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^1H , ^{13}C , and ^{15}N backbone resonance assignments of the L124D mutant of StAR-related lipid transfer domain protein 4 (StARD4)

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Abstract

Protein-mediated cholesterol trafficking is central to maintaining cholesterol homeostasis in cells. START (Steroidogenic acute regulatory protein-related lipid transfer) domains constitute a sterol and lipid binding motif and the START domain protein StARD4 typifies a small family of mammalian sterol transport proteins. StARD4 consists of a single START domain and has been reported to act as a general cholesterol transporter in cells. However, the structural basis of cholesterol uptake and transport is not well understood and no cholesterol-bound START domain structures have been reported. We have undertaken the study of cholesterol binding and transport by StARD4 using solution state NMR spectroscopy. To this end, we report nearly complete ^1H , ^{15}N , and ^{13}C backbone resonance assignments of an inactive but well behaved mutant (L124D) of StARD4.

Keywords

START domain; StARD4; sterol transport; cholesterol

Biological context

Cholesterol trafficking within cells is critical for proper regulation of its uptake and synthesis and proceeds by both vesicular and non-vesicular (i.e. protein-mediated) pathways. Proteins involved in transporting cholesterol and other sterols include OSBPs (oxysterol-binding proteins) and START-domain (StAR-related lipid transfer domain) proteins. Of the latter, StARD4 has recently been demonstrated to act as an important general cholesterol carrier in the cell (Mesmin et al., 2011).

StAR (Steroidogenic Acute Regulatory protein), the founding member of the START-domain protein family, was discovered in mouse tumor Leydig cells as the product of a gene upregulated during stimulated steroid hormone biosynthesis (Clark et al., 1994). StAR is responsible for delivering cholesterol to the mitochondrial inner membrane where it is processed by P450 side-chain cleavage enzymes into pregnenolone. StAR contains a mitochondrial targeting sequence and a ~210-residue sterol transfer domain, which is the prototypical START-domain and provided the basis for the identification of the START domain family. START domains are found in proteins from bacteria, fungi, plants, and animals, often as a part of larger multi-domain proteins. START domains from mammalian proteins can bind sterols, oxysterols, sphingolipids, and phospholipids, and are found in proteins containing domains such as Rho-GTPase, thioesterase, and kinesin motor domains (Clark, 2011).

StARD4 and the closely related proteins StARD5 and StARD6 consist of a single START domain without any other domain or targeting sequence. StARD4 was originally discovered as a protein downregulated in livers of mice fed a high-cholesterol diet (Soccio et al., 2002).

Recently, it was demonstrated that overexpression of StARD4 in cells leads to equilibration of the fluorescent cholesterol analogue dehydroergosterol (DHE) throughout different membrane compartments, and StARD4 was proposed to be a general cholesterol transporter (Mesmin et al., 2011). StARD4 was also shown in the same study to efficiently transport DHE between liposomes in vitro.

The structure of StARD4 in the ligand-free state has been solved by X-ray crystallography. The structure shows an α/β helix-grip fold: a half- β -barrel surrounding a hydrophobic cavity which is capped by two α -helices, one near the N terminus and one at the C terminus. The cavity is posited to hold the cholesterol ligand, while the C-terminal helix and the so-called $\Omega 1$ loop may act as a lid that mediates cholesterol uptake and release (Romanowski et al., 2002). The structures of several other START-domain proteins have been solved, including the close homologue StARD5, and all show a similar fold (Tsujishita and Hurley, 2000; Roderick et al., 2002; Kudo et al., 2010; Thorsell et al., 2011). In addition, some progress has been made toward the study of several START-domain protein structures by solution-state NMR spectroscopy (Barbar et al., 2009; Mercier et al., 2009; Lorin et al., 2012).

Some of the currently existing START domain structures were co-crystallized with ligands or inhibitors occupying the central hydrophobic cavity, though as of yet no structure of a START-domain protein with a sterol ligand has been solved. To this end, we have undertaken the study of the structural basis of cholesterol binding and transport by StARD4 using solution-state NMR spectroscopy. Here we report the ^1HN , $^{13}\text{C}^\alpha$, $^{13}\text{C}^\beta$, $^{13}\text{C}^\gamma$, ^{15}N , and $^1\text{H}^\alpha$ chemical shift assignments of the L124D mutant of mouse StARD4 in its ligand-free form. The L124D mutant was used because the wild-type protein appears to undergo intermolecular interactions and/or conformational exchange processes that prevent efficient magnetization transfer in triple-resonance experiments. It is expected that these assignments will be largely transferable to the wild-type protein, and will therefore provide the basis for studying the structural determinants of StARD4 cholesterol binding.

Materials and experiments

Protein expression and purification

The cDNA for mouse StARD4 was kindly provided by Dr. Jan Breslow, Rockefeller University. The cDNA was subcloned into the pET-SUMO vector (Invitrogen), resulting in a StARD4 construct with an N-terminally fused hexahistidine-tagged yeast SUMO protein for enhanced solubility, expression, and purification. Mutation of the leucine at position 124 to aspartate was accomplished with a QuikChange Mutagenesis Kit (Stratagene). *E. coli* BL21(DE3) cells transfected with this plasmid were used to express ^{15}N - and ^{13}C , ^{15}N -labeled protein by the media swap method (Marley et al., 2001). Cells were first grown at 37°C in rich LB medium until an OD_{600} of 0.6. Cells were then washed and transferred to a smaller volume of M9 minimal medium supplemented with 1 g/L ^{15}N ammonium chloride, or 1 g/L ^{15}N ammonium chloride and 2 g/L ^{13}C glucose (Cambridge Isotope Laboratories). Expression of the fusion protein was induced with isopropyl-1-thio- β -D-galactopyranoside (IPTG) followed by incubation with shaking for 3–4 hours at 37°C. Cells were harvested by centrifugation and the pellets stored at –20°C until purification.

Cell pellets were resuspended in lysis buffer (350mM NaCl, 20mM Tris pH 8, 20mM imidazole, 1mM phenylmethylsulfonyl fluoride (PMSF), 0.2mM ethylenediaminetetraacetic acid (EDTA), 2mM dithiothreitol (DTT), 1.5mM β -mercaptoethanol (BME)) and lysed by sonication. The lysate was clarified by centrifugation at 40,000 rpm for 40 minutes and the supernatant was loaded onto a Ni-NTA column pre-equilibrated with equilibration buffer (350mM NaCl, 20mM Tris pH 8, 20mM imidazole, 2mM DTT, 1.5mM BME), then washed with equilibration buffer. The fusion protein was eluted with elution buffer (350mM NaCl,

20mM tris pH 8, 250mM imidazole, 2mM DTT, 1.5mM BME). The eluate was dialyzed overnight against cleavage buffer (150mM NaCl, 20mM tris pH 8, 1mM DTT) and then incubated with Ulp1 protease for 2 hours at 4°C to cleave off the SUMO tag. The resultant mixture was passed over a Ni-NTA column and the flowthrough was collected. The protein was subjected to a final cleanup step on a Superose6 gel filtration column (GE Healthcare) using 20mM NaCl, 20mM tris, 5mM DTT, pH 6.4 buffer. The protein was then concentrated to between 0.5 and 1 mM and supplemented with 10% D₂O to produce the final NMR samples.

NMR spectroscopy

NMR experiments were performed on a Varian INOVA 600 MHz and a Bruker AVANCE 800 MHz spectrometer, both equipped with cryoprobes. Sequence-specific backbone resonance assignments were obtained from standard triple-resonance experiments all performed at 296 K. The experiments performed, with spectral widths and points collected, are detailed in Table 1. NMR data were processed with NMRPipe (Delaglio et al., 1995) and analyzed with NMRViewJ (Johnson and Blevins, 1994). Spectra were referenced indirectly to DSS. Secondary structural analysis was performed with TALOS+ (Shen, Delaglio, et al., 2009).

Assignments and data deposition

Backbone resonance assignments were obtained for 200/213 ¹H^N and ¹⁵N, 217/224 ¹³C^α and ¹³C^β, 200/224 ¹³C' and 194/224 ¹H^α nuclei. We note that many (10) of the missing resonances are for residues located in a flexible 23-residue N-terminal region that does not give rise to electron density in the crystal structure. Figure 1 shows ¹H^N and ¹⁵N assignments annotated onto an HSQC spectrum.

Chemical shifts were submitted to TALOS+ (Shen, Delaglio, et al., 2009) for secondary structure prediction, with results that agree well with the published crystal structure of the wild-type StARD4 protein (Figure 2) (PDB code 1JSS) (Romanowski et al., 2002). Assignments have been deposited in BioMagResBank under accession number 18647 (<http://www.bmrb.wisc.edu>).

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Figure 1. ^1H , ^{15}N -HSQC spectrum of L124 StARD4 with amide cross-peaks labeled with the assigned residues (A). The red box denotes the region expanded in (B).

Figure 2. TALOS+-predicted secondary structure for each residue. Negative values denote extended structure such as β -strands and positive values denote α -helical structure. Stable helices and strands inferred from the TALOS+ prediction as well as those seen in the wild-type StARD4 crystal structure are indicated.

Table 1

Triple resonance experiments performed, with spectral widths and number of points collected.

Experiment Name	Field (MHz)	Direct ¹ H Dimension		Indirect ¹⁵ N Dimension		Indirect ¹³ C Dimension	
		SW (ppm)	Pts	SW (ppm)	Pts	SW (ppm)	Pts
HNCA	600	20	1024	30.4	84	25.2	136
HN(CO)CA							
HNCO	600	20	1024	30.4	92	9.95	68
HBHA(CO)NH	600	20	1024	30.4	84	10.8	128
HNCACB	800	12	1024	30	50	65	76
HN(CO)CACB	800	12	1024	30	50	65	64