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Molecular differences between human liver fatty acid binding protein and its T94A variant in their unbound and lipid-bound states

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ABSTRACT

Liver fatty acid binding protein (L-FABP) is an abundant cytosolic protein playing a central role in intracellular lipid trafficking. The L-FABP T94A variant, originating from one of the most common polymorphisms in the FABP family, is associated with several lipid-related disorders. However, the molecular factors that determine the observed functional differences are currently unknown.

In our work, we performed a high resolution comparative molecular analysis of L-FABP T94T and L-FABP T94A in their unbound states and in the presence of representative ligands of the fatty acid and bile acid classes. We collected residue-resolved NMR spectral fingerprints of the two variants, and compared secondary structures, backbone dynamics, side chain arrangements, binding site occupation, and intermolecular contacts. We found that threonine to alanine replacement did not result in strongly perturbed structural and dynamic features, although differences in oleic acid binding by the two variants were detected. Based on chemical shift perturbations at sites distant from position 94 and on differences in intermolecular contacts, we suggest that long-range communication networks in L-FABP propagate the effect of amino acid substitution at sites relevant for ligand binding or biomolecular recognition.

1. Introduction

Liver fatty acid binding protein (liver FABP, L-FABP, or FABP1) is an intracellular lipid binding protein abundantly expressed in hepatocytes, and also found in the intestine, pancreas, lung, stomach, and kidney [1]. L-FABP mediates the cellular uptake, transport and metabolism of fatty acids (FA), and is involved in the regulation of gene expression and cell differentiation [2–4]. L-FABP displays the widest ligand repertoire among all members of the FABP family: in addition to long chain FA, it also binds intermediates of FA oxidation and glyceride synthesis, as well as lysophospholipids, cholesterol, bile acids, eicosanoids, retinoids, heme and bilirubin [2,5,6]. The extensive ligand binding property of L-FABP suggests that this protein has multiple key functional roles in lipid transport and metabolism, energy regulation, and cytoprotection. In addition to possessing affinity for endogenous substrates, L-FABP also binds to a variety of xenobiotic drugs, including beta-blockers, non-steroidal anti-inflammatory drugs, fibrates, benzodiazepines, and lipid-based contrast agents [7–9], thereby constituting a target of active medical interest.

A common human L-FABP genetic variation at sequence position 94, a threonine to alanine replacement (T94A), has been identified [10]. The T94A variant is one of the most frequently occurring polymorphisms in the FABP protein family, with a 26–38% minor allele frequency (8.3% homozygous) in human populations [11]. The variant is associated with altered body mass index, clinical dyslipidemias (elevated plasma triglycerides and LDL cholesterol), atherothrombotic cerebral infarction, and non-alcoholic fatty liver disease [11]. The results of recent investigations indicate that the T94A substitution does not abolish L-FABP function but rather produces functional perturbations associated with altered structure and structural response to binding [12]. In particular, differences in secondary structures and in their changes occurring upon ligand binding were reported [12]. These differences did not result in substantial changes in binding affinities for long chain FA and intermediates of triglyceride synthesis, although L-FABP T94A variant displayed a threefold increased affinity towards cholesterol [12]. These observations spur further molecular characterizations of the two L-FABP forms, aimed at elucidating the determinants of altered function and drug responsiveness associated with the T94A

Abbreviations: CSP, chemical shift perturbation; FA, fatty acid; FABP, fatty acid binding protein; GCA, glycocholic acid; HSQC, heteronuclear single quantum coherence; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; OLA, oleic acid

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substitution.

High resolution tertiary structures are available for the L-FABP T94T wild-type form only. The structure of the protein, similar to that of all other FABPs, consists of ten antiparallel β-strands (βA-βJ) organized into two nearly orthogonal sheets that wrap around a solvent accessible cavity in a clam shell-like fold or twisted β-cylinder [13,14]. Hydrogen bonds join adjacent strands except for a gap between βD and βE [14]. The cylinder is closed off at one end by side chain packing, at the other end by a helix-turn-helix (αI-αII) motif formed between strands βA and βB. The capacious internal binding pocket offers accommodation for up to two long chain FA without requiring significant structural adaptation [13,14]. The structure of oleate(OLA)-bound L-FABP reveals that one ligand molecule is in a bent conformation above a cluster of hydrophobic side chains, with the carboxylate group pointing inwards to form polar contacts with arginine and serine residues. The second molecule adopts a more extended conformation with the carboxylate being positioned at the opening of the binding cavity and exposed to the solvent [14], in agreement with structures of rat L-FABP [15,16]. Unexpectedly, the corresponding molecule in palmitate-bound L-FABP displays reversed orientation with the carboxylate buried inside the cavity [13].

Structural data of L-FABP T94A were only obtained for the glycocholate (GCA)-bound protein. NMR data-driven docking calculations [6] indicated that GCA bound to the internal cavity of the protein with the steroid nucleus occupying a region that mostly overlaps with the binding site of the internal OLA molecule in the T94T form, and the polar side chain extending towards the second OLA binding site. Although no direct comparison of ligand-bound L-FABP forms can be made, it must be noted that threonine 94 is located at the C-terminal end of strand βG with its side chain oriented towards the protein exterior. This arrangement suggests that substitutions in this position may not directly affect ligand binding. Thus, a more detailed investigation of the structural and binding features of the two variants is necessary to better understand their functional differences.

In this work, we aimed at a high resolution comparative molecular characterization of L-FABP T94T and L-FABP T94A in their unbound states and in the presence of representative ligands of the FA and bile acid classes. We collected residue-resolved NMR spectral fingerprints of the two isoforms and mapped the differences onto the three-dimensional structure. Secondary structures, backbone dynamics, side chain arrangements, and intermolecular contacts were also compared. The binding of OLA was followed by ligand-observed experiments, establishing individual binding site occupation upon increasing ligand concentration. From analysis of our experimental data it emerges that differences in ligand binding do not originate from large structural or dynamic changes between protein isoforms, but rather may be attributed to small long-range effects that propagate changes in the site of substitution to more distant regions.

2. Materials and methods

2.1. Protein expression and purification

The expression plasmid carrying the gene coding for the variant L-FABP T94T was obtained using the Quickchange (Stratagene) mutagenesis kit on the plasmid coding for L-FABP T94A. The plasmids were transformed in *E. coli* SG (Qiagen) cells. The expression and purification of both protein variants were achieved as previously described [9,17,18]. M9 minimal medium supplemented with 1 g/l $^{15}NH_4Cl$ or $^{15}NH_4Cl$ and 4 g/l ^{13}C-labeled glucose was used to obtain ^{15}N- or ^{15}N,^{13}C-labeled proteins, respectively. All the samples were prepared in 10 mM Na_2HPO_4/NaH_2PO_4 buffer at pH 6.5, containing 0.02% NaN_3.

2.2. Preparation of protein-ligand complexes

Sodium oleate (OLA), and sodium glycocholate (GCA) were purchased from Sigma-Aldrich. $^{13}C_1$-OLA was from Cambridge Isotope Laboratories (Andover, MA). Solutions of 20 mM OLA or 50 mM GCA in 10 mM Na_2HPO_4/NaH_2PO_4 buffer at pH 6.5 were added to L-FABP dissolved in the same buffer to obtain complexes at the desired protein:ligand ratios.

2.3. NMR spectroscopy experiments

NMR experiments were recorded at 25 °C on a Bruker Avance III spectrometer operating at a proton Larmor frequency of 600.13 MHz, equipped with a triple resonance TCI cryogenic probe. Typical samples contained 0.3–0.8 mM L-FABP in phosphate buffer, 90% H_2O/10% D_2O.

^1H-^{15}N HSQC spectra were acquired using a spectral window of 12 ppm and 2048 complex points in the ^1H dimension, and a spectral window of 36 ppm and 256 complex points in the ^{15}N dimension. A total number of 4–8 transients were acquired for each spectrum with an interscan delay of 1.2 s. A standard sequence scheme with pulsed field gradients was used to achieve suppression of the solvent signal and spectral artifacts.

Constant time (CT) ^1H-^{13}C HSQC spectra were acquired optimizing for either the aliphatic or the aromatic spectral regions. Aliphatic spectra were recorded in a matrix of 2048 × 676 complex points on spectral windows of 16 ppm and 80 ppm in the ^1H and ^{13}C frequency dimensions, respectively, with the carbon carrier placed at 40 ppm. Spectra of aromatic signals were recorded in a matrix of 2048 × 182 complex points on spectral windows of 16 ppm and 40 ppm in the ^1H and ^{13}C frequency dimensions, respectively, with the carbon carrier placed at 120 ppm. The recycle delay was set to 2 s. The constant time evolution period was set to $1/J_{CC}$ in order to determine opposite phase for nuclei coupled to an even or odd number of carbon atoms.

Standard triple resonance experiments, HNCA, HN(CO)CA, HNCACB, CBCA(CO)NH, HNCO, and ^{15}N HSQC-NOESY (100 ms mixing time) were recorded on ^{15}N,^{13}C–labeled samples to achieve sequence-specific backbone atom resonance assignments of L-FABP T94T in its unbound form and in complex with GCA. Chemical shift data were deposited in the Biological Magnetic Resonance Data Bank, BMRB, under accession numbers 27,129 (unbound form) and 27,136 (complex with GCA). Backbone amide chemical shift assignments for L-FABP T94T bound to OLA were obtained from the BMRB (Entry 17,303). Assignments for L-FABP T94A were obtained in our previous work [6].

In order to detect intermolecular NOEs for the L-FABP:OLA complex, 2D [^{15}N,^{13}C]-filtered NOESY experiments were performed on samples containing ^{15}N,^{13}C–labeled protein and unlabeled ligand, in 98% D_2O. A mixing time of 100 ms, a recycle delay of 1.2 s, 12 ppm spectral windows in both dimensions, 64 or 80 scans, and 576 points in the F_1 dimension were employed. Two spectra were acquired with isotope filters in either F_2 or both in F_1 and F_2 frequency dimensions. For the F_2-filtered experiment, a frequency-swept adiabatic inversion pulse of 1.75 ms with a 10.5 kHz sweep frequency centered at 70 ppm was employed to purge signals of protons bound to ^{13}C. The WATERGATE pulse scheme was used for water signal suppression.

[^{13}C]-1D experiments were performed to observe the signals of $^{13}C_1$-labeled OLA in ligand titration experiments. These experiments were acquired with inverse-gated decoupling using a 30° flip angle for the carbon pulse. A pulse interval of 1.5 s, spectral width of 60 ppm centered at 170 ppm and 16,384 time domain points were used for 6656 spectral accumulations. A line-broadening factor of 10 Hz was used in data processing.

Protein backbone ^{15}N T_1 and T_2 were recorded on [^{15}N]L-FABP T94T. Experiments were performed in gradient-selected sensitivity-enhanced mode and in interleaved fashion, using a matrix of 2048 (^1H) × 128 (^{15}N) complex data-points for each relaxation delay and spectral widths of 13 and 33 ppm in the ^1H and ^{15}N dimensions, respectively. Optimal water signal suppression was obtained with a flip-back pulse. Recycle delays of 3 s were used for both T_1 and T_2. T_1