Biochimica et Biophysica Acta 1838 (2014) 874–881

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Membrane attachment and structure models of lipid storage droplet protein 1

Penghui Lin [a], Xiao Chen [b], Hem Moktan [a], Estela L. Arrese [b], Lian Duan [a], Liying Wang [a,c], Jose L. Soulages [b], Donghua H. Zhou [a,*]

[a] Department of Physics, 230 L Henry Bellmon Research Center, Oklahoma State University, Stillwater, OK 74078, USA
[b] Department of Biochemistry and Molecular Biology, Oklahoma State University, Stillwater, OK 74078, USA
[c] State Key Laboratory of Magnetic Resonance and Atomic Molecular Physics, Wuhan Center for Magnetic Resonance, Key Laboratory of Magnetic Resonance in Biological Systems, Wuhan Institute of Physics and Mathematics, Chinese Academy of Sciences, Wuhan 430071, China

ARTICLE INFO

Article history:
Received 23 August 2013
Received in revised form 29 November 2013
Accepted 5 December 2013
Available online 12 December 2013

Keywords:
Lipid storage droplet protein
Triglyceride lipolysis
Proton spin diffusion
Magic-angle spinning
Solid-state NMR
MD simulation

ABSTRACT

Neutral lipid triglycerides, a main reserve for fat and energy, are stored in organelles called lipid droplets. The storage and release of triglycerides are actively regulated by several proteins specific to the droplet surface, one of which in insects is PLIN1. PLIN1 plays a key role in the activation of triglyceride hydrolysis upon phosphorylation. However, the structure of PLIN1 and its relation to functions remain elusive due to its insolubility and crystallization difficulty. Here we report the first solid-state NMR study on the *Drosophila melanogaster* PLIN1 in combination with molecular dynamics simulation to show the structural basis for its lipid droplet attachment. NMR spin diffusion experiments were consistent with the predicted membrane attachment motif of PLIN1. The data indicated that PLIN1 has close contact with the terminal methyl groups of the phospholipid acyl chains. Structure models for the membrane attachment motif were generated based on hydrophobicity analysis and NMR membrane insertion depth information. Simulated NMR spectra from a *trans*-model agreed with experimental spectra. In this model, lipids from the bottom leaflet were very close to the surface in the region enclosed by membrane attachment motif. This may imply that in real lipid droplet, triglyceride molecules might be brought close to the surface by the same mechanism, ready to leave the droplet in the event of lipolysis. Juxtaposition of triglyceride lipase structure to the *trans*-model suggested a possible interaction of a conserved segment with the lipase by electrostatic interactions, opening the lipase lid to expose the catalytic center.

1. Introduction

Animals store most of excess energy in the form of neutral lipid triglycerides for later use as metabolic fuel. The hydrophobicity of triglycerides allows them to be densely packed into lipid droplets, providing an energy density 10 times that of hydrated proteins and carbohydrates [1]. The lipid droplets are composed of a triglyceride core surrounded by a monolayer of phospholipids and a variety of proteins [2]. Utilization of the stored triglycerides requires enzymatic breakdown (lipolysis) by lipases, while the surface layer of the droplet controls the accessibility of lipases to the stored triglycerides. Among the proteins surrounding the lipid droplet surface, proteins in the PAT family (named after three earliest members) have raised great interest in recent years. The PAT family consists of the mammalian perilipin, ADRP, TIP47, S3-12, and OXPAT, as well as insect lipid storage droplet protein 1 (Lsd1) and 2 (Lsd2) [3]. Perilipin, ADRP, and Lsd1 constitutively attach to the lipid droplets, and they maintain fat storage and regulation of lipolysis. TIP47, S3-12, OXPAT, and arguably Lsd2 bind reversibly to the droplets; hypothetically they are responsible for the packaging of newly synthesized triglycerides into lipid droplets [3]. A new nomenclature has been recently proposed for the PAT-family of proteins [4]. Accordingly, from now on we will refer Lsd1 and Lsd2 as PLIN1 and PLIN2, respectively. Mammals and insects share significant conservation in the molecular mechanism of lipid droplet metabolism, highlighting the tremendous potential of using genetic technical advantages of insects to discover novel features of lipid homeostasis [3]. Studies of fruit fly models have established a correlation between triglyceride accumulation and the level of PLIN2 expression [5]. PLIN1 is found exclusively associated with lipid droplets [6]. It dynamically interacts with lipid droplet to control access of lipase to triglycerides thus regulates the lipids homeostasis. In contrast to mouse perilipin, which protects triglycerides from hydrolysis [7,8], depletion of PLIN1 leads to adult-onset obesity [9] while overexpression of PLIN1 induces lipid droplet to shrink and

Abbreviations: Lsd1, lipid storage droplet protein 1; PAT family, perilipin, ADRP, TIP 47 family; MD, molecular dynamics; DMPG, 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol; MAS, magic-angle spinning; CP, cross polarization; DARR, dipolar-assisted rotational resonance

* Corresponding author. Tel.: +1 405 744 3277.
E-mail address: donghua@okstate.edu (D.H. Zhou).

0005-2736/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbamem.2013.12.003

aggregate [10]. PLIN1 serves as a lypolytic switch, which upon protein kinase A (PKA) mediated phosphorylation, promotes the activation of triglyceride lipolysis [11].

The association of these proteins on the surface of the lipid droplets is critical to their ability to properly regulate both storage and release of the triglycerides in the droplets. Despite the pressing need to understand the interaction between these proteins and the lipid droplets, progress has been hampered by the scarcity of three-dimensional structure information for these proteins. The first and so far the only structure determined was for the C-terminal TIP47 (residues 191–437) at 2.8 Å resolution using X-ray crystallography [12]. The structure consists of an α/β domain and a four-helix bundle that resembles the receptor-binding domain of apolipoprotein E. The deep hydrophobic cleft between the α/β domain and the four-helix bundle is consistent with binding to hydrophobic proteins and small molecules, rather than to the extended phospholipid membrane. This C-terminus construct was selected from one dozen truncations for soluble protein expression. Regrettably, it does not have the N-terminal 11-mer helical repeats that are probably responsible for reversible binding to lipid membranes.

Meanwhile, *in vitro* systems have recently become available for structural and functional studies. Recombinant PLIN1 has been purified and reconstituted in lipid droplet-like particles [13,14]. Using an *in vitro* system, it was shown that phosphorylation of PLIN1 enhances the triglyceride lipase activity, demonstrating the direct connection between PLIN1 phosphorylation and the activation of lipolysis [13]. Hypothetically, PLIN1 phosphorylation causes changes on the droplet surface, making the internal triglycerides more accessible to the lipase [11]. The ability to reconstitute PLIN1 in lipoprotein particles opens the possibility to design structural studies to advance our understanding of the mechanisms of lipolysis regulation. Nevertheless, these lipoprotein particles are too large (~20 nm diameter) for solution NMR studies, and they are very difficult if not impossible to form diffraction quality single crystals for crystallography studies. Fortunately, several other structural techniques could be applied to this type of samples. For example, topologies of lipoprotein complexes have been determined using solid-state NMR [15,16], fluorescence spectroscopy [15–18] and electron paramagnetic resonance (EPR) [19]. Among these, solid-state NMR is particularly suitable to study these lipoprotein complexes because three-dimensional structure details could be obtained [22–29]. Here we report both NMR experimental data and structural models that could be useful to advance our understanding of protein targeting to lipid droplet and the function of PLIN1.

2. Material and methods

2.1. Protein expression and purification

Isotopically enriched (^{13}C, ^{15}N) ISOGRO, $^{15}NH_4Cl$ and uniformly labeled ^{13}C-glucose, were purchased from Sigma-Aldrich (St. Louis, MO). Lipid 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG) was purchased from Avanti Polar Lipids (Alabaster, AL). Benzonase was purchased from EMD Millipore (Billerica, MA). The over-expression of PLIN1 (CG10374, NP_732904.2) as a fusion protein with thioredoxin-$[His]_6$-Stag was carried out as previously reported [13] with slight modifications to incorporate stable isotopes for NMR studies. Transformed *Escherichia coli* Rosetta cells with the recombinant plasmid (pET32-CG10374) were grown in 200 mL Luria broth medium at 37 °C until optical density 0.8 at 600 nm. The bacteria pellet was collected by centrifugation, and cultured in 1 liter M9 minimal medium containing reagents enriched with NMR-active stable isotopes (^{13}C-glucose and $^{15}NH_4Cl$). The medium was supplemented with properly labeled algae extracts (ISOGRO) to boost protein yield. When optical density reached 0.8, protein expression was induced by addition of 1 mM IPTG. After 6 h, cells were harvested by centrifugation. Thioredoxin–Lsd1 fusion protein was purified essentially as previously described [12]. The final protein pellet was resuspended in 20 mM Tris, pH 8.0, 6 M Urea, 150 mM NaCl, 10 mM dithiothreitol and a solution of protein stock (1.7 mg/mL) was stored in the freezer.

2.2. Reconstitution of thioredoxin–PLIN1 in lipoprotein particles and thrombin cleavage

Thioredoxin–PLIN1/DMPG complexes were prepared as previously described with a final lipid to protein ratio of 70:1 [12]. After exhaustive dialysis, thioredoxin–PLIN1/DMPG complexes were brought to 60% (w/v) sucrose and subjected to ultracentrifugation in a sucrose density gradient (30 to 60% (w/v)). The distinct white band floating at a density of 1.17 g/mL was collected as a single fraction. Complexes were sedimented by ultracentrifugation in an aqueous buffer and resuspended in a buffer containing 5 mM Na_2HPO_4, 0.15 M NaCl, 0.1% octylglucoside at pH 7.4 and incubated with thrombin (1 unit/mg of protein) for 15 h at 4 °C to cleave the thioredoxin-$[His]_6$ tag. After centrifugation, the pellet containing PLIN1/DMPG complex was washed with 5 mM phosphate buffer (pH 7.5) and excess of water was removed in the speed vac for 1 h. Based on proton NMR signal intensities, the sample contained about 35% wt of water. These complexes were previously reported to have apparent diameter of 20 nm [13], and they are likely small unilamellar vesicles (SUVs) [20]. The head groups of anionic lipids, such as DMPG, facilitate interaction with positively charged protein side chain groups and they are important for membrane attachment of the protein. Neutral lipid DMPC was also tested, but it resulted in dramatic protein loss by sticking to the dialysis membranes and the centrifuge tubes. DMPG provided a cost effective way to obtain isotopically enriched PLIN1 lipoprotein complexes.

2.3. NMR spectroscopy

All solid-state NMR experiments were carried out on a 600 MHz Varian INOVA spectrometer and a triple resonance magic-angle spinning (MAS) probe with a 1.6 mm spin module. All spectra were acquired with a MAS rate at 13.3 kHz. For ^{13}C 1D and ^{13}C–^{13}C 2D experiments, the proton 90° pulse was 2.2 μs, cross polarization (CP) contact time 0.7 ms, locking fields of 73 kHz on ^1H and 80 kHz on ^{13}C channels, 100 kHz two pulse phase modulation (TPPM) decoupling [21], and dipolar-assisted rotational resonance (DARR) recoupling [22]. For proton spin diffusion experiment, the proton-detected NHH pulse sequence (Fig. S1 in Supplementary data) was modified from the CHH sequence [23] with MISSISSIPPI solvent suppression [24] and an additional T_2 filter (300 μs) [25] to suppress signals from the less mobile protein molecule (Fig. S2 in Supplementary data). The CP locking fields were 73 and 60 kHz on ^1H and ^{15}N channels, respectively. Contact time for the first CP (^1H to ^{15}N) was 1 ms, and 0.6 ms for the second CP (^{15}N to ^1H). Other experimental details can be found in figure captions.

2.4. MD simulation and spectral simulation

All MD simulations were performed on up to 600 processors on a Linux cluster supercomputer using software GROMACS 4.5.5 and GROMOS96 54A7 force field combined with DMPG lipid interaction parameters with simple point charge-extended (SPCE) water model [36,37]. The non-bonded van der Waals interactions were estimated using Lennard–Jones potential with cutoff value of 1.2 nm and the bonds were constrained by linear constraint solver (LINCS) algorithm [26]. Electrostatic forces and energies were calculated using Particle-Mesh Ewald (PME) summation algorithm with cutoff value of 1.2 nm too [27].

Two equilibration phases, constant volume (NVT) and constant pressure (NPT) ensembles, were subsequently carried out, each with 1 fs time steps. In the first phase, the system was coupled to a strong temperature bath using V-rescale coupling [28] with temperature coupling constant of $\tau_T = 0.1$ ps to maintain system temperature at 300 K. In the