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Protocols and pitfalls in obtaining fatty acid-binding proteins for biophysical studies of ligand-protein and protein-protein interactions

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ABSTRACT

Adipocyte fatty acid-binding protein (AFABP: FABP4) is a member of the intracellular lipid-binding protein family that is thought to target long-chain fatty acids to nuclear receptors such as peroxisome proliferator-activated receptor gamma (PPARγ), which in turn plays roles in insulin resistance and obesity. A molecular understanding of AFABP function requires robust isolation of the protein in liganded and free forms as well as characterization of its oligomerization state(s) under physiological conditions. We report development of a protocol to optimize the production of members of this protein family in pure form, including removal of their bound lipids by mixing with hydrophobically functionalized hydroxypropyl dextran beads and validation by two-dimensional NMR spectroscopy. The formation of self-associated or covalently bonded protein dimers was evaluated critically using gel filtration chromatography, revealing conditions that promote or prevent formation of disulfide-linked homodimers. The resulting scheme provides a solid foundation for future investigations of AFABP interactions with key ligand and protein partners involved in lipid metabolism.

1. Introduction

Fatty acid-binding proteins (FABPs) are low molecular weight (~15 kDa) members of the intracellular lipid-binding protein (iLBP) family with 20–70% sequence identity [1] and the ability to bind both long-chain fatty acids (LCFA) and other hydrophobic ligands reversibly. Functional studies implicate FABPs in trafficking and targeting of LCFAs to the nucleus, where their interactions with *e.g.* the peroxisome proliferator-activated receptors (PPARs) have been demonstrated both in vitro and for hepatocyte cells [2–5]. Some FABPs exhibit a nuclear localization signal in their three-dimensional fold; when activated by particular ligands, translocation of the FABP from the cytosol to the nucleus and delivery of the ligand to its PPAR partner are promoted [6,7]. Together these proteins play important roles in signal transduction, cell growth, cell cycle and differentiation [8]; the action of adipose FABP, for instance, has been linked to hyperglycemia, insulin resistance, and obesity [9]. In addition, some reports have correlated the circulating level of adipocyte FABP (AFABP; FABP4) to the development of Type-2 diabetes [9,10]. While typically considered to exist as monomers, several FABPs appear to have a tendency to self-associate [7,11].

To define the molecular mechanisms underlying AFABP function, it is first essential to isolate and characterize the protein under near-physiological conditions. An ideal preparative method should be efficient and reproducible; it should yield an unliganded (apo) FABP that retains its native solution-state fold and oligomerization state without rapid deterioration; it should be amenable to probing with respect to three-dimensional conformation and binding to ligands or

Abbreviations: AFABP, adipose fatty acid-binding protein; ESI-MS, Electrospray Ionization Mass Spectrometry; FABP, fatty acid-binding protein; GF, Gel filtration chromatography; HSQC, [^{1}H–^{15}N] heteronuclear single quantum correlation spectroscopy; LCFA, Long-chain fatty acid; MALDI-TOF, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; NMR, Nuclear Magnetic Resonance; NOESY, 2D nuclear Overhauser spectroscopy; PPAR, peroxisome proliferator-activated receptor; TCEP, tris(2-carboxyethyl)phosphine; TEV, Tobacco Etch Virus; TOCSY, 2D Total correlation spectroscopy

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other protein partners. These goals can be elusive even for the small soluble iLBPs, due to the affinity of FABPs for the *E. coli* cellular constituents associated with their expression and/or the reported tendency of a few family members to form dimers or multimers [7,11,12].

This Report outlines protocols that optimize the production of AFABP and can be extended to other members of this large protein family. A newly developed scheme is presented to obtain efficient expression and purification, also achieving removal of bound lipids and/or fatty acids with validation made by two-dimensional NMR spectroscopy. We define conditions that promote or prevent formation of AFABP homodimers, either by self-association or covalent bonding though disulfide bridges. These procedures provide a macromolecular entity that is suitable for subsequent molecular-level studies of protein-ligand and protein-protein interactions, both of which are involved in metabolic signaling in mammalian tissues.

2. Materials and methods

2.1. Molecular cloning, expression, and purification of recombinant AFABP

Scheme S1 and the accompanying commentary describe cloning of the murine AFABP DNA including an N-terminal His_6-tag and a Tobacco Etch Virus (TEV) protease cleavage site, protein overexpression in *E. coli* cells and purification using HistidineTrap and gel filtration chromatography.

2.2. Delipidation of AFABP

Hydrophobic materials that are bound tightly to AFABP such as endogenous lipids generated during *E. coli* growth were removed from association with the protein via preferential binding to a Lipidex-5000 resin [13] (Sigma, H6383) which consisted of a lipophilic Sephadex LH–20–100 (hydroxypropyl beaded dextran) that was substituted with C13–C18 alkyl ethers. In our implementation of this strategy, a 5-gram portion of dry beads was activated by immersing in 5 mL of gel filtration (GF) buffer (described in the Supplementary information), then shaking in a conical Falcon tube at 37 °C for 2.5 h. To the resulting suspension was added 40–45 mL of protein solution (50 mg of protein), followed by shaking at 225 rpm and 37 °C for 2 h; no packed columns were used. The beads were removed using a 0.22-μm Millipore Amicon filter (Mahopac, NY). Two cycles of this treatment were carried out to ensure completeness of the delipidation. Similar procedures were followed for the intestinal and liver-type FABPs.

2.3. Isolation and development of apo-AFABP oligomers

To verify the oligomerization state of the AFABP protein, a prepacked XK26/40 column of Superdex 75 (GE Healthcare) was used for gel filtration chromatography. Protein samples, diluted serially from 460 to 11.5 μM, were injected and eluted with GF buffer at a flow rate of 0.6 mL/min on XK26/40 for large loading amounts (20 mL) or 0.25 mL/min on a Superdex 75 10/300 column for small loading amounts (100 μL). Molecular weight calibration used a protein standard kit spanning the range of 6.5 – 75 kDa; Blue Dextran (2000 kDa) served to determine the column void volume. Elution profiles were monitored for volumes of 0–25 mL using the Absorbance at 280 nm. Relative areas of monomer (lagging) and dimer (leading) fractions were determined in triplicate for each sample by calculating the respective peak areas. To test for disulfide linkages in the oligomers recovered from GF, standard SDS-PAGE was compared with gels run in the presence of 25% β-mercaptoethanol that can break such bonds.

The role of exposure to oxygen gas in the formation of putative disulfide-bonded AFABP dimers was examined by monitoring the dimer proportion as a function of time. The recovered monomer (lagging) fraction from GF of freshly prepared AFABP was split into two portions. One portion was treated with oxygen-free nitrogen gas, which was passed through a reservoir containing 100 mM vanadium sulfate, 25 mL sulfuric acid and 10 g zinc metal for one hour; the protein sample was then sealed with a rubber cap and Parafilm. The second portion was left with the cap open to the air. Both samples were maintained at 4 °C for a total time period of one month, including collection and testing of aliquots by GF at roughly one-week intervals. The dimer proportion was calculated by calculating the peak areas as a function of development time.

2.4. Mass spectrometry

Electrospray Ionization Mass Spectrometry (ESI-MS) was performed on a Bruker Maxis II ETD instrument (Bruker Biospin, Billerica, MA). Samples were exchanged with 200 mM ammonium acetate by using 10 kDa Amicon Ultra-15 filters, then concentrated to 10 μM before injection into the mass spectrometer at 3 μL/min. Typical native ESI-MS runs used a source temperature of 150 °C, dry nitrogen gas kept at 4 L/min, and a collision cell voltage optimized to 5 V. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) was conducted with a Bruker Autoflex Speed-High-Performance System using protein samples concentrated to 10 μM and mixed at 1:1 (v/v) with 10 mg/mL of a sinapic acid matrix.

2.5. Solution-state NMR spectroscopy

The two- and three-dimensional NMR experiments [14] were performed at 20 °C on a Bruker Avance I spectrometer operating at 500 MHz and equipped with a 5-mm TXI cryoprobe (Bruker Biospin, Billerica, MA). The AFABP sample (400 μM, assuming all proteins are monomers) was prepared in a solution that contained 10% (v/v) D_2O, 10 mM potassium phosphate, 150 mM potassium chloride and 0.2 g/L sodium azide, adjusted to pH 7.4. The 2D ^1H–^{15}N heteronuclear single quantum correlation (HSQC) spectra were acquired with respective spectral widths of 14 ppm and 32 ppm in the ^1H and ^{15}N dimensions, requiring 8–128 scans (0.7–11 h) in separate experiments at a range of protein concentrations. For 2D nuclear Overhauser and total correlation spectroscopy (^1H–^{15}N NOESY-HSQC and ^1H–^{15}N TOCSY-HSQC) experiments, the typical mixing and spin-lock times were 150 ms and 70 ms, respectively. The triple-resonance experiments (HNCO, HN(CA)CO, HNCACB and CBCA(CO)NH) were conducted using typical acquisition and processing parameters described previously [15,16]. The resulting data were processed using NMRPipe software [17] and analyzed by NMRViewJ software [18].

3. Results and discussion

3.1. Apo-AFABP purification has been optimized and validated

Following the protocol outlined in Section 2, recombinant murine AFABP was overexpressed in *E. coli* and purified to obtain ~30 mg protein per liter of LB culture (unlabeled samples) or ~25 mg per liter of minimal media (^{15}N- or ^{15}N,^{13}C-enriched protein samples for NMR spectroscopy). After sequential purification by affinity and size exclusion chromatography, TEV protease cleavage conducted with expedited removal of the linker yielded a protein of the expected 15 kDa molar mass that was verified to have excellent purity by both SDS-PAGE and MALDI-TOF MS methods (Fig. 1). As compared with prior purifications of FABPs [16,19], this method required half the time of schemes using size exclusion and ion exchange chromatography while maintaining a robust overall yield of ≥25 mg for a 1-L culture.

Prior publications on related members of the FABP family, including several reports from our own laboratories, have described the removal of bound lipids by column chromatography with Lipidex-1000 beads (hydroxyalkoxypropyl Sephadex), hydrophobic interaction column chromatography (phenyl Sepharose), or acidic precipitation. Such