Journal of Structural Biology 190 (2015) 261–270

Contents lists available at ScienceDirect

Journal of Structural Biology

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

Structural insights on cholesterol endosynthesis: Binding of squalene and 2,3-oxidosqualene to supernatant protein factor

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ARTICLE INFO

Article history:
Received 25 February 2015
Received in revised form 8 May 2015
Accepted 11 May 2015
Available online 16 May 2015

Keywords:
Cholesterol biosynthesis
CRAL-TRIO
Free energy perturbation
Lipid binding
SEC-14 like

ABSTRACT

We present the crystal structures of the SEC14-like domain of supernatant protein factor (SPF) in complex with squalene and 2,3-oxidosqualene. The structures were resolved at 1.75 Å (complex with squalene) and 1.6 Å resolution (complex with 2,3-oxidosqualene), leading in both cases to clear images of the protein/substrate interactions. Ligand binding is facilitated by removal of the Golgi-dynamics (GOLD) C-terminal domain of SPF, which, as shown in previous structures of the apo-protein, blocked the opening of the binding pocket to the exterior. Both substrates bind into a large hydrophobic cavity, typical of such lipid-transporter family. Our structures report no specific recognition mode for the epoxide group. In fact, for both molecules, ligand affinity is dominated by hydrophobic interactions, and independent investigations by computational models or differential scanning micro-calorimetry reveal similar binding affinities for both ligands. Our findings elucidate the molecular bases of the role of SPF in sterol endo-synthesis, supporting the original hypothesis that SPF is a facilitator of substrate flow within the sterol synthetic pathway. Moreover, our results suggest that the GOLD domain acts as a regulator, as its conformational displacement must occur to favor ligand binding and release during the different synthetic steps.

1. Introduction

Cholesterol and related sterol analogs are major lipid components of eukaryotic cell membranes and essential to evolution of higher organisms Bloch (1983). Cholesterol can be taken up from the diet or alternatively be endogenously synthesized (Ikonen, 2008). Under western diet conditions, roughly two thirds of the total body cholesterol are of endogenous origin (Dietschy and Wilson, 1970). Deregulation of cholesterol levels in the human body has been directly associated to vascular diseases (Ikonen, 2008), which, in turn, are enlisted among the first causes of mortality in developed countries. It is thus of major importance to understand the molecular mechanisms that regulate the cholesterol synthesis (Maxfield and Tabas, 2005).

The late stages of the committed cholesterol synthesis involve the formation of squalene, its oxygen-dependent epoxidation to 2,3-oxidosqualene, and the subsequent cyclization of the epoxide leading to the sterol backbone (Chugh et al., 2003). The epoxidation reaction by the microsomal squalene monooxygenase has been extensively studied for more than twenty years by Konrad Bloch and colleagues (Yamamoto and Bloch, 1970). Initially, rat liver microsomes were reported to require supernatant fraction (S105) for monooxygenase activation (Tchen and Bloch, 1957). Subsequent studies identified and characterized specific activators of the reaction, NADPH-cytochrome-c reductase, NADPH, FAD, phospahtidylserine, phosphatidylglycerol and a heat-labile protein (Tai and Bloch, 1972; Yamamoto and Bloch, 1970). The heat-labile protein was later purified from rat cytosolic fraction and termed "supernatant protein factor" (Ferguson and Bloch, 1977). Purified

Abbreviations: CD, circular dichroism; CRALBP, cellular retinaldehyde-binding protein; ER, endoplasmatic reticulum; FEP, free-energy perturbation; GOLD, Golgi dynamics; MD, molecular dynamics simulations; microDSC, differential scanning micro-calorimetry; NF1, Neurofibromatosis-inducing factor 1; RMSD, root-mean-square deviation; SEM, standard error of the mean; SPF, supernatant protein factor; α-TTP, α-tocopherol transfer protein; vdW, van-der-Waals.

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http://dx.doi.org/10.1016/j.jsb.2015.05.001
1047-8477/

rat SPF actively promoted squalene transfer across membranes *in vitro* and it was proposed to be directly involved in substrate transfer towards squalene monooxygenase (Friedlander et al., 1980).

In 1999 we identified a 46 kDa protein by incubating cytosol of bovine liver with radioactively labeled α-tocopherol (Stocker et al., 1999). The human homolog of this protein was cloned and initially termed tocopherol associated protein (TAP) due to its ability to bind α-tocopherol (Zimmer et al., 2000). One year later Arai and co-workers showed that TAP was identical to SPF (Shibata et al., 2001). They also reported the primary structure of SPF, its *in vitro* squalene transfer activity and its *in vivo* stimulatory role for sterol production in SPF-transfected hepatoma cells. By use of a SPF-deficient mouse model, the same group confirmed that SPF plays an *in vivo* role in hepatic cholesterol synthesis under fasting conditions (Shibata et al., 2006). Recent investigations on human SPF in rat hepatoma cells also show that phosphorylation may play a role on its activation (Mokashi and Porter, 2005).

We reported recently the crystal structure of SPF revealing its two-domain architecture consisting of a SEC14-like lipid-binding core-domain and a C-terminal jelly-roll barrel with putative functions in Golgi dynamics and in secretion (Anantharaman and Aravind, 2002; Stocker et al., 2002). Surprisingly, no evidence for direct squalene binding by SPF has been reported to date (Chin and Bloch, 1985; Shibata et al., 2001; Stocker and Baumann, 2003; Stocker et al., 1999). Thus, the *in vivo* ligand specificity of SPF and its physiological role are still unresolved.

Here we present high-resolution X-ray structures of the SEC14-like domain of SPF in complex with its proposed natural ligands squalene and 2,3-oxidosqualene. The removal of the C-terminal jelly-roll barrel of SPF has proven favorable for ligand-binding and allowed us to experimentally assess the thermal stability by circular dichroism (CD) spectroscopy and measure the thermodynamic parameters of binding by differential scanning micro-calorimetry. Ligand binding was also studied by molecular dynamics simulations (MD) and free-energy perturbation calculations, following protocols successfully applied to investigate substrate selectivity by α-tocopherol transfer protein (α-TTP) (Helbling et al., 2012, 2014). Our calculations show that SPF has no significant preferentiality for either squalene or 2,3-oxidosqualene, in agreement with experimental data. Overall, our results support the hypothesis originally proposed by Bloch and co-workers that SPF facilitates cholesterol synthesis by acting as dual substrate carrier that demonstrate the ability of SPF to stimulate squalene monooxygenase. However, the binding promiscuity of SPF for other lipids evidences the need for further studies to fully understand its function.

2. Materials and methods

2.1. Expression and purification

SPFs (residues Met1 to Lys275) was cloned into the pET28a vector (Novagen) by site directed mutagenesis using the full length SPF sequence as template. The oligonucleotides (Microsynth) used for PCR were 5′-GGGAATTCCATATGAGCGGCAGAGTC-3′ and 5′-GTGTTCCTCGAG**CTA**TTTCACCTGGTC-3′ with the NdeI and XhoI sites underlined and the introduced stop codon in bold. The construct was transformed into *Escherichia coli* BL21(DE3)Lemo (NEB). Protein expression was carried out following standard auto-induction protocols using ZYP-5052 media supplemented with antibiotics in a HT Minifors Fermenter (Studier, 2005). An additional lactose feed was introduced and protein was expressed at 22 °C. The cells were harvested at 5000 rpm for 25 min at 4° C and resuspended in lysis buffer (20 mM imidazole, 300 mM NaCl, 20 mM Tris–HCl, 1 mM PMSF, pH 7.4). Disruption of the cells was performed by sonication cooled on ice, after which the debris were removed by centrifugation at 50,000 rpm for 30 min at 4 °C. SPFs was purified by affinity chromatography using a His-Trap^{FF} column from GE Healthcare following manufacturer's instructions. After loading, the His-Trap^{FF} column was washed with lysis buffer, followed by a second wash with 6% elution buffer (500 mM imidazole, 300 mM NaCl, 20 mM Tris–HCl, pH 7.4). Protein was eluted with 100% elution buffer. The eluate was exchanged into final buffer (40 mM NaCl, 20 mM Tris–HCl, pH 7.4) using a HiPrep 26/10 desalting column from GE Healthcare. The His-tag was cleaved with thrombin protease (5 units per mg of SPFs) at 4 °C overnight. The tag and minor impurities were removed by size exclusion chromatography using a HiLoad 16/60 Superdex™ 75 column (GE Healthcare). SPFs was concentrated using Vivaspin-15 concentrators (Sartorius Stedim Biotech). Protein concentration was determined at 280 nm by UV absorbance (ε_{280} = 45,380 M^{-1} cm^{-1}) using a Nanodrop2000 from Thermo Scientific. SPFs was flash frozen in liquid nitrogen and stored at −18 °C. SPF full length was expressed and purified following the same procedures.

2.2. Preparation of protein–ligand complexes

The natural ligands squalene and 2,3-oxidosqualene were purchased from Sigma (⩾98% and ⩾92% purity, respectively). For solubilization, 60 mg of sodium cholate were overlaid with 1.5 mg of corresponding ligand and centrifuged at room temperature for 5 min at 13,600 rpm yielding an oil in detergent matrix. 250 µl of final buffer were added and the resulting suspension containing 557.6 mM sodium cholate and 14.6 mM ligand was sonicated in a water bath, until it became transparent. The solubilized ligand was mixed with purified SPFs yielding 27.9 mM sodium cholate, 1 mg/ml protein with a 25-fold molar excess of ligand over protein. This mixture was dialysed 3 times for 5 h against final buffer at 4 °C. Eventually forming precipitates were removed by centrifugation at 5000 rpm and the remaining ligand–protein complex was concentrated and unbound ligand removed by size exclusion chromatography. Complex formation was checked using GC–MS (squalene) and nano-ESI-MS (2,3-oxidosqualene). In both cases 0.2 mg of complex were lyophilised and extracted with methanol. As negative control, apo SPFs was analyzed under equal conditions. GC-chromatograms of squalene were carried out on an Agilent Technologies, 7820 A GC system on a Supelco 28045-4 column (15 m × 250 µm × 25 µm). A temperature ramp of 180–250 °C (5 °C/min) and a flow rate of 1.5 ml/min were applied. The detection was carried out using a FID detector at 310 °C. Data acquisition was done at a data rate of 50 Hz/0.004 min. A peak at 13.6 min could be readily seen in squalene controls as well as in the extracted samples (Fig. S2A–C). These were submitted to MS (Finnigan Trace MS, Thermo Quest) and the obtained fragments were compared with the SDBS-Mass database (Anantharaman and Aravind, 2002), confirming the presence of squalene in the complex (Fig. S2D). As shown in Fig. S2E–G, nanoESI (Thermo Scientific LTQ Orbitrap XL) in positive ion mode was used to detect 2,3-oxidosqualene. Resulting spectra showed a mass peak at m/z = 449.38 indicating the singly charged sodium adduct of 2,3-oxidosqualene.

2.3. Crystallization

Protein–ligand complexes were concentrated to 12 mg/ml (squalene) and 17 mg/ml (2,3-oxidosqualene) using the Vivaspin-15 concentrators (Sartorius Stedim Biotech). The crystals were obtained by hanging drop vapor diffusion method at 18 °C, whereby drops were set up by mixing 1 µl of sample solution with 1 µl of reservoir solution (squalene: 0.2 M Lithium sulfate, 0.1 M