.953 3.833	65.339		
Gln ¹²	117.059	8.651	5.460	55.295	2.380 2.276	33.160	γ 2.487	γ 34.465
Glu ¹³	121.119	9.401	4.865	55.431	2.136	34.106	γ 2.289,2.289	γ 36.462
Asn ¹⁴	117.678	9.349	4.504	54.740	3.730 3.215	36.549		
Phe ¹⁵	119.963	8.556	3.602	62.777	3.000	40.551		
Glu ¹⁶	117.201	9.673	3.809	62.411	2.179	27.151	γ 2.316	γ 37.829
Pro ¹⁷			4.249	65.490			γ 2.104; δ 3.681,3.564	γ 28.173; δ 42.619
Phe ¹⁸	119.562	7.314	3.890	62.617	2.804 2.684	40.692		
Met ¹⁹	117.091	8.373	4.033	56.720	1.685 1.386	31.279	γ 1.749, 1.525	γ 32.610
Lys ²⁰	121.249	8.319	3.949	59.712	1.769	32.449	γ 1.487,1.326; δ 1.627; ϵ 2.906	γ 25.708; δ 29.279; ϵ 41.906
Ala ²¹	124.812	7.565	4.014	54.861	1.313	18.087		
Met ²²	116.202	7.596	4.191	55.037	2.213 2.072	32.398	γ 1.896,1.819	
Gly ²³	107.670	7.652	4.110 3.676	45.455				
Leu ²⁴	124.992	7.693	4.401	54.059	1.367 1.210	42.141	γ 1.633; δ^1 0.837; δ^2 0.691	γ 27.141; δ^1 25.500; δ^2 24.407
Pro ²⁵			4.450	63.148	2.450 2.078	32.938	γ 2.122,2.122; δ 3.995,3.497	γ 28.047; δ 51.017
Glu ²⁶	123.972	8.833	3.858	60.150	2.099 2.035	29.951	γ 2.339,2.339	γ 36.362
Asp ²⁷	117.333	8.964	4.362	57.142	2.645 2.614	39.999		
Leu ²⁸	120.650	7.211	4.196	57.579	1.805 1.586	41.725	γ 1.714; δ^1 0.963; δ^2 0.881	γ 27.478; δ^1 25.319; δ^2 23.882
Ile ²⁹	121.192	7.801	3.524	66.667	1.994	38.066	γ^1 1.661,0.383; γ^2 0.918; δ 0.662	γ^1 29.529; γ^2 17.844; δ 13.811
Gln ³⁰	116.997	8.186	3.951	58.825	2.152	28.162	γ 2.513, 2.026	γ 33.622
Lys ³¹	117.753	7.697	4.183	58.959	1.897	33.834	γ 1.654,1.508; δ 1.688; ϵ 2.958	γ 25.944; δ 29.565; ϵ 41.906
Gly ³²	106.405	8.593	3.957 3.538	47.065				

Table 3.7 -----Continued.

Spin	¹⁵ N	¹ HN	¹ H ^α	¹³ C ^α	¹ H ^β	¹³ C ^β	Other ¹ H	Other ¹³ C
Lys ³³	120.928	7.844	3.006	59.346	1.895	32.002	γ 1.460; δ 1.717,1.717	γ 23.899; δ 30.422
Asp ³⁴	118.186	7.622	4.791	54.592	2.839 2.464	42.230		
Ile ³⁵	122.206	7.277	3.962	61.441		39.746	γ ¹ 1.725,1.111	γ ¹ 28.147
Lys ³⁶	128.458	8.711	4.363	54.207	1.851	30.870	γ 1.462,1.302; δ 1.595; ε 2.969	γ 24.407; δ 28.634; ε 41.982
Gly ³⁷			3.750 3.014	45.270				
Val ³⁸	120.456	8.428	5.095	61.053	1.966	36.174	γ ¹ 0.935; γ ² 0.953	γ ¹ 21.941; γ ² 21.941
Ser ³⁹	120.974	9.356	5.597	55.633	3.621 3.595	67.198		
Glu ⁴⁰	126.921	9.308	5.272	55.621	2.140 1.913	32.811	γ 2.138,2.096	γ 36.896
Ile ⁴¹	127.588	9.786	5.125	60.612	2.226	41.063	γ ¹ 1.652,0.978; γ ² 0.792; δ 0.875	γ ¹ 29.065; γ ² 18.100; δ 13.469
Val ⁴²	131.506	9.423	4.202	63.290	2.074	33.505	γ ¹ 0.945; γ ² 0.970	γ ¹ 20.813; γ ² 20.032
His ⁴³	132.867	9.024	5.525	53.071	3.348 2.491	34.680		
Glu ⁴⁴	131.681	9.009	4.346	55.020	1.851	31.879	γ 2.046,2.046	γ 36.223
Gly ⁴⁵	118.525	8.738	3.967 3.544	47.415				
Lys ⁴⁶	126.614	9.013	4.590	56.445	2.267 1.836	33.707	γ 1.618,1.540; δ 1.770,1.770; ε 3.026	γ 25.729; δ 29.614; ε 41.472
Lys ⁴⁷	121.332	8.251	4.741	56.290	1.940	33.674	γ 1.438; δ 1.658; ε 2.715	γ 25.238; δ 29.154; ε 41.999
Val ⁴⁸	125.674	8.830	4.540	61.457	0.426	33.976	γ ¹ 0.393; γ ² 0.436	γ ¹ 22.173; γ ² 21.125
Lys ⁴⁹	126.430	8.763	4.736	55.528	1.843	34.434	γ 1.439,1.349; δ 1.645; ε 2.884	γ 25.500; δ 29.875; ε 41.906
Leu ⁵⁰	127.960	9.062	5.366	53.597	1.773 1.201	46.495	γ 1.524; δ ¹ 0.897	γ 25.553; δ ¹ 24.474
Thr ⁵¹	128.005	9.518	5.206	62.311	4.061	70.988	γ 1.051	γ 22.766
Ile ⁵²	131.088	9.231	4.973	60.922	1.696	41.352	γ ¹ 1.661, γ ² 0.949; δ ¹ 0.787	γ ¹ 28.782; γ ² 17.889 δ ¹ 13.925
Thr ⁵³	124.927	9.252	5.130	62.439	3.952	70.415	γ 1.102	γ 21.272
Tyr ⁵⁴	132.690	9.536	4.538	57.120	3.195	41.188		
Gly ⁵⁵	115.276	8.816	4.104 3.599	47.365				
Ser ⁵⁶	124.529	9.393	4.382	59.453	4.034 3.886	64.221		
Lys ⁵⁷	125.934	8.168	4.525	55.506	1.970	32.990	γ 1.432,1.432; δ 1.731; ε 2.967	γ 25.217; δ 28.698; ε 42.499
Val ⁵⁸	126.493	8.357	4.984	61.139	1.856	34.437	γ ¹ 0.637; γ ² 0.885	γ ¹ 21.120; γ ² 21.125
Ile ⁵⁹	126.249	9.181	4.532	60.098	1.692	41.593	γ ¹ 1.727,1.400; γ ² 0.752; δ 0.793	γ ¹ 27.688; γ ² 18.276 δ 13.469
His ⁶⁰	130.402	9.427	5.257	56.043	3.147 2.988	32.709		
Asn ⁶¹	122.601	8.617	5.218	53.262	2.525 2.525	45.473		
Glu ⁶²	121.750	9.029	5.203	55.649	2.025	32.570	γ 2.167,1.995	γ 36.449
Phe ⁶³	120.616	8.643	5.088	55.816	3.269	41.517		
Thr ⁶⁴	118.891	8.879	5.017	61.562	3.918	70.240	γ 1.213	γ 21.917
Leu ⁶⁵	130.405	9.694	4.463	57.134	1.897 1.666	42.231	δ ¹ 0.978	δ ¹ 26.494

Table 3.7 -----Continued.

Spin	¹⁵ N	¹ HN	¹ H ^α	¹³ C ^α	¹ H ^β	¹³ C ^β	Other ¹ H	Other ¹³ C
Gly ⁶⁶	110.674	9.585	4.405 3.450	45.772				
Glu ⁶⁷	120.168	7.985	4.807	54.515	2.162 1.955	32.517	γ 2.221	γ 36.438
Glu ⁶⁸	125.416	9.013	4.453	57.937	2.028 1.959	30.516	γ 2.165	γ 37.900
Cys ⁶⁹	124.361	9.419	5.176	56.367	3.453 3.107	32.406		
Glu ⁷⁰	120.233	8.479	5.034	55.721	1.890	32.215	γ 2.133,1.976	γ 38.081
Leu ⁷¹	126.955	9.160	4.613	53.243	1.193 0.847	44.311	γ 1.365; δ ¹ 0.223; δ ² 0.457	γ 26.594; δ ¹ 25.232; δ ² 23.313
Glu ⁷²	126.115	9.901	4.812	55.866	1.948	31.288	γ 2.441,2.054	γ 36.438
Thr ⁷³	113.703	8.152	4.353	60.654	4.523	69.634	γ 1.108	γ 21.672
Met ⁷⁴								
Thr ⁷⁵	108.942	7.859	4.347	61.064	4.527	69.554	γ 1.163	γ 22.252
Gly ⁷⁶	110.976	7.742	4.362 3.860	45.351				
Glu ⁷⁷	122.181	7.437	4.201	56.475	1.815 1.815	30.641	γ 2.245,2.106	γ 36.428
Lys ⁷⁸	124.549	8.639	5.353	54.871	1.766 1.528	33.543	γ 1.518,1.304; δ 1.608; ε 2.968	γ 24.953; δ 29.212; ε 41.906
Val ⁷⁹	120.041	9.105	4.667	59.117	2.115	35.018	γ ¹ 0.634; γ ² 0.859	γ ¹ 19.732; γ ² 22.219
Lys ⁸⁰	124.327	8.535	5.085	56.131	1.812 1.701	33.020	γ 1.549,1.398; δ 1.652; ε 2.946	γ 24.953; δ 28.782; ε 41.906
Ala ⁸¹	127.119	8.963	4.839	51.489	1.209	23.704		
Val ⁸²	118.874	8.395	4.232	62.335	1.919	34.623	γ ¹ 0.703; γ ² 0.854	γ ¹ 20.891; γ ² 21.043
Val ⁸³	134.258	9.525	4.520	61.220	1.593	32.175	γ ¹ -0.122; γ ² 0.575	γ ¹ 18.267; γ ² 22.081
Lys ⁸⁴	125.997	9.043	4.937	54.709	1.773	36.823	γ 1.363; δ 1.664; ε 2.947	γ 25.460; δ 29.328
Met ⁸⁵	120.631	8.561	5.441	54.082	2.825 2.626	32.139	γ 2.367,2.015	γ 31.516
Glu ⁸⁶	127.286	8.790	4.557	56.148	1.775	32.466	γ 2.122,2.085	γ 36.852
Gly ⁸⁷	114.182	8.622	4.196 3.775	45.727				
Asp ⁸⁸	119.300	8.699	4.755	55.438	2.715 2.715	41.972		
Asn ⁸⁹	115.048	8.142	5.183	53.314	3.786 2.802	40.113		
Lys ⁹⁰	118.024	7.390	6.004	54.861	2.240 1.512	36.386	γ 1.486,1.123; δ 1.516; ε 2.853	γ 25.339; δ 29.875; ε 40.813
Met ⁹¹	120.450	8.962	5.279	54.336		32.286	γ 2.442,2.113	γ 33.703
Val ⁹²								
Thr ⁹³			4.841	61.788	4.030	69.814	γ 0.936	γ 18.176
Thr ⁹⁴	118.691	8.220	5.520	60.003	4.026	71.505	γ 1.080	γ 20.414
Phe ⁹⁵	122.457	8.194	4.834	56.796	3.259 2.785	39.757		
Lys ⁹⁶	119.692	9.579	3.907	57.611	2.126 1.842	30.887	γ 1.601; δ 1.653,1.766; ε 2.992	γ 25.374; δ 28.235; ε 40.648
Gly ⁹⁷	105.573	8.464	4.009 3.518	45.584				
Ile ⁹⁸	123.566	8.420	4.382	60.497	1.537	39.810	γ ¹ 1.470,0.819; γ ² 0.729; δ 0.836	γ ¹ 28.959; γ ² 18.655; δ 14.563
Lys ⁹⁹	126.472	8.109	4.860	55.513	1.857 1.692	34.519	γ 1.340,1.246; δ 1.614; ε 2.855	γ 25.435; δ 29.373; ε 41.707

Table 3.7 -----Continued.

Spin	¹⁵ N	¹ HN	¹ H ^α	¹³ C ^α	¹ H ^β	¹³ C ^β	Other ¹ H	Other ¹³ C
Ser ¹⁰⁰	123.460	9.192	5.442	56.738	3.744 3.237	64.977		
Val ¹⁰¹	130.631	9.030	4.639	62.273	2.019	34.615	γ ¹ 0.830; γ ² 0.936	γ ¹ 21.305; γ ² 21.055
Thr ¹⁰²	130.335	9.519	4.778	61.850	3.807	70.036	γ 0.515	γ 23.860
Glu ¹⁰³	127.202	9.128	5.035	54.796	2.018	32.584	γ 2.119, 1.984	γ 36.984
Phe ¹⁰⁴	126.432	8.803	4.498	57.841	2.950 2.699	40.250		
Asn ¹⁰⁵	124.528	8.469	5.024	52.692	2.789 2.568	40.176		
Gly ¹⁰⁶	113.860	8.574	4.227 3.572	47.839				
Asp ¹⁰⁷	127.886	8.938	4.840	55.111	3.042 2.855	41.527		
Thr ¹⁰⁸	113.504	7.861	5.311	61.477	4.091	72.534	γ 1.160	γ 21.758
Ile ¹⁰⁹	123.451	8.896	4.702	59.738	0.976	41.985	γ ¹ 0.860, 0.176; γ ² 0.150; δ -0.277	γ ¹ 28.235; γ ² 18.003; δ 13.302
Thr ¹¹⁰	120.619	8.680	5.156	60.978	3.947	70.915	γ 1.086	γ 21.311
Asn ¹¹¹	127.345	9.209	5.731	52.758	2.715 1.749	44.189		
Thr ¹¹²	123.140	8.874	4.873	61.649	3.819	70.661	γ 0.978	γ 22.019
Met ¹¹³	127.979	9.211	5.464	55.029	1.908 1.780	36.449	γ 2.458, 2.302	γ 33.156
Thr ¹¹⁴	119.067	8.845	5.302	62.008	3.969	71.172	γ 1.160	γ 21.758
Leu ¹¹⁵	131.430	9.051	4.675	54.297	1.684 1.236	44.856	γ 1.334; δ ¹ 0.477; δ ² 0.684	γ 27.688; δ ¹ 26.163; δ ² 22.814
Gly ¹¹⁶	119.088	9.166	3.929 3.612	47.662				
Asp ¹¹⁷	127.292	8.763	4.615	54.268	2.739 2.685	41.197		
Ile ¹¹⁸	123.603	8.357	4.092	61.929	2.210	39.014	γ ¹ 1.702, 1.079; γ ² 0.783; δ 0.993	γ ¹ 27.141; γ ² 14.094; δ 18.284
Val ¹¹⁹	128.143	8.490	4.793	61.628	1.953	33.176	γ ¹ 0.875; γ ² 0.732	γ ¹ 20.846; γ ² 21.865
Tyr ¹²⁰	131.390	9.241	5.448	54.398	2.637 1.971	39.457		
Lys ¹²¹	132.534	8.268	5.249	54.360	1.682 1.502	37.030	γ 1.301, 1.106; δ 1.515; ε 2.713	γ 24.953; δ 29.636; ε 41.359
Leu ¹²²	124.174	8.900	5.108	54.192	1.656 1.545	46.500	γ 1.643; δ ¹ 0.777, δ ² 0.771	γ 27.669; δ ¹ 26.047 δ ² 26.875
Val ¹²³	123.314	8.576	4.893	61.483	2.030	34.493	γ ² 0.944	γ ² 20.855
Ala ¹²⁴	131.131	9.737	5.276	50.168	1.051	22.683		
Lys ¹²⁵	120.844	8.539	5.466	54.062	1.907 1.780	36.770	γ 1.512; δ 1.699; ε 2.958	γ 24.971; δ 29.182; ε 41.906
Arg ¹²⁶	127.226	8.863	4.400	57.376	1.758	31.692	γ 1.579, 1.449; δ 3.390, 3.104	γ 27.270; δ 43.709
Ile ¹²⁷	130.805	8.581	4.227	62.969	1.890	40.660	γ ¹ 1.222, 0.822; γ ² 0.928; δ 0.813	γ ¹ 26.594; γ ² 18.812; δ 14.694

Chemical Shift Index (CSI)

The chemical shift index (CSI) is a quick and simple protocol for determining secondary structure in proteins from the chemical shifts of ^1H and ^{13}C nuclei. It is based on methods developed originally by Wishart et al. for the analysis of $^1\text{H}^\alpha$ chemical shifts (Wishart et al., 1992). By extending the chemical shift index to include $^{13}\text{C}^\alpha$, $^{13}\text{C}^\beta$ and ^{13}CO chemical shifts, it is now possible to use four independent chemical-shift measurements to identify and locate protein secondary structures. There is a strong correlation between chemical shift and secondary structure. Both $^{13}\text{C}^\alpha$ and ^{13}CO produce an upfield shift when they are in β -sheets and downfield shift when they are in α -helices (Siato, 1986; Wishart et al., 1991). In contrast, $^1\text{H}^\alpha$ and $^{13}\text{C}^\beta$ experience a downfield shift when they are in β -sheets and an upfield shift when they are in α -helices (Spera and Bax, 1991). Thus, the chemical shifts of H^α , C^α , C^β and CO are compared with their random coil range values and then assigned an index of 1 if they have a downfield shift beyond this range and -1 with upfield shifts (Wishart et al., 1992). By combining three or more types of shifts for a particular residue, a consensus estimate of secondary structure for the protein of interest can be calculated. In general, if a stretch of -1 values is observed for a region in the consensus plot, a helix is predicted for this region of the protein. Conversely, if a region displays +1 indices, a beta strand is predicted.

The backbone assignment of $^1\text{H}\alpha$, $^{13}\text{C}\alpha$, and $^{13}\text{C}\beta$ nuclei for the apo form and holo form (complexed with oleate) of R122L/S124A-LFABP were used to derive their secondary structural elements employing the chemical shift index program. Both forms of the mutant LFABP displayed similar secondary structure as wild-type LFABP, with 10 β -strands and 2 α -helices spanning the region between β -strand A and B (Figures 3.17A

and 3.18A). The gaps between each structural element represent the loops connecting each of the 10 β -strands and the 2 α -helices in the three dimensional structure. The average of all three types of shifts was calculated generating the consensus secondary structure for the mutant protein (Figures 3.17 and 3.18). The chemical shift-derived secondary structure also serves as an important restraint on the interpretation of otherwise ambiguous NOEs used in the initial stage of tertiary structure determination.

Since the K31L-LFABP mutant has strikingly similar backbone amide peak positions in both apo and holo forms, its structures in these two forms as well as their resonances must be similar as well. Thus a detailed resonance analysis and structural calculation have not been pursued for the K31L-LFABP.

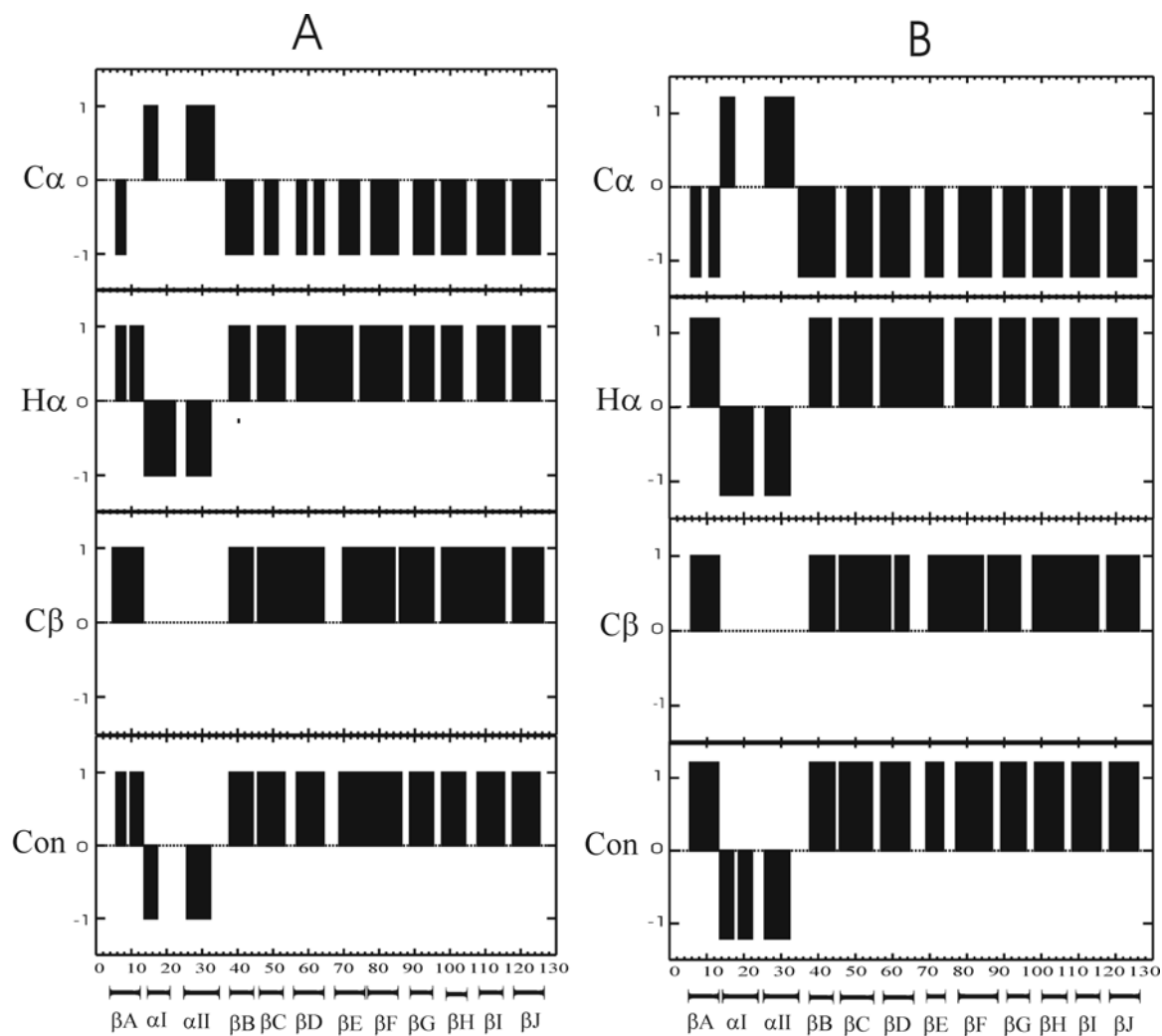


Figure 3.17 Chemical shift index (CSI). (A) apo R122L/S124A LFABP and (B) apo wild-type LFABP.

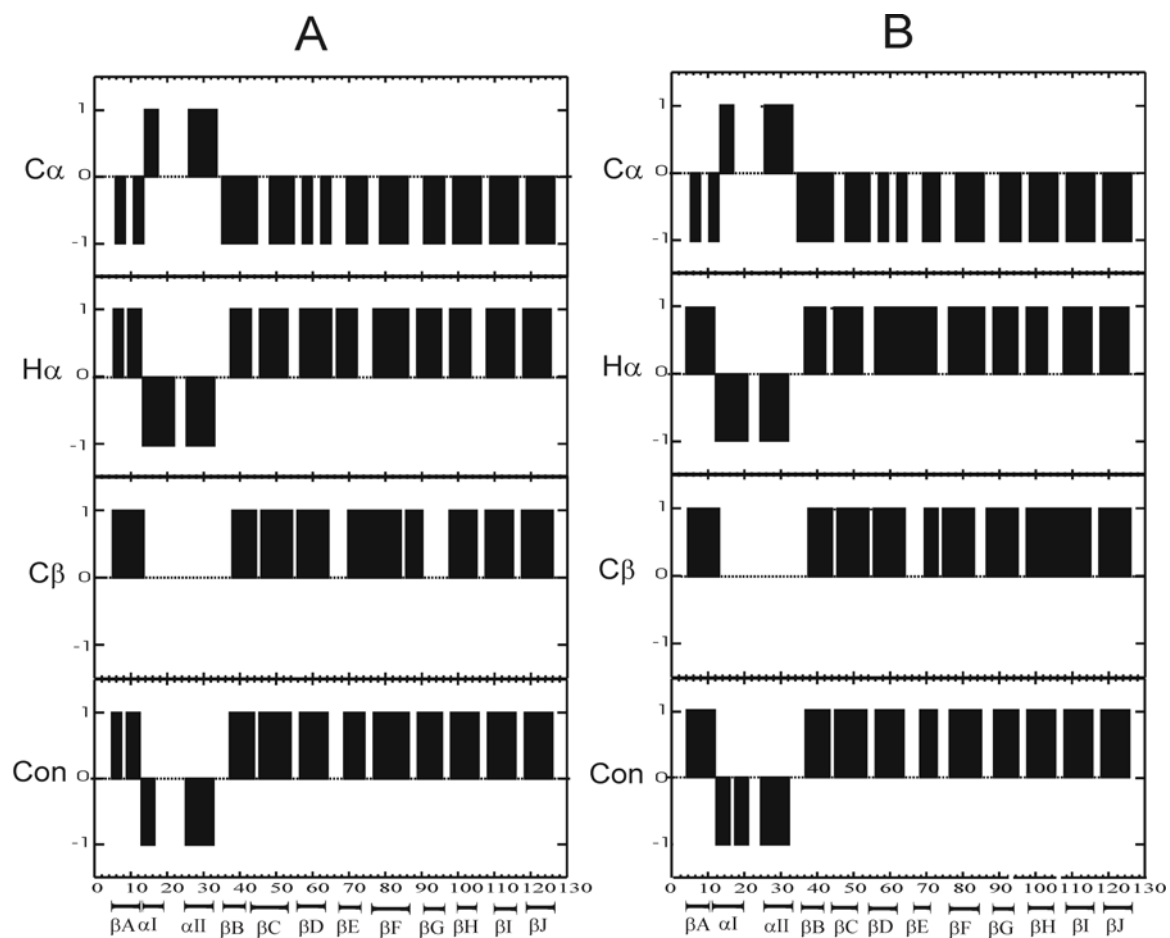


Figure 3.18 Chemical shift index (CSI). (A) holo R122L/S124A LFABP and (B) holo wild-type LFABP.

3D/4D-NOESY assignments

Cross peaks in NOESY spectra provide important structural information for biomolecules in solution. Typically a NOE is observed between any set of hydrogen atoms if they are located at a distance shorter than 5.0 Å from each other. In order to determine the specific distance constraint between these hydrogen atoms, first the sequence-specific resonance assignment has to be complete, and then the distance constraints can be specified for specific sites along the protein chain and accordingly the three-dimensional structure can be calculated.

The following three- and four-dimensional NMR experiments were performed to establish the structure: NOESY3DC (NOE interaction between a bonded C-H and any other proton in proximity), NOESY3DN (NOE interaction between a bonded N-H and any other proton in proximity), NOESY4DCC (NOE interaction between C-H and C-H), and NOESY4DCN (NOE interaction between C-H and N-H) (*Reid, 1997; Wang et al., 1998*). Extending the three-dimensional NOESY into a fourth dimension by employing isotope-editing has reduced markedly the problem of overlap resulting from proton chemical shift degeneracy and thus allowed for confident assignments.

The intensity of the NOE, corresponding to the volume of the cross peak in a NOESY spectrum, is related to the distance between the two interacting spins (*Kumar et al., 1980*). Secondary structures can also be characterized by regular patterns of NOEs (*Wuthrich, 1986*). Thus the sequential and medium-range interproton distances derived from the NOESY data set can corroborate with the CSI results and confirm the elements of regular secondary structure in apo R122L/S124A-LFABP, in holo R122L/S124A-LFABP, and in apo K31L-LFABP as illustrated in Figures 3.19, 3.20, and 3.21,

respectively. The apo and holo forms of R122L/S124A-LFABP clearly show the presence of two α -helical regions ($d_{\alpha N}(i, i+3)$, $d_{\alpha\beta}(i, i+3)$) spanning residues 14 to 36. Due to the lack of detailed assignment effort for K31L-LFABP the $d_{\alpha\beta}(i, i+3)$ information is not available. But the $d_{\alpha N}(i, i+3)$ in the α II-helix region suggests that the helix structure remains in this segment. However, both mutants display a strong $d_{\alpha N}(i, i\pm 1)$ but slightly weaker $d_{NN}(i, i+1)$ and $d_{\beta N}(i, i+1)$ connectivities throughout the protein's sequence, indicating β -strands.

For a less localized but more comprehensive view of the structural changes occurring in the mutants, the β -sheet network was deduced from backbone NOEs (Figures 3.22, 3.23, and 3.24). Generally speaking, there were no significant changes in the β -sheet structure of apo K31L-LFABP mutant compared to that of the apo wild-type protein (Figures 3.22). Upon analogous examination of apo R122L/S124A-LFABP (Figure 3.23), the majority of NOEs throughout the β -sheet were similar to that of wild-type protein, but there were two main regions in which the NOEs were absent. One region that had extensive NOEs in the wild-type protein but none in the R122L/S124A-LFABP mutant was located between β -strand E (residues 70, 71, and 72) and β -strand F (residues 78 and 79). Another was located between β -strand G (residues 94 and 95) and β -strand H (residues 98, 99, and 100). In addition, the number of NOEs between the β -strands of holo R122L/S12A-LFABP were similar to those of holo wild-type protein in the majority of the β -sheet, but again some regions, particularly between β G and β H, displayed fewer NOEs. It is noted with interest that V92, T93 crosspeaks in HSQC spectrum have not been located. This could result in incomplete NOE assignments in that

region of the preliminary structure, so the preliminary structure (see below) is currently being used to make additional assignments and permit further structural refinement.

Distance restraints for apo and holo R122L/S124A-LFABP were classified using the DYANA program (Figures 3.25 and 3.26, respectively). A combined total of 2149 NOE restraints obtained from the 3D and 4D NOESY datasets were used as input files to the distance calibration for apo R122L/S124A-LFABP. Fewer NOE restraints (2032) were available for holo-R122L/S124A-LFABP due to incomplete NOE assignments, but additional NOE identifications will be forthcoming as more ARIA/CNS calculations are carried out. The numbers of distance restraints classified as intraresidue, sequential, medium-range, and long-range are shown as a function of protein sequence for apo- and holo-R122L/S124A-LFABP in Figures 3.25 B and 3.26B, respectively.

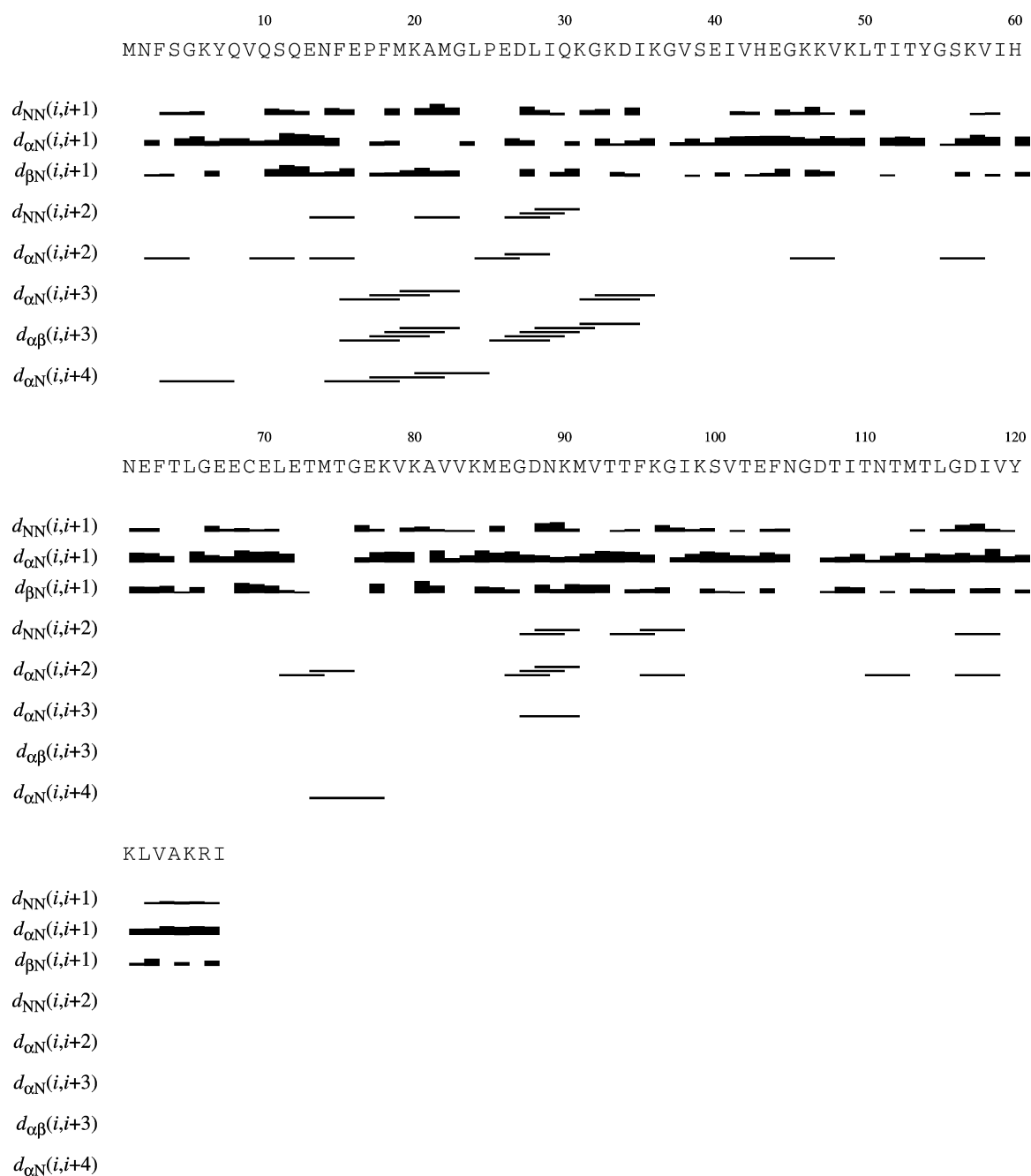


Figure 3.19 Amino acid sequence and summary of NOEs used to define the sequential and medium-range NOEs and to identify elements of secondary structure in apo R122L/S124A-LFABP. Plots were obtained from the DYANA program.

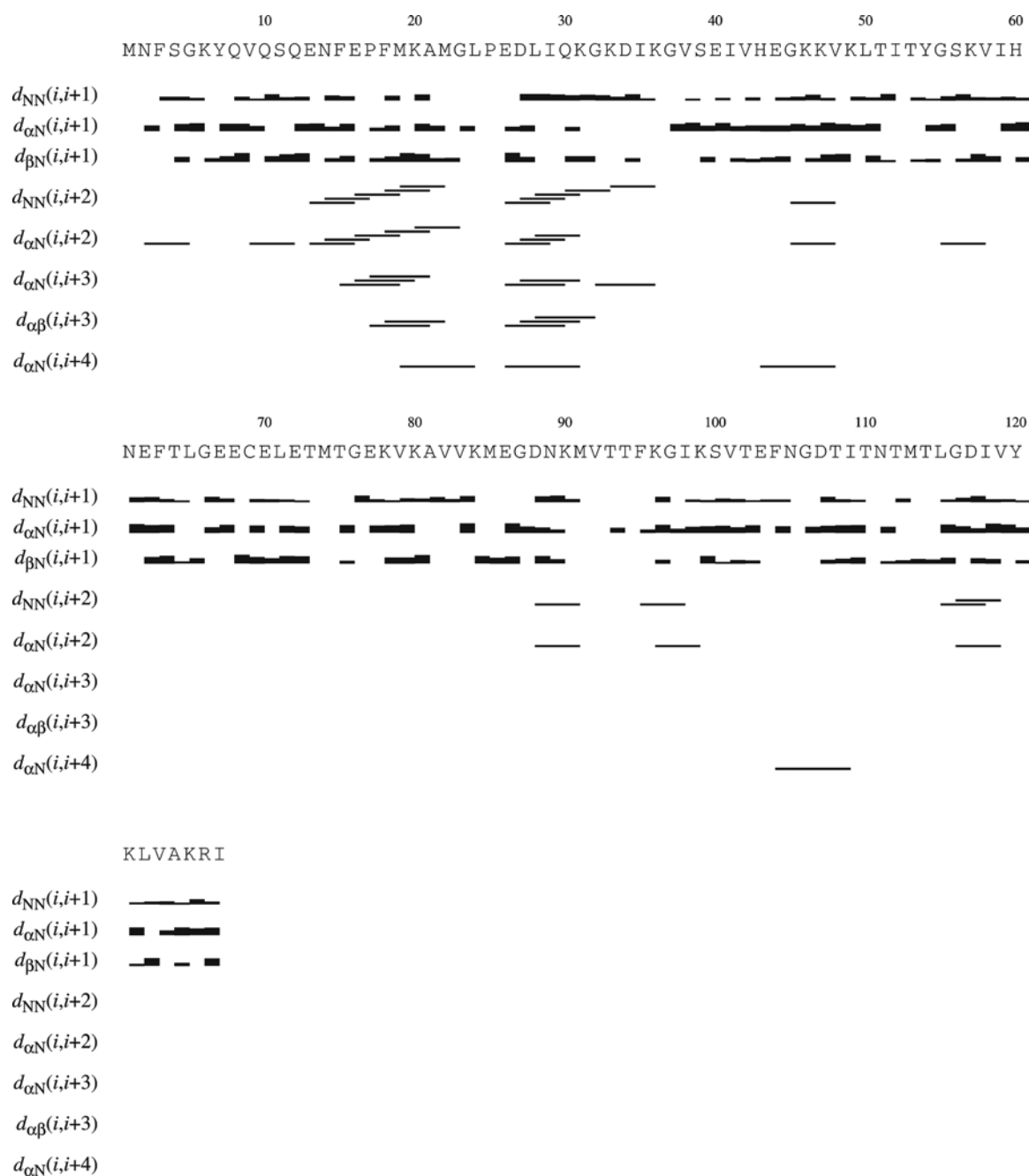


Figure 3.20 Amino acid sequence and summary of NOEs used to define the sequential and medium-range NOEs and to identify elements of secondary structure in holo R122L/S124A-LFABP. Plots were obtained from the DYANA program.

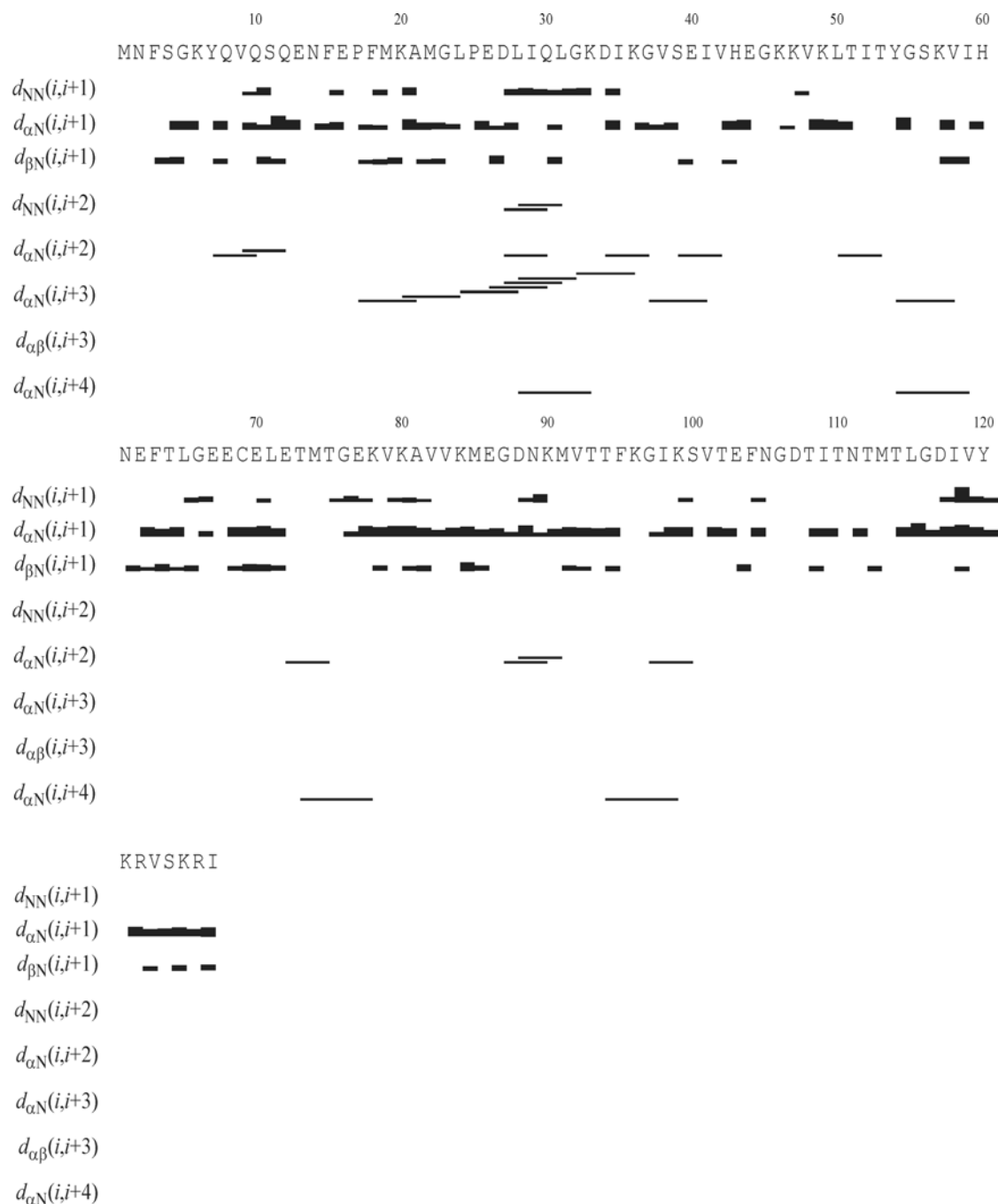


Figure 3.21 Amino acid sequence and summary of NOEs used to define the sequential and medium-range NOEs and to identify elements of secondary structure in apo K31L-LFABP. Plots were obtained from the DYANA program.

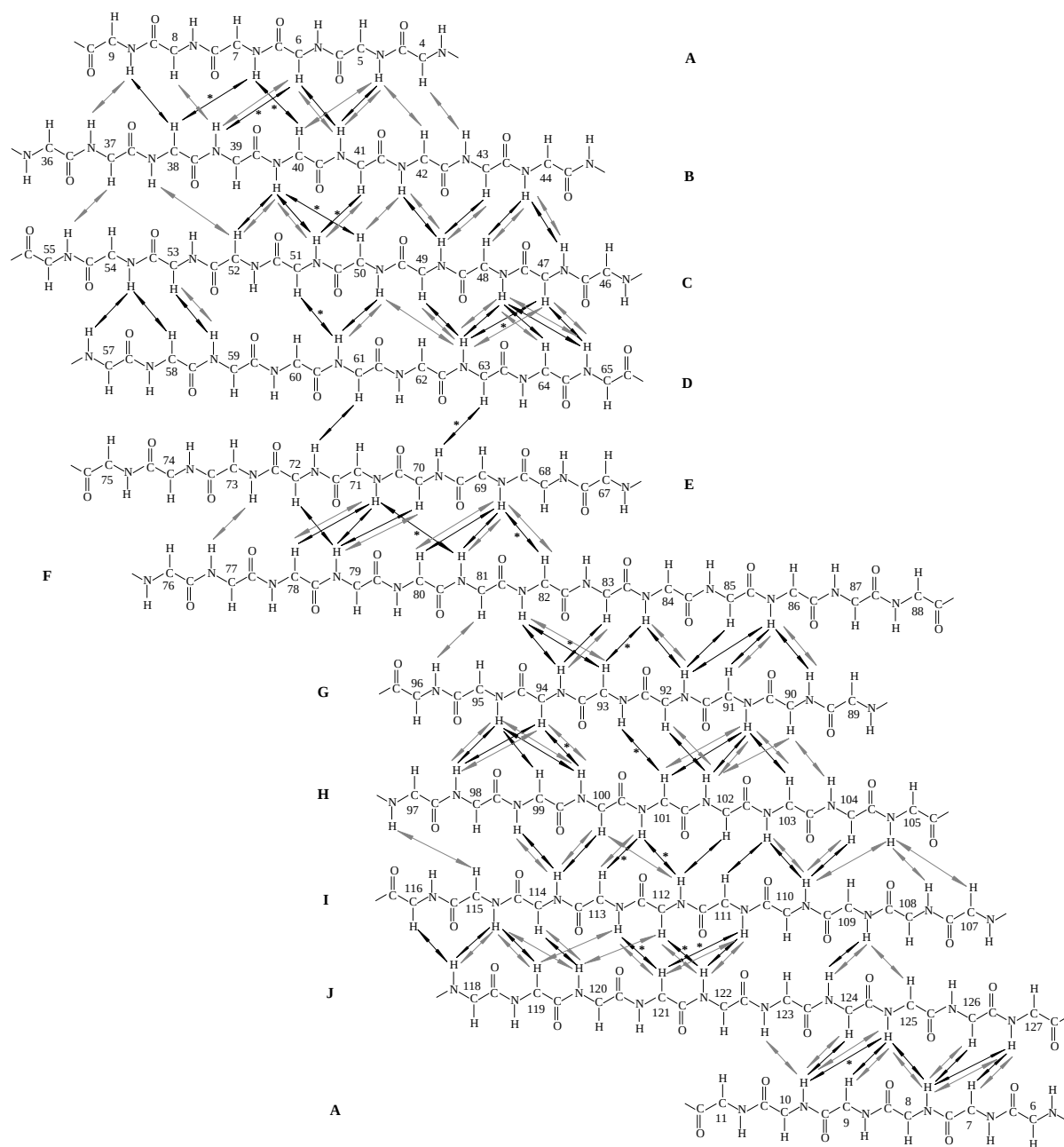


Figure 3.22 Interstrand β -sheet (β A- β J) hydrogen-bonding network between pairs of proton spins assembled from backbone NOE connectivities derived from the NOESY-HSQC (^{15}N) spectrum. ($\leftrightarrow$ for apo-LFABP, $\rightarrow$ for apo-K31L-LFABP).

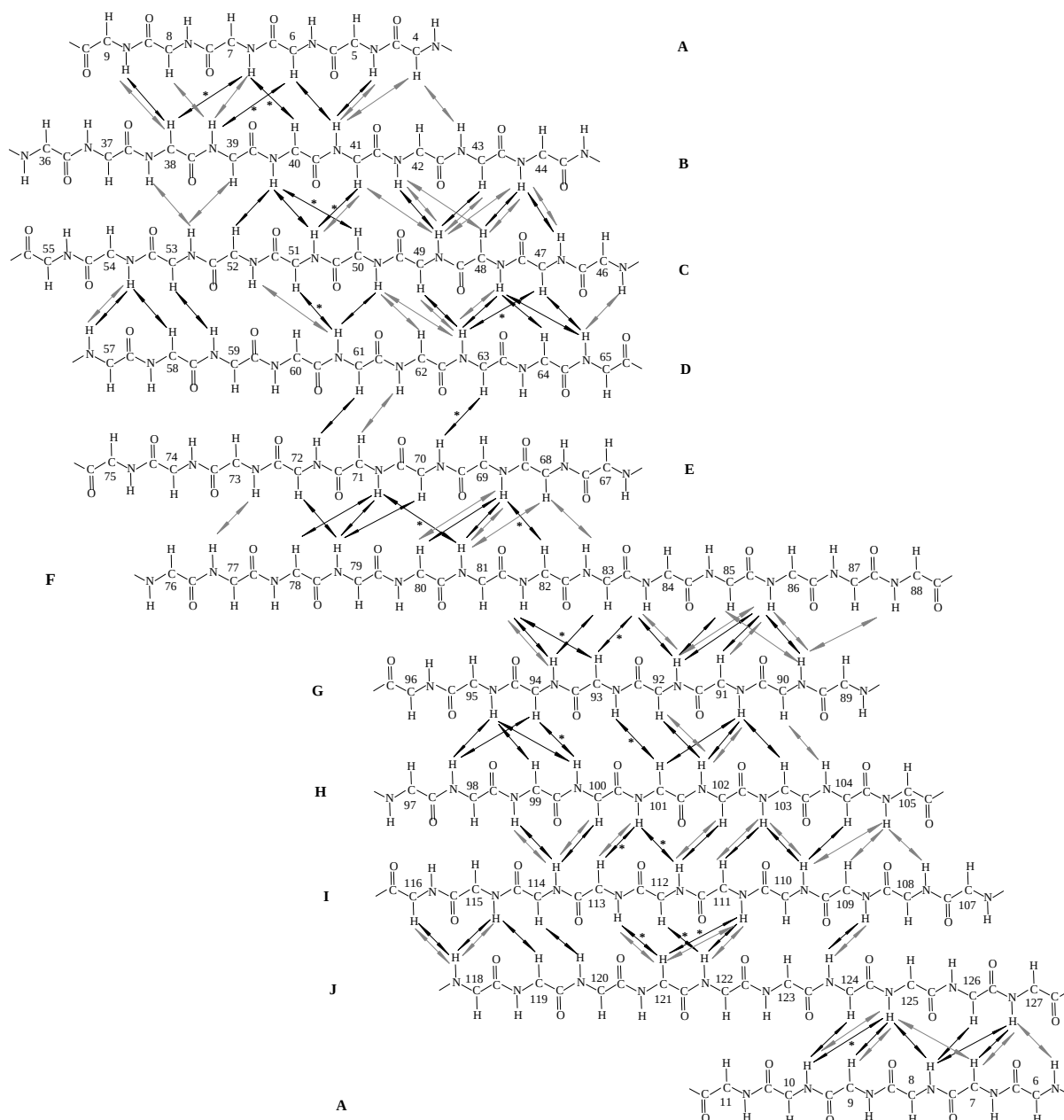


Figure 3.23 Interstrand β -sheet (β A- β J) hydrogen-bonding network between pairs of proton spins assembled from backbone NOE connectivities derived from the NOESY-HSQC (^{15}N) spectrum. ($\leftrightarrow$ for apo-LFABP, $\leftrightarrow$ for apo-R122L/S124A-LFABP).

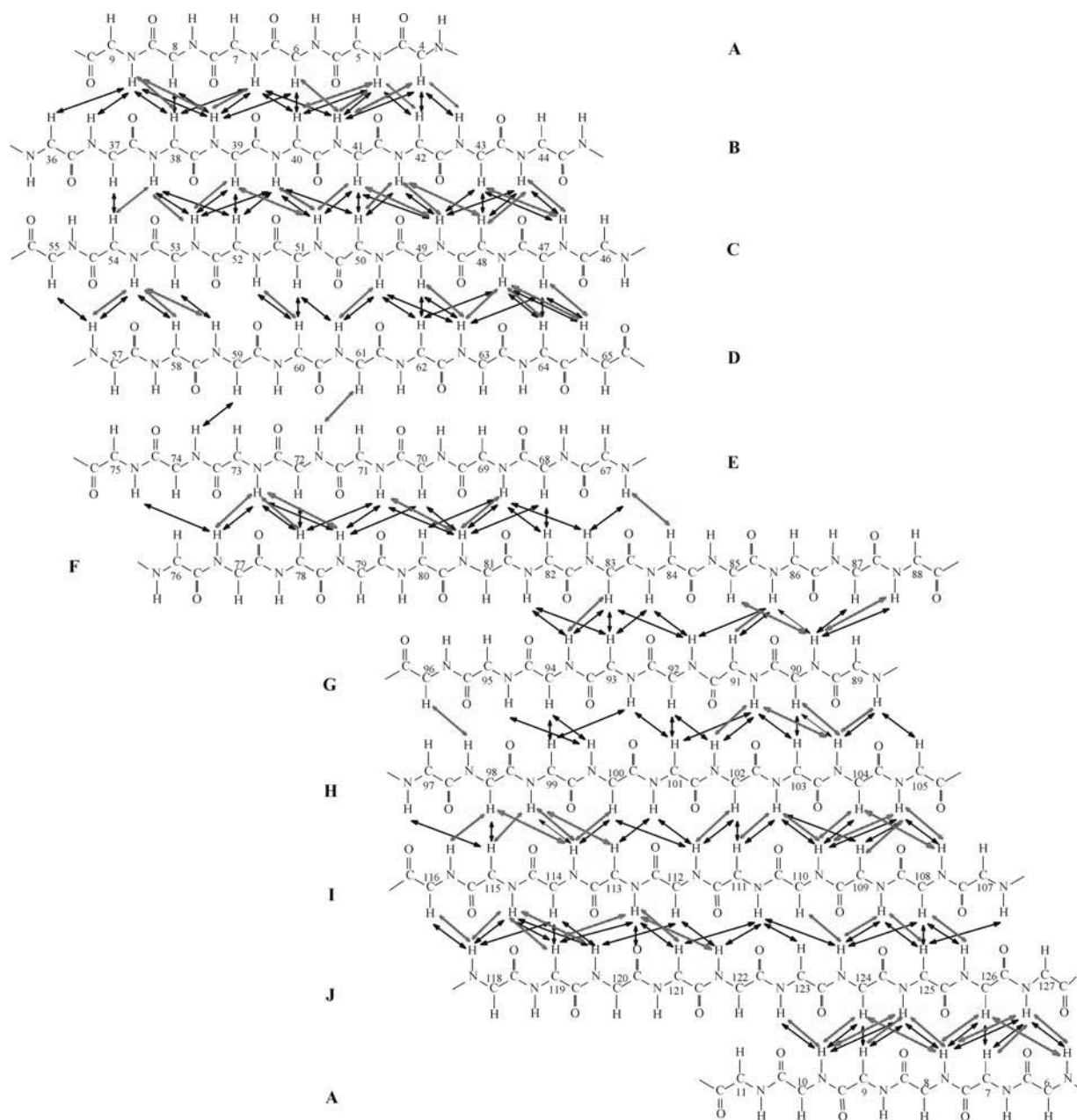


Figure 3.24 Interstrand β -sheet (β A- β J) hydrogen-bonding network between pairs of proton spins assembled from backbone NOE connectivities derived from the NOESY-HSQC (^{15}N) spectrum. ($\leftrightarrow$ for holo-LFABP, $\leftrightarrow$ for holo-R122L/S124A-LFABP).

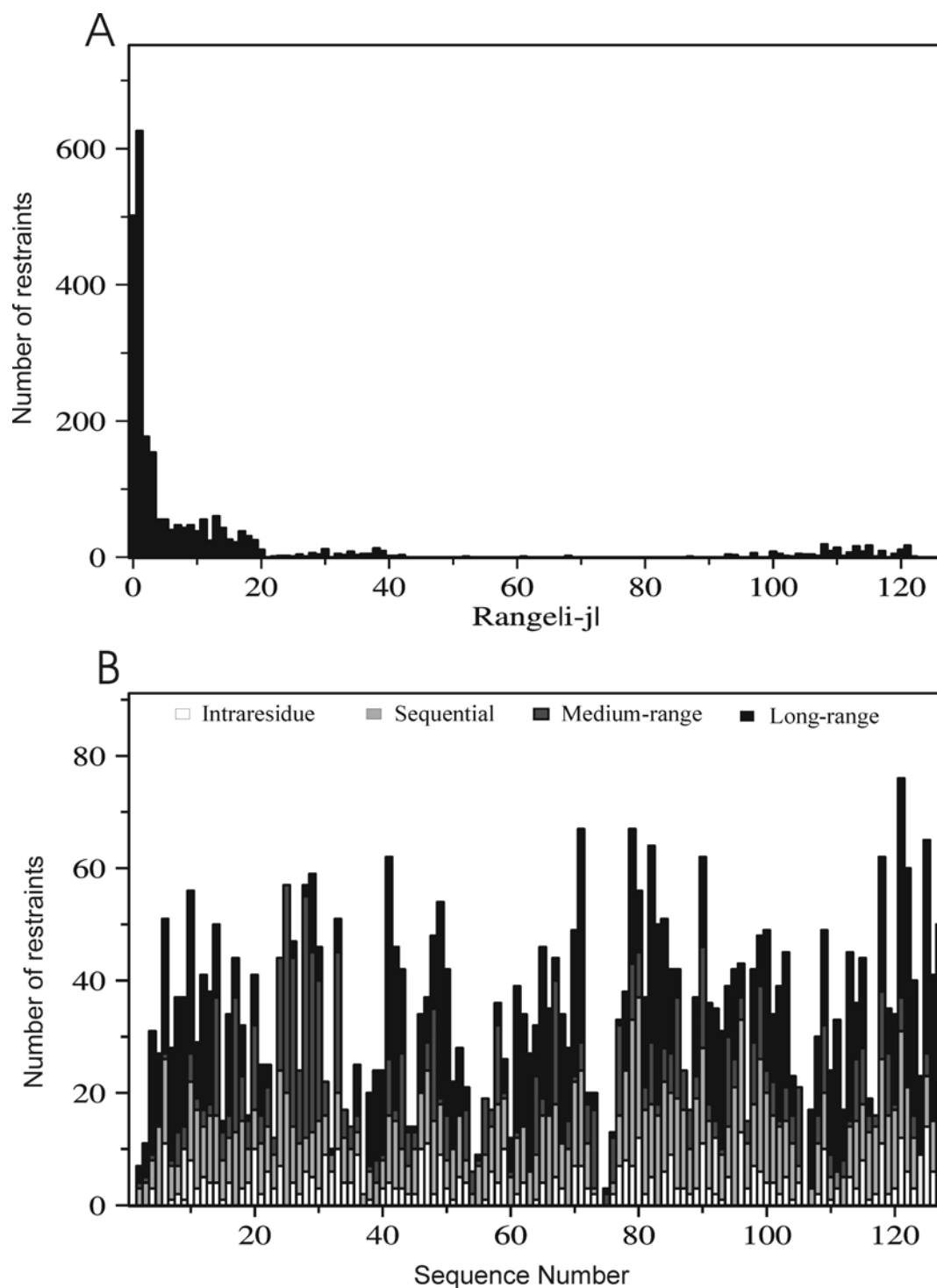


Figure 3.25 Distribution of the distance restraints used to obtain the final 20 solution structures for apo R122L/S124A-LFABP. (A) number of restraints vs. the range of constraints along the protein sequence. (B) number and type of restraints vs. the protein sequence.

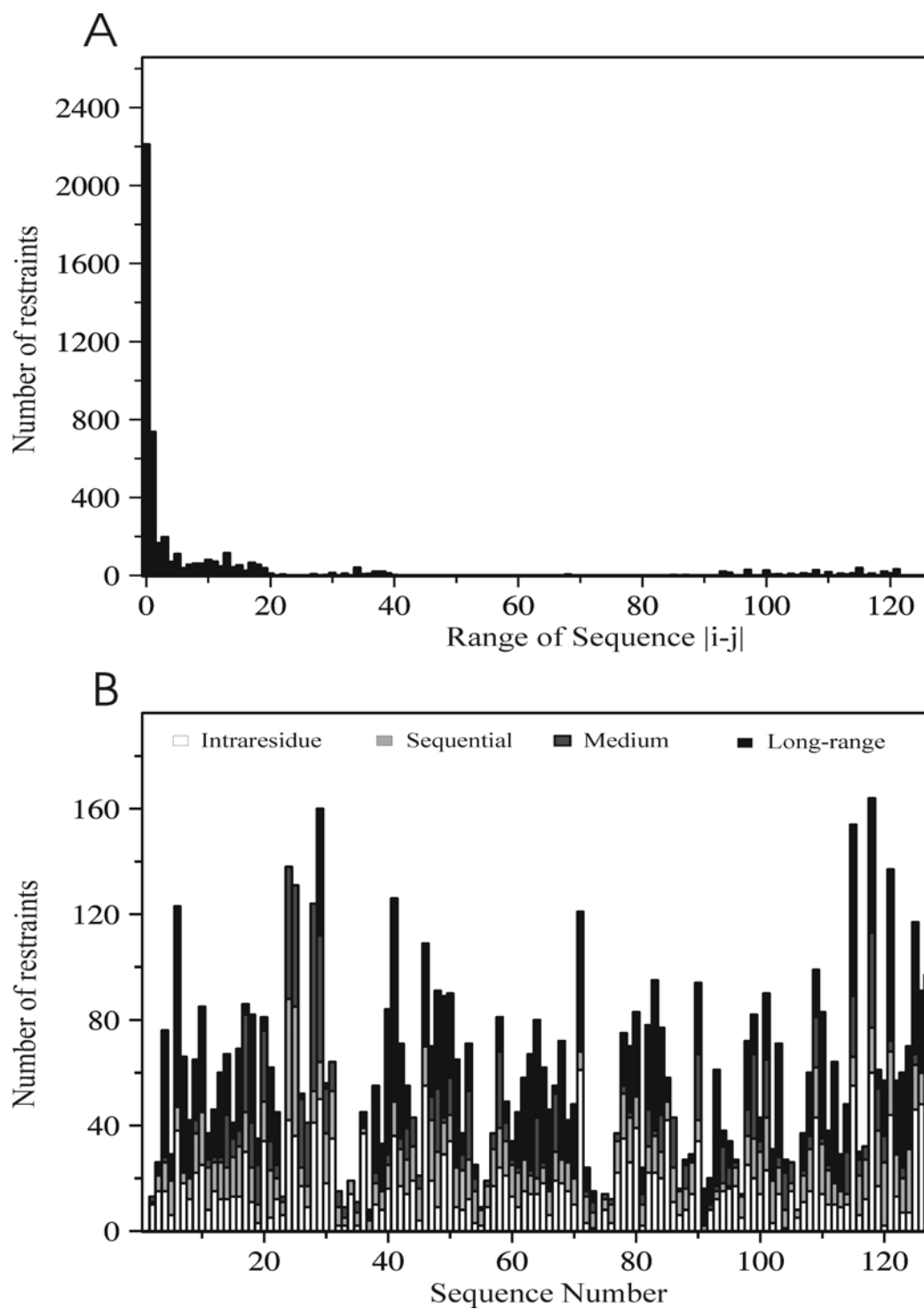


Figure 3.26 Distribution of the distance restraints used to obtain the final 20 solution structures for holo R122L/S124A-LFABP. (A) number of restraints vs. the range of constraints along the protein sequence. (B) number and type of restraints vs. the protein sequence.

Intermolecular NOE assignment

Isotope-filtered techniques were used to identify intermolecular NOEs that exist between the ligand (oleate) and the R122L/S124A-LFABP as described in the “Materials and Methods” section. One experiment measures NOEs between protons attached to aliphatic carbons of a $^{15}\text{N}/^{13}\text{C}$ -labeled protein (R122L/S124A-LFABP) in D_2O and all other protons in unlabeled oleic acid. The other experiment measures NOEs between protons in ^{15}N -labeled protein (R122L/S124A-LFABP) and protons attached to carbons in ^{13}C -labeled oleic acid. Analysis of these experiments revealed that there is one oleate binding with the carboxylate end interacting with K96 and I98 near the opening of the protein, and the hydrocarbon tail interacting with S39, I41, T51, and I109 (Figure 3.27). This finding corroborates our previous results from NMR titration studies in which one resonance was observed for each of the $\alpha\text{-CH}_2$ and the $\omega\text{-CH}_3$ of ^{13}C -oleate bound to R122L/S124A-LFABP (Figures 3.13 and 3.14). NMR titration studies demonstrate that the $\alpha\text{-CH}_2$ resonance for the oleate complexed with the R122L/S124A mutant lies close to the $\alpha\text{-CH}_2$ resonance of the second binding site in wild-type LFABP suggesting that they are in similar environments (near the surface). This hypothesis is confirmed by the intermolecular NOE result revealing that the carboxylate end of oleate is interacting with K96 and I98 near the opening of the protein.

In subsequent calculations, intermolecular NOE data will be incorporated into the structure determination of the R122L/S124A-LFABP complexed with oleate, to give us a better view of the location of the oleate in the protein.

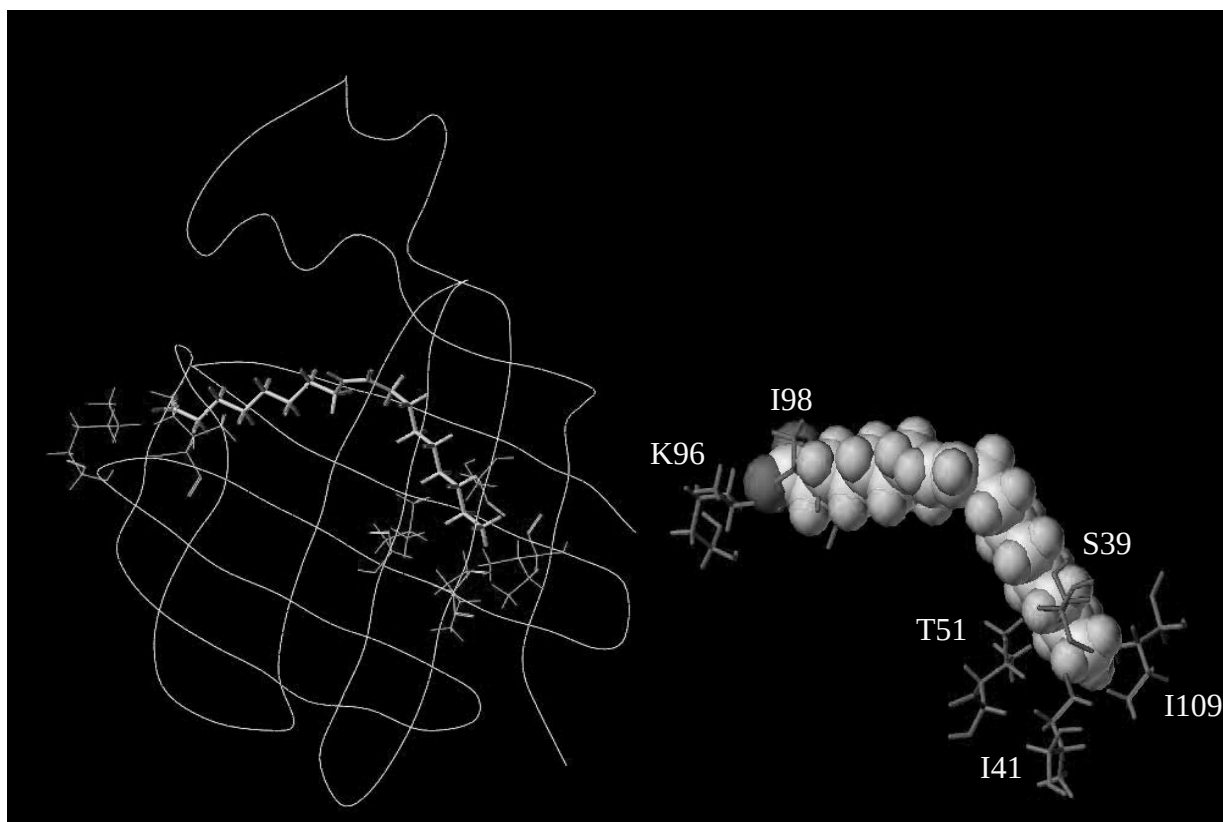


Figure 3.27 Tentative conformation and location for oleate complexed to R122L/S124A-LFABP as deduced from intermolecular NOE measurements.

Structure Calculations

The apo R122L/S124A-LFABP three-dimensional structure was determined employing an iterative procedure utilizing ARIA/CNS program, based solely on the use of experimental NMR data. The input for the ARIA/CNS program consisted of a resonance assignment table, manually edited and assigned peak volumes for the 3D ^{15}N -edited, 3D ^{13}C -edited, 4D $^{13}\text{C}/^{13}\text{C}$, and 4D $^{13}\text{C}/^{15}\text{N}$ NOESY spectra including 2149 NOEs, 30 hydrogen bond restraints deduced from NOE connectivities in ^{15}N -edited NOESY-HSQC, 79 values of $^3J(\text{H}^{\text{N}}-\text{H}^{\alpha})$ from HNHA experiment, and 126 dihedral angle

constraints derived from chemical shift data using the program TALOS. The frequency window tolerance for matching NOESY peaks with resonance assignments within the NOESY assign module of the program were set to ± 0.04 ppm for the proton and ± 0.5 ppm for nitrogen and carbon shifts, respectively. The program automatically corrects the chemical shifts of aliased peaks. This information was used to establish the molecular topology of an initial NMR-derived apo R122L/S124A-LFABP structure in solution and to provide a model to allow additional NOESY peaks to be assigned.

Twenty initial structures were generated by distance geometry and simulated annealing protocols in ARIA/CNS. Structures from each ARIA iteration were used to automatically derive residue-specific assignments of previously unassigned NOEs. A total of 20 structures in the last (eighth) iteration were analyzed and their structural quality was verified using PROCHECK, which revealed that 89 % of the residues are located in the most favored region of Ramachandran space. All 20 structures converged to the same general fold. The 10 lowest-energy structures from the last (eighth) iteration were further refined in a shell of water molecules included in the structure calculation and also checked for quality by PROCHECK. The final ensemble of 10 structures is characterized statistically in Table 3.9. The very few dihedral angle violations (0.456 deg.) and the large percentage of residues (89 %) in the most favored region of Ramachandran space confirm the high quality of the NMR-derived structures.

The preliminary holo R122L/S124A-LFABP three-dimensional structure was determined employing the same principles as used for apo R122L/S124A-LFABP. 2032 NOEs, 43 hydrogen bond restraints deduced from NOE connectivities in ^{15}N -edited NOESY-HSQC, 86 values of $^3J(\text{H}^{\text{N}}-\text{H}^{\alpha})$ were used for the preliminary structure

calculation. All other parameter settings were the same as for the apo R122L/S124A-LFABP. The quality of the final 20 structures was also verified using PROCHECK. 70 % of the residues are located in the most favored region of Ramachandran space compared to 89 % for residues of apo R122L/S124A-LFABP. The smaller percent of residues in the most favored region may be attributed to the incomplete but ongoing nature of the NOE assignments

Table 3.9 Structural statistics for NMR Structures of Apo R122L/S124A-LFABP

Restraints	
NOEs	
Intraresidue	913
Sequential/short	460
Medium-range ($i < 5$)	266
Long-range ($i \geq 5$)	510
Total NOEs	2149
Other	
Dihedral angles	126
Hydrogen bonds	30
^3J -couplings	79
RMSD (Å)	
Secondary structure (backbone)	0.26 ± 0.06
Secondary structure (heavy atoms)	0.74 ± 0.12
All residues (backbone)	0.47 ± 0.16
All residues (heavy atoms)	0.91 ± 0.15
RMSD (covalent geometry)	
bonds (Å)	0.0098 ± 0.00019
angles (deg.)	0.456 ± 0.020
impropers (deg.)	0.421 ± 0.064
Ramachandran space	
Most favored (%)	89.2
Additionally allowed (%)	9.27
Generously allowed (%)	0.63
Disallowed (%)	0.9

Table 3.10 Structural statistics for (preliminary) NMR Structures of Holo R122L/S124A-LFABP

Restrains	
NOEs	
Intraresidue	761
Sequential/short	405
Medium-range ($i < 5$)	273
Long-range ($i \geq 5$)	593
Total NOEs	2032
Other	
Hydrogen bonds	43
^3J -couplings	86
RMSD (Å)	
Secondary structure (backbone)	0.24 ± 0.07
Secondary structure (heavy atoms)	0.62 ± 0.07
All residues (backbone)	0.64 ± 0.12
All residues (heavy atoms)	1.09 ± 0.16
RMSD (covalent geometry)	
bonds (Å)	0.0091 ± 0.0002
angles (deg.)	0.90 ± 0.018
impropers (deg.)	0.79 ± 0.017
Ramachandran space	
Most favored (%)	70.3
Additionally allowed (%)	25.5
Generously allowed (%)	3.51
Disallowed (%)	0.72

The apo R122L/S124A-LFABP structures are represented by superimposed traces through the backbone C^α coordinates of the protein (Figure 3.28A) and worm plots using the trace thickness to show the backbone RMSD of each element of the protein (Figure 3.28B). The conformation of the protein backbone appears to be well determined. The average backbone root-mean-square deviation (RMSD) for the ensemble is $0.47\text{\AA}$, while the RMSD for the heavy atoms is $0.91\text{\AA}$. The reported RMSD values correspond to the

entire protein sequence. Regions with the most variability include the N-terminus (residues 1-3), β C- β D loop (residues 52-58), β E- β F loop (residues 72-78) and β H- β I loop (residues 104-108).

The NMR structures of apo R122L/S124A-LFABP and apo wild-type LFABP are displayed side-by-side in Figure 3.29 for comparison. The portal region (helix) in the wild-type protein exists in a more open conformation made possible by a kink at residue 13, whereas the R122L/S124A mutant exists in a more closed conformation due to the lack of that kink at residue 13 and the extension of the β A strand in a direction parallel to β J. In addition, the β E- β F loop of the R122L/S124A mutant appears in a bent inward conformation, while the same loop in the wild-type protein seems to be bent outward. Therefore, the cavity opening in the R122L/S124A mutant gives the impression of being partially blocked by the β E- β F loop. There are some regions in the R122L/S124A mutant that look more disordered than wild-type such as the N-terminal region (residues 1-3), β C- β D loop (residues 52-58) and β H- β I loop (residues 104-108). Nevertheless, the overall RMSD of the backbone for the mutant (0.47 Å, all residue) is better than that of wild-type (0.73 Å, all residues) suggesting a better resolved and perhaps a more stable (less flexible) structure.

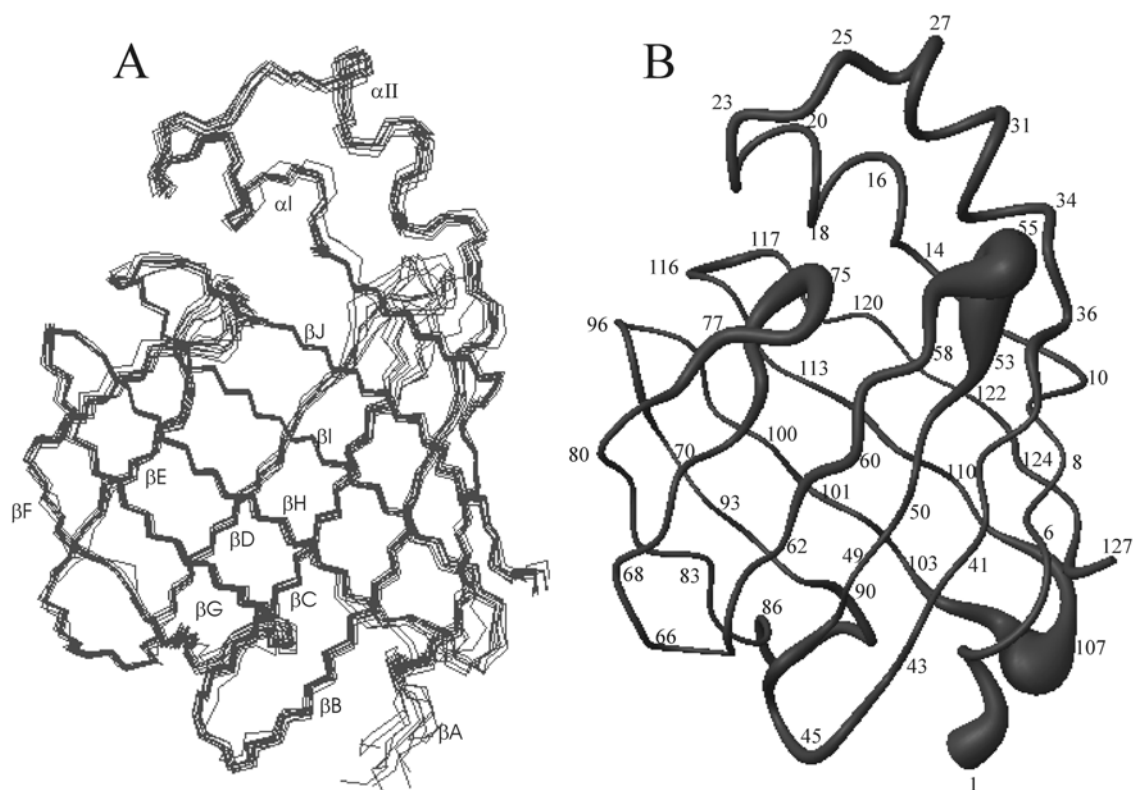


Figure 3.28 Comparison of NMR-derived conformations for apo R122L/S124A-LFABP. (A) diagram of 10 refined superimposed backbone C^α traces representing the final ensemble of NMR structures for apo R122L/S124A-LFABP. (B) Worm plot of the β -clam structures of apo R122L/S124A-LFABP using trace thickness to show the backbone RMSD of each element of the protein. These structures were calculated using ARIA/CNS protocol as detailed in Materials and Methods. The average pairwise C^α RMSD of this family is 0.47Å. This figure as well as Figure 3.28 and 3.29 were generated using MOLMOL.

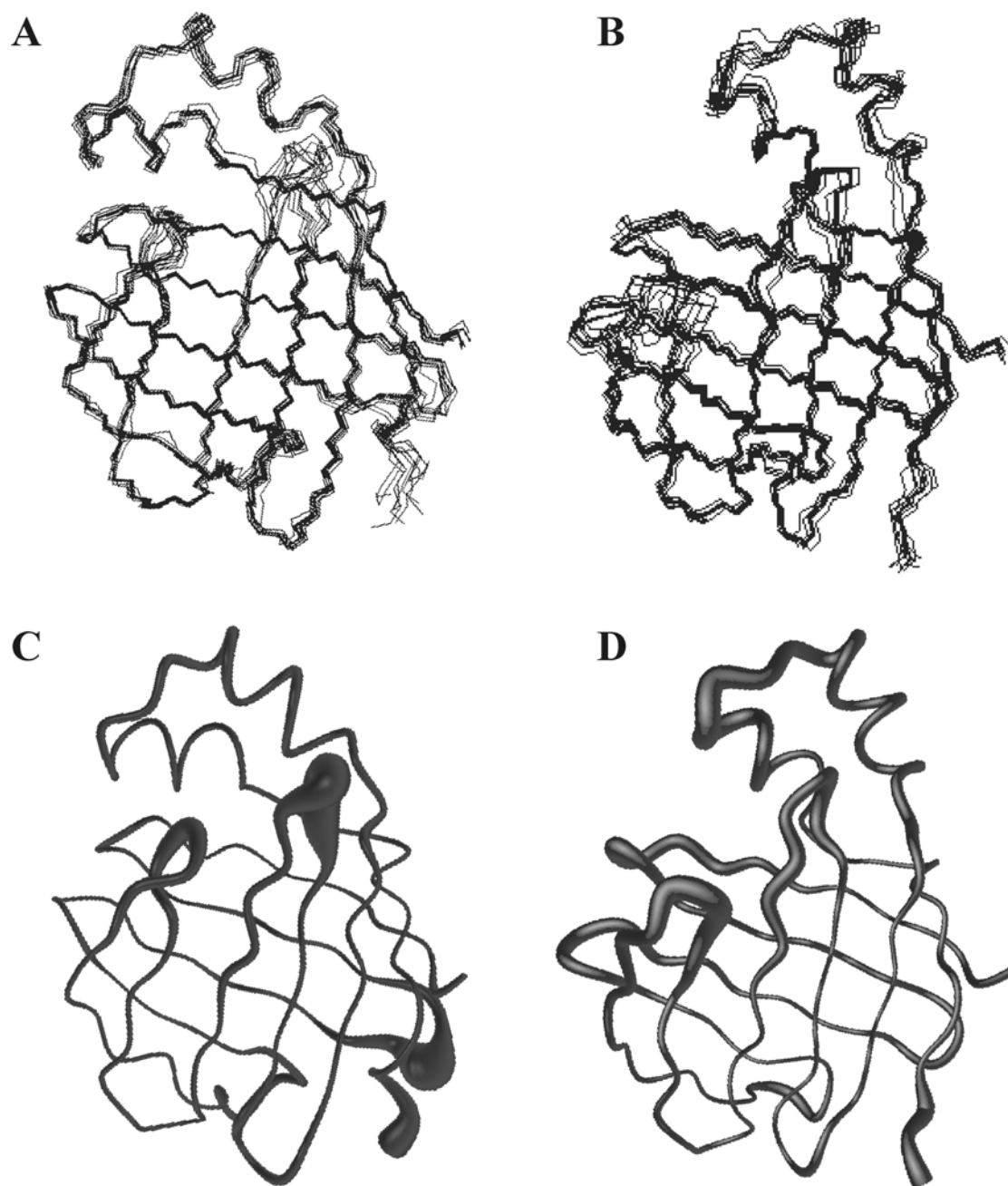


Figure 3.29 Comparison of apo R122L/S124A-LFABP with wild-type LFABP

The 10 lowest-energy refined structures for apo R122L/S124A-LFABP were superimposed (A), as well as apo wild-type LFABP (B). (C) Worm plot of the β -clam structures of apo R122L/S124A-LFABP and apo wild-type LFABP (D) using trace thickness to show the backbone RMSD of each element of the protein.

The preliminary holo R122L/S124A-LFABP structures are represented by superimposed traces through the backbone C^α coordinates of the protein (Figure 3.30A). The conformation of the protein backbone appears to be well determined. The average backbone root-mean-square deviation (RMSD) for the ensemble is 0.64 Å, whereas the RMSD for the heavy atoms is 1.09 Å. The reported RMSD values correspond to the entire protein sequence. Regions with the greatest variability include the N-terminus (residues 1-3), the loops and the αII-βB region. This structure is in the process of being refined further along with restraints involving oleate-protein interactions.

The holo R122L/S124A-LFABP structure is very similar to that of the apo R122L/S124A-LFABP, with the most variable regions near the αII-helix and the loop areas, as evidenced by superimposed traces of the backbone C^α atoms (Figure 3.30B). However, the difference between the holo R122L/S124A-LFABP and holo wild-type structures is more noticeable (Figure 3.30C). One half of the β-clam (βG, βH, βI, and βJ) is superimposable with that of the holo wild-type structure, but the other half of the β-clam (βA, βB, βC, βD, βE and βF) is not. In addition, the overall spatial relationship of the helix motif relative to the β-clam is similar to what is found in the holo wild-type structure. Since the holo R122L/S124A-LFABP structure is similar to that of apo R122L/S124A-LFABP and not too different from holo wild-type LFABP, it is possible to assume that the binding site in this mutant is already preformed. This situation contrasts with wild-type LFABP, for which the apo protein is in a more open conformation and shifts to a more closed structure on ligand binding.

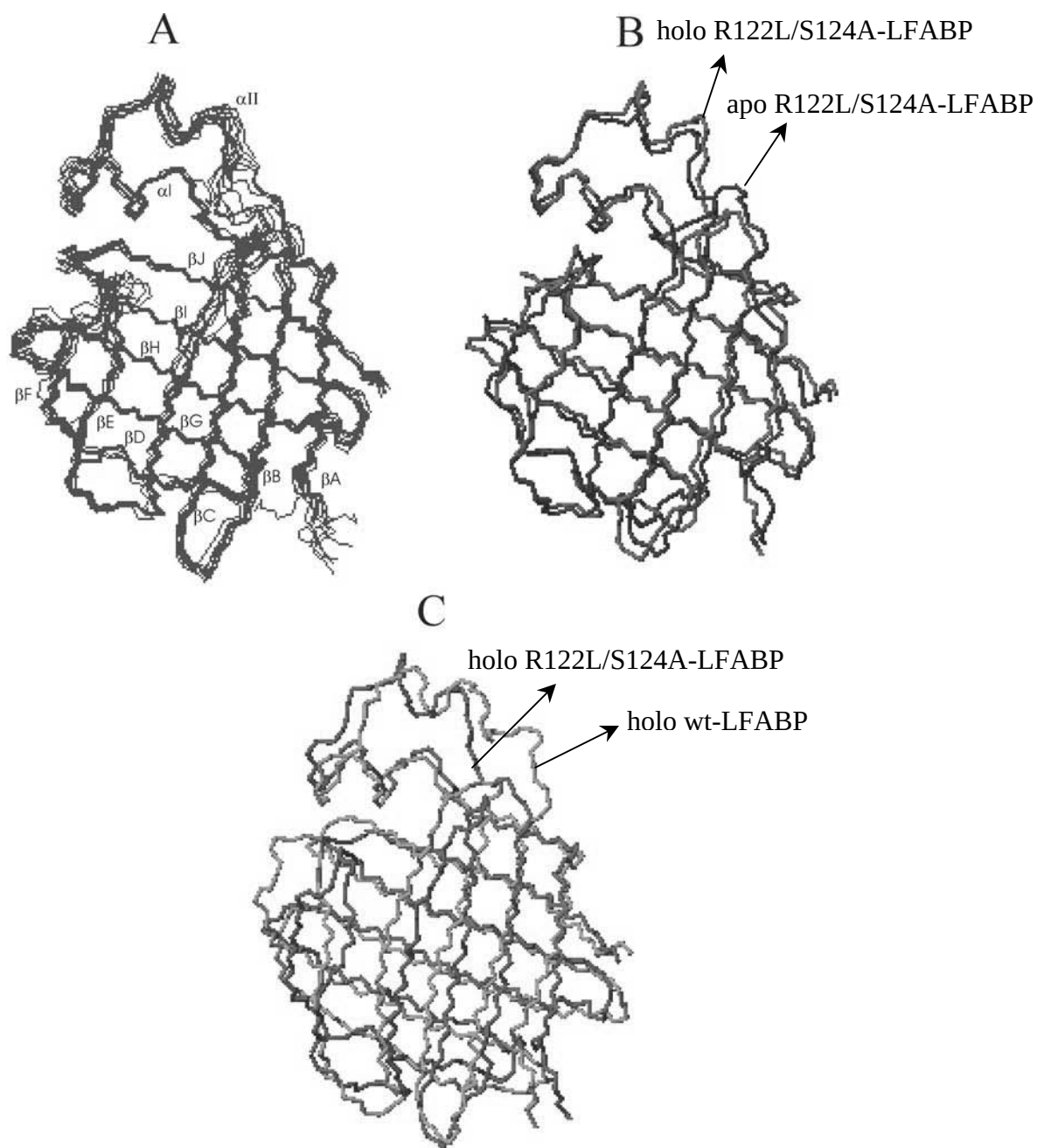


Figure 3.30 Comparison of NMR-derived conformations for holo LFABPs. (A) diagram of 10 refined superimposed backbone C^α traces representing the final ensemble of NMR structures for holo R122L/S124A-LFABP. (B) mean backbone C^α trace of holo R122L/S124A-LFABP superimposed with apo R122L/S124A-LFABP. (C) mean backbone C^α trace of holo R122L/S124A-LFABP superimposed with holo wild-type LFABP.

OVERALL SUMMARY AND DISCUSSION

Electrostatic interactions between LFABP and oleate (MCCE modeling)

By calculating the ionization energies of all residues throughout the LFABP protein using the MCCE method, we have gained useful information about the nature of the interaction between the ligands and the residues in the binding cavity. MCCE calculations, in conjunction with functional assays and a published X-ray structure, have guided us in the construction of site-specific mutant LFABPs. It was deduced that R122 and S124 contributed the most towards the ionization of the first oleate that binds LFABP, making them important residues for binding. In addition, K31 contributed the most towards the ionization of the second oleate that binds, suggesting that it could be important for the binding of the second oleate. As a result, R122 plus S124, and K31, were mutated to hydrophobic residues of similar size (in separate experiments) to eliminate those essential electrostatic interactions and favored contribution to binding.

Assessment of overall secondary structure for LFABP mutants

Protein expression and purification were successful in producing sufficient amounts of excellent quality LFABP for structural and functional studies. This result tells us that introducing these mutations has no adverse effect on protein folding. Although the protein was folded and functional, its overall secondary structure required verification before proceeding to further studies since prior reports by Thumser et al. showed that a T102Q/R122Q mutation changed LFABP to a predominantly α -helical protein (*Thumser et al., 1996*). For R122L/S124A- and K31L-LFABP, CD spectra verified that no overall folding modifications occurred in the mutant LFABP structures, rather, the mutants

maintained an overall β -sheet structure similar to wild-type protein (Figure 3.5). Since no major modification has occurred by mutating these residues, further functional and structural studies could be undertaken.

Determining the transfer mechanism of the LFABP mutants

One function of LFABP is to act as a buffer by solubilizing LCFA from membranes to maintain the unbound fatty acid concentration in the cytosol at a normal level (*Paulussen et al., 1990*). Other FABPs transfer these fatty acids to acceptor membranes for further processing by interacting directly with these membranes either via ionic interactions between lysine residues and acidic phospholipids (*Hsu et al., 1996; Herr et al., 1995; Corsico et al., 1998*) or by interaction with specific high affinity membrane receptors (*Burton et al., 1994*).

In order to investigate the transfer mechanism of the mutant LFABPs, transfer of 12-(9-anthroyloxy)oleic acid (12AO) from R122L/S124A-LFABP and K31L-LFABP (using wild-type LFABP as a control) was monitored with: (1) increasing concentrations of neutral EPC vesicles (Figure 3.3) and (2) increasing negative charge on the phospholipid headgroup of the acceptor vesicles (Figure 3.4). Both mutants, like the wild-type protein, exhibited essentially no increase in transfer rate, suggesting that the transfer process is still diffusion-mediated through the aqueous solution.

Corsico et al. have previously reported changes in the transfer mechanism from collisional to diffusional for a chemically modified IFABP mutant in which surface lysine residues were acetylated to eliminate positive surface charges (*Corsico et al., 2005*). Another study has shown that the helical domain in FABPs mainly dictates the transfer mechanism (*Corsico et al., 2004*). Those investigations have measured the rate of fatty

acid transfer from chimeric LFABP and IFABP that have their helical domains swapped. The chimeric $\alpha\text{L}\beta\text{IFABP}$ (helix from LFABP and β -sheet from IFABP) exhibited a diffusion-mediated mechanism (slower rate), whereas the chimeric $\alpha\text{I}\beta\text{LFABP}$ (helix from IFABP and β -sheet from LFABP) exhibited a collision-mediated mechanism (faster rate) (*Corsico et al., 2004*). The difference may be due to the different number and distribution of charged residues on the helices. Previous studies suggest that the αII -helix is important in determining the absolute fatty acid transfer rates, whereas the αI -helix may be important in regulating protein sensitivity to the negative charge of membranes (*Liou et al., 2002*). It is believed that all FABPs except LFABP have a net positive potential on the surface of their helix-turn-helix region that facilitates their interaction with the membranes. In contrast, the helix-turn-helix region in LFABP has both positive and negative potentials on its surface and that may be the reason for the lack of interaction with the membranes (*LiCata and Bernlohr, 1998*). In addition, amphipathicity in the helix motif, which is a characteristic present in FABPs that employ a collisional mechanism for the transfer of fatty acids, is also important in targeting the proteins to membranes (*Scapin et al., 1992*). However, LFABP is not amphipathic with hydrophilic residues dominating the helix-turn-helix region of the protein surface. This lack of hydrophobicity in the region provides an additional rationale for why LFABP does not collisionally interact with membranes during fatty acid transfer (*LiCata and Bernlohr, 1998*). FABPs that utilize a collisional mechanism for transfer have a characteristic increase in the transfer rate with a proportional increase of acceptor vesicles, whether they are neutral or charged. Therefore, although electrostatic interactions are essential for

collisional transfer of fatty acids to membranes, the hydrophobic interactions may also be of equal importance.

Given these recently discerned trends, it is not surprising that the R122L/S124A mutation did not alter the transfer mechanism, since such changes have been observed previously in IFABP only with mutations in the helix region (*Corsico et al., 1998; Corsico et al., 2005*). On the other hand, K31 is in the helix, so that mutating it to a non-polar residue (leucine) will eliminate that positive charge. Nevertheless, since the LFABP transfer mechanism is already slow and diffusion-controlled, it is difficult to see whether this single mutation further disfavors collisional FA transfer. Although these mutations had no effect on the transfer mechanism, which suggest that the off-rate (K_{off}) of the ligand is the same for the mutant proteins as that for the wild type, they may influence the protein's binding affinity to ligands (see above) or the on-rate (K_{on}) of the ligand. An interesting follow up experiment might involve mutations of acidic helical residues such as glutamate and aspartate to lysine, testing if a collisional mechanism can be induced in LFABP.

Ascertaining the binding affinity of oleate to mutant LFABPs

The binding affinity of R122L/S124A-LFABP and K31L-LFABP to oleate, compared to the wild-type protein, was measured using the fluorescent probe ADIFAB (Table 3.4). As expected, the R122L/S124A mutation caused a significant decrease in binding affinity towards the first oleate ($K_{\text{d1}} = 452 \mu\text{M}$), given that Arg122 and Ser124 are important for the interaction (electrostatic) with the ligand. They provide a favorable environment for the ligand, so that if these interactions are abolished then the ligand might not bind in that position or may bind only weakly. In fact, it is possible that

whereas the first oleate binds to LFABP at an interior site, the assay fails to reveal whether the oleate binds to the double mutant closer to the aqueous interface. Conversely, the K31L mutation was not significant enough to change the binding affinity to the first oleate that binds: the binding constant for the K31L mutant ($K_{d1} = 0.052 \mu\text{M}$) was close to that of wild-type protein ($K_{d1} = 0.043 \mu\text{M}$). The K31L mutation was not expected to change the binding affinity of the first ligand since it only interacts with the carboxylate of the second oleate on the surface of the protein.

The number of binding sites on the protein was determined using the same assay. The first binding site (n_1) had a stoichiometry of about 1 for both mutants, similar to that of wild-type LFABP. However, the stoichiometry for the second binding site (n_2) was not well determined since this assay was not sensitive enough to give an accurate binding constant for the second site. Consequently, we could not deduce whether any of the mutations caused any change in the affinity for the binding of a second oleate or prevented it from binding. Yet, we know from NMR binding studies that the K31L mutant, like wild-type LFABP, is still binding two oleates in its cavity but the R122L/S124A mutant binds only one oleate. Further NMR analysis of intermolecular NOE data between the ligand (oleate) and the R122L/S124A-LFABP revealed that there is one oleate binding. This oleate is binding in a different orientation than those in the wild-type protein. The carboxylate end of the fatty acid is interacting with K96 and I98 near the opening of the protein, whereas the hydrocarbon tail is interacting with S39, I41, T51, and I109.

As a complementary measure of the binding affinity of oleate to the LFABP mutants, the relative partitioning of 12AO between LFABP mutants and membranes was

examined. This assay supported our preceding finding from the binding assay using ADIFAB, in which oleate showed a significantly weaker binding to the R122L/S124A-LFABP mutant but the K31L-LFABP binding affinity was similar to the wild-type protein. Since the dissociation constant (K_d) is represented by the ratio of the off-rate (K_{off}) to the on-rate (K_{on}) of the ligand. These kinetic studies suggest that the ligand (oleate) has a hard time binding to the R122L/S124A-LFABP mutant (lower K_{on}) but it dissociates from its binding site at the same rate it dissociates from the wild-type protein (same K_{off}).

The electrostatic distribution for the R122L/S124A-LFABP cavity compared to wt-protein

As much as the binding affinity is important in understanding the function of these proteins, it is also essential to understand how the ligand enters and exits the LFABP cavity. One way to gain insight into this process involves examining the electrostatic potential maps of the inner surface (residues 1-37 & 81-127) (Figure 4.1) of the apo and holo R122L/S124A-LFABP compared to apo and holo wild-type LFABP structures. The electrostatic distribution for the cavity of apo wild-type LFABP reveals two positively charged groups on the inner surface, K90 and R122 (Figure 4.1 (B)). In contrast to holo wild-type LFABP (Figure 4.1 (C)), apo R122L/S124A-LFABP (Figure 4.1 (A)), and holo R122L/S124A-LFABP (Figure 4.1 (D)), the K90 side chain in apo wild-type LFABP is located within the cavity rather than at the surface. It is speculated that K90, with its positive charge, in apo wild-type LFABP aids in the recruitment of the first oleate to the cavity along with R122. Due to its location (between βF and βG), it may be able to swivel easily back and forth between the cavity and the outer surface. Thus, once the oleate comes in and interacts with R122, S124, and S39, the side chain of

K90 could be pushed outward to avoid proximity to the enhanced hydrophobic environment (oleate's hydrocarbon chain). Finally, entry of the second oleate could be facilitated by hydrophobic interactions between the first oleate and I59, bringing the helix domain closer to the β -barrel and covering the opening of the β -barrel. In the case of R122L/S124A-LFABP the R122 and S124 are replaced by leucine and alanine, respectively. Since this replacement may plausibly create a more hydrophobic environment in the cavity than that of wild-type protein, the positively charged side chain of K90 could be forced to swivel outward facing the surface, where the electrostatic environment is more favorable. Looking at the electrostatic distribution surface of the mutant's inner cavity (Figure 4.1 (A) and (D)) makes us believe that due to the lack of positive charge, the first oleate cannot bind in the same conformation as the wild-type, for which the carboxylate is buried deep in the cavity. However, oleate can now bind to K96, which is more exposed to the inner surface in the mutant than in the wild-type protein. Therefore, analysis of the electron distribution map further confirms our finding from the intermolecular NOE data that there is one oleate in the cavity and its carboxylate is interacting with K96.

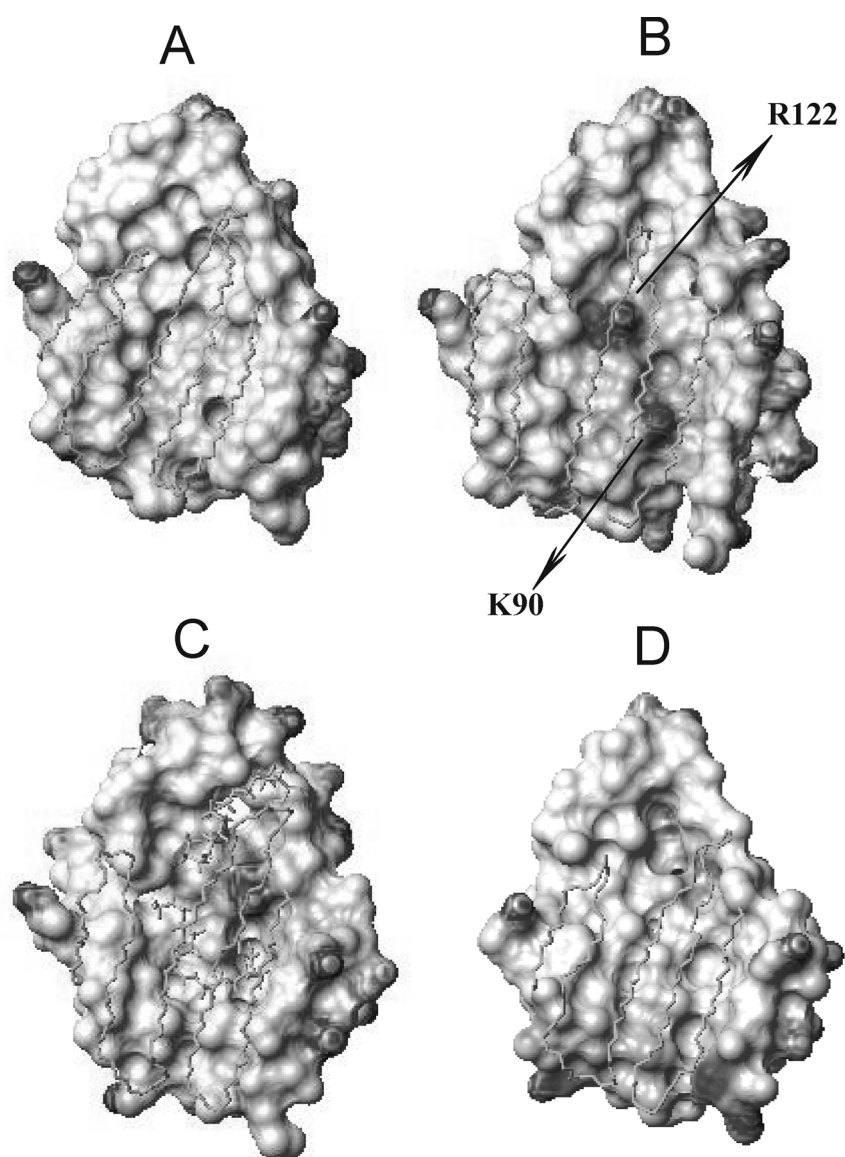


Figure 4.1 Electrostatic distributions within the cavities of (A) apo R122L/S124A-LFABP, (B) apo wt-LFABP, (C) holo wt-LFABP, and (D) holo R122L/S124A-LFABP. The inner surfaces are displayed for residues 1-37 and 81-127, respectively. Surface charges are displayed in blue for positive, red for negative and white for neutral. Protein backbone traces of the opposite β -sheet are shown in cyan and ligands are shown in gold.

Comparison of R122L/S124A-LFABP secondary and tertiary structure with wt-LFABP

The backbone ^1H , ^{15}N , $^{13}\text{C}^\alpha$ and $^{13}\text{C}^\beta$ resonances of R122L/S124A-LFABP were initially assigned by utilizing a combination of CBCA(CO)NH, HNCACB and ^1H - ^{15}N HSQC experiments using uniformly labeled $^{13}\text{C}/^{15}\text{N}$ protein. Later, we extended the assignments to resonances of the aliphatic side-chains. As a result, confirmation of the sequential connectivities was achieved. Chemical shifts are sensitive to secondary structure. Therefore, $^1\text{H}^\alpha$, $^{13}\text{C}^\alpha$, and $^{13}\text{C}^\beta$ nuclei for the apo form and the holo form (complexed with oleate) of R122L/S124A-LFABP were used to derive their secondary structure using the chemical shift index (CSI) program. Plots of the $^1\text{H}^\alpha$, $^{13}\text{C}^\alpha$, and $^{13}\text{C}^\beta$ chemical shift deviations from random coil positions versus primary sequence are shown in Figures 3.17 and 3.18. We have previously shown that both holo- and apo-R122L/S124A LFABP proteins in solution have α -helix and β -strand structural elements along the polypeptide chain that compare closely with the wild-type LFABP (Figure 3.17 and 3.18).

The assignments allowed for the identification of 2149 NOE interactions and 30 hydrogen bonds that were used as constraints for the calculation of a set of preliminary structures. Later 79 values of $^3J(\text{H}^\text{N}-\text{H}^\alpha)$ from HNHA experiment and 126 dihedral angle constraints derived from chemical shift data using the program TALOS were incorporated into the structure calculation to introduce more constraints. The 10 lowest-energy apo R122L/S124A-LFABP solution structures shown previously in Figure 3.27 display essentially the same secondary structural elements as those deduced from chemical shift indices, including 10 β -strands and two α -helices. This is also consistent with the structural motif common to iLBP family members, comprising ten antiparallel β -strands

forming a β -barrel, and a helix-turn-helix near the N-terminal covering one end of the barrel.

Considering the whole protein sequence, the average pairwise RMSDs for these 10 structures are 0.47 and 0.91 Å for backbone and all heavy atoms, respectively (Table 3.8), consistent with a well-defined three-dimensional structure, compared to the apo wild-type LFABP average pairwise RMSDs for backbone (0.73 Å with RDC) and all heavy atoms (1.26 Å with RDC). The corresponding worm plot (Figure 3.20 and 3.21) confirms the excellent definition of the β -barrel but reveals structurally variable loops. On the basis of the pairwise backbone root-mean-square deviation (RMSD), residues from the N-terminus (residues 1-3), β C- β D loop (residues 52-58), β E- β F loop (residues 72-78) and β H- β I loop (residues 104-108) appear to be considerably more disordered than the majority of the residues in the apo R122L/S124A-LFABP. These observations are consistent with the very few long-range NOE interactions in these areas of the protein (Figure 3.19), giving rise to structurally variable segments in the set of calculated structures.

The first FABP structure determined in solution by NMR spectroscopy was that of bovine HFABP (*Lassen et al., 1995*). During the course of the ^1H and ^{15}N resonance assignments, very pronounced spin-system heterogeneities were observed (*Lucke et al., 1992*). The majority of the heterogeneities were found in the portal region (helix region), where the fatty acid ligand enters and exits the protein molecule (*Sacchettini et al., 1992*), suggesting several subtle but distinct conformational states in this region. Various FABPs exhibit some conformational variability in the portal region upon ligand binding but not as drastic as that for LFABP (*He et al., to be submitted*). For LFABP, like CRBP II (*Lu et*

al., 1999), CRABPI (*Thompson et al.*, 1995), and CRABPII (*Wang et al.*, 1998), the helix-turn-helix, C/D, E/F, and I/J turns exist in an open state conformation but changes to a closed state upon ligand binding.

An important conclusion of the current study is that although the overall global folds are the same for R122L/S124A-LFABP and wild-type LFABP structures and the α -helices with respect to each other have the same orientation (Figure 4.2) for apo-, holo-R122L/S124A-LFABP and apo wild-type LFABP, they differ in several structurally variable elements. For example, the two α -helices are closer to the β -barrel in the mutant than in the apo wild-type protein (Figure 4.3), giving the appearance of a more closed portal that may be the reason for the lower on-rate of the ligand. This contrast appears to be due to some localized secondary structure difference between the two. For instance, in apo wild-type LFABP residues 9-12 are in coil conformation and residue 13 is in a bridge conformation, giving rise to a more open state. However, in apo R122L/S124A-LFABP residues 9-13 have a β -sheet conformation similar to holo wild-type LFABP, making the β A strand longer than the apo wild-type protein by five residues. Another secondary structural difference than in wild-type protein concerns β E- β F region: residues 70-72 in the β E strand and 78-80 in the β F strand form a much shorter β -sheet, whereas residues 73-77 form a much longer and tighter wound left-hand turn, which results in a smaller lipid cavity entry than in apo wild-type protein. The conformational variability of the β E- β F loop was confirmed previously in the solution structure of apo and holo wild-type LFABP and also in the crystal structure of the holo wild-type LFABP by large temperature factors of the M74 side chain (*Thompson et al.*, 1997).

Examination of the hydrogen-bonding network for both apo K31L-LFABP (Figure 3.22) and apo R122L/S124A-LFABP (Figure 3.23) reveals a lack of interstrand hydrogen bonds between β -strands D and E, creating a gap that is also present in the wild-type protein and a common feature in the intracellular lipid-binding protein (iLBP) family. This gap is not large enough to provide access to the ligand-binding cavity (*Banaszak et al., 1994*), but water molecules can form bridges between backbone atoms in β D and β E strands occupying some of the space, and the remainder of the space is filled with amino acid side-chains. It is not clear whether the amino acid side-chains present in the gap are pushing β D and β E apart or if the β F strand is pulling β E towards it, creating the gap. Hence, despite the fact that this gap is present in all of the iLBPs, neither the structural nor functional basis of the gap is evident.

The most striking structural difference between the double mutant and the wild-type LFABP is the orientation of the helix-turn-helix element with respect to the β -barrel. The helix of the apo wild-type protein stands out from the β -barrel and exposes the protein cavity to ligands and/or solvent molecules. In contrast, the helix motif of the apo and holo R122L/S124A-LFABP is closer to the β -barrel making it harder for the ligand to enter the cavity and thus giving rise to a lower on-rate. In addition, as noted above, the orientation of the helices with respect to each other is nevertheless nearly perpendicular in the apo wt-LFABP, apo R122L/S124A-LFABP, and holo R122L/S124A-LFABP, unlike that of the holo wild-type LFABP where they are in a parallel orientation to each other (Figure 4.2).

Despite all the changes in the orientation of loops and helices, we can conclude that the three-dimensional solution-state structure of apo R122L/S124A-LFABP is well

determined by NMR and exhibits the expected β -clam structural motif as other FABPs. Moreover, the transfer mechanism stayed diffusional as the wild-type protein. However, due to the more closed conformation of the helix, the E/F loop partially blocking the cavity, and the elimination of some crucial electrostatic interactions, the binding of oleate was hindered. This was evident with the functional assays (binding assay and partition assay) showing the reduced affinity of R122L/S124A-LFABP to oleate. In addition, prior ^{13}C NMR results on oleate bound to R122L/S124A-LFABP show one resonance for the α -CH₂ and one for the ω -CH₃ suggesting the possibility of one oleate binding. Additional structural information on R122L/S124A-LFABP complexed with oleate is needed to fully understand the protein-ligand interaction and fatty acid targeting in this mutant. This crucial piece of information will continue to be investigated in our laboratory.

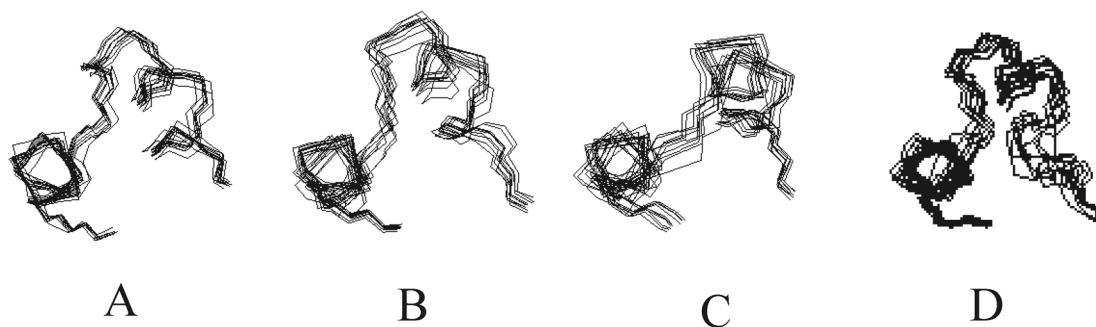


Figure 4.2 Comparison of the α -helix relative orientations for apo R122L/S124A-LFABP (A), apo wt-LFABP (B), holo wt-LFABP (C) and holo R122L/S124A-LFABP (D).

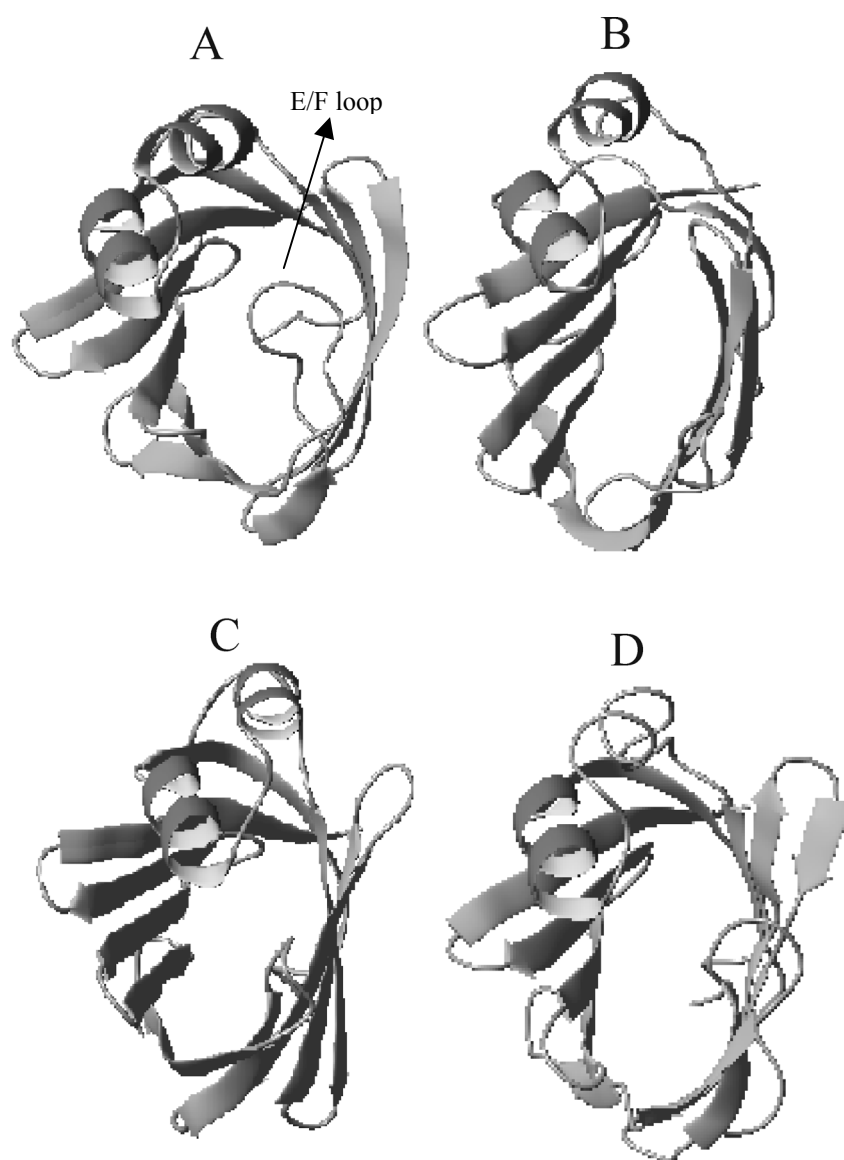


Figure 4.3 NMR-derived three-dimensional structure (cavity view) of (A) apo R122L/S124A -LFABP, (B) apo wild-type LFABP, (C) holo wild-type LFABP, and (D) holo R122L/S124A -LFABP

Future perspectives

The three-dimensional structure of R122L/S124A-LFABP complexed with oleate is still being refined using both intermolecular NOE measurements and protein restraints. The resulting structure should confirm the location of the oleate bound to the protein and what protein residues the ligand interacts with. Subsequently, these results can be rationalized using multi-conformational continuum electrostatics (MCCE) modeling. The MCCE method uses the protein-oleate complex coordinates to identify the factors and specific sites involved in stabilizing the oleate binding to the R122L/S124A-LFABP mutant.

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