Journal of Structural Biology 192 (2015) 418–425

Contents lists available at ScienceDirect

Journal of Structural Biology

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

Structural and biochemical insights into the DNA-binding mode of *Mj*Spt4p:Spt5 complex at the exit tunnel of RNAPII

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ARTICLE INFO

Article history:
Received 12 June 2015
Received in revised form 27 September 2015
Accepted 30 September 2015
Available online 14 October 2015

Keywords:
Transcription factor
Archaea
Fluorescence polarization assays
NusG
Spt5
Crystal structure

ABSTRACT

Spt5 (NusG in bacteria) is the only RNA polymerase-associated factor known to be conserved in all three domains of life. In archaea and eukaryotes, Spt5 associates with Spt4, an elongation factor that is absent in bacteria, to form a functional heterodimeric complex. Previous studies suggest that the Spt4:Spt5 complex interacts directly with DNA at the double-stranded DNA exit tunnel of RNA polymerase to regulate gene transcription. In this study, the DNA-binding ability of Spt4:Spt5 from the archaeon *Methanocaldococcus jannaschii* was confirmed via nuclear magnetic resonance chemical shift perturbation and fluorescence polarization assays. Crystallographic analysis of the full-length *Mj*Spt4:Spt5 revealed two distinct conformations of the C-terminal KOW domain of Spt5. A similar alkaline region was found on the Spt4:Spt5 surface in both crystal forms, and identified as double-stranded DNA binding patch through mutagenesis-fluorescence polarization assays. Based on these structural and biochemical data, the Spt4:Spt5-DNA binding model was built for the first time.

1. Introduction

Gene expression, a complex process that involves transcription, translation, and post-translational modifications, is mediated by the activity of several multi-subunit protein complexes. The transfer of genetic information from DNA to RNA is carried out by RNA polymerases (RNAPs), which was generally conserved across the three domains of life (Werner and Grohmann, 2011). Numerous transcription factors have been identified to regulate the processes of transcription initiation, elongation, and termination. However, the Spt5/NusG protein family represents the only transcription regulators known to be universally conserved across all three domains of life (Harris et al., 2003).

Eukaryotic Spt5 consists of an NusG N-terminal (NGN) domain, 4–6 copies of KOW domains, and a C-terminal repeat (ctr) which is subjected to post-translational phosphorylation (Guo et al., 2008; Hartzog et al., 1998; Wada et al., 1998a). Archaeal and bacterial Spt5/NusG is composed of an NGN domain and only one KOW domain (Hirtreiter et al., 2010; Klein et al., 2011; Knowlton et al., 2003; Mooney et al., 2009). Spt5 intimately interacts with Spt4 via the NGN domain to form the heterodimeric Spt4:Spt5 complex in archaea and eukaryotes (also called DSIF in metazoans) (Guo et al., 2008; Wada et al., 1998b; Zhou et al., 2009). However, NusG functions as a monomer in bacteria as the Spt4 homologue does not exist in this domain of life (Ponting, 2002).

NusG and Spt4:Spt5 are widely accepted as elongation factors that stimulate transcription elongation and RNAP processivity (Kwak et al., 2003; Mooney et al., 2009; Yamada et al., 2006; Zhu et al., 2007); however, these proteins have also been reported to suppress transcription elongation under certain conditions (Core and Lis, 2008; Margaritis and Holstege, 2008; Sevostyanova et al.,

Abbreviations: RNAPs, RNA polymerases; NGN, NusG N-terminal; KOW domain, Kyprides, Ouzounis, Woese domain; DSIF, DRB sensitivity-inducing factor; NMR, nuclear magnetic resonance; CSP, chemical shift perturbation; FP, fluorescence polarization; EMSA, electrophoretic mobility shift assay; FL, full length; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; HSQC, heteronuclear single quantum coherence; CD, circular dichroism.

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http://dx.doi.org/10.1016/j.jsb.2015.09.023
1047-8477/

2008; Yakhnin et al., 2008). In bacteria, NusG suppresses pausing and increases elongation rate (Burova and Gottesman, 1995; Torres et al., 2004), and is also necessary for rho-dependent transcription termination (Cardinale et al., 2008; Pasman and von Hippel, 2000; Sullivan and Gottesman, 1992). In eukaryotes, the Spt4:Spt5 complex is believed to promote elongation by reducing the frequency of transcription pausing or arrest (Wier et al., 2013; Zhu et al., 2007), and additionally, in some cases, suppress elongation by negatively regulating the anti-arrest transcript cleavage factor TFIIS (Palangat et al., 2005).

The *Sc*Spt4:Spt5 complex has been proven to physically associate with *Sc*RNAPII (Hartzog et al., 1998). Using a mutagenesis-based approach, Hirtreiter et al. identified a hydrophobic pocket on the Spt5 NGN domain as the binding site for *Mj*RNAP, and reciprocally, the *Mj*RNAP clamp coiled-coil motif as the binding site for Spt4:Spt5 (Hirtreiter et al., 2010). This binding model was confirmed by the crystal structure of Spt4:NGN complexed with the RNAP clamp domain, which revealed that Spt4:Spt5 interacts directly with the clamp and contributes one side of the RNAP active center cleft (Martinez-Rucobo et al., 2011). A structural model of the entire RNAP-Spt4:Spt5 complex, constructed using data from cryoelectron microscopy analysis, shows that the Spt4:Spt5 complex closes the active center cleft by bridging and sealing the clamp, protrusion, and lobe of RNAP (Klein et al., 2011). In the absence of Spt4:Spt5, the re-annealed upstream DNA exhibits increased mobility in the exit tunnel of the polymerase (Andrecka et al., 2009), which may in turn distort the conformation of the substrate DNA at the catalytic center of RNAP.

Several working models of the exit tunnel for upstream DNA on RNAP have been proposed (Andrecka et al., 2009; Klein et al., 2011; Martinez-Rucobo et al., 2011), in which Spt4:Spt5 has been suggested to interact with DNA (Klein et al., 2011; Martinez-Rucobo et al., 2011); however, this has not been experimentally evidenced. Herein, the crystal structure of full length Spt4:Spt5 was determined, and nuclear magnetic resonance (NMR) chemical shift perturbation (CSP) and mutagenesis-fluorescence polarization (FP) assays were performed in order to locate the dsDNA binding area on Spt4:Spt5 and construct a Spt4:Spt5-DNA binding model.

2. Methods

2.1. Protein expression and purification

The *Mj*Spt4 (UniProtKB ID: Q57839) gene was inserted between the *Bam*HI and *Not*I sites of pRSFDuet-1 MSC1 (Novagen), and *Mj*Spt5 (UniProtKB ID: Q57818) (or the *Mj*NGN domain containing residues 1–87) gene was inserted between the *Nde*I and *Xho*I sites of pRSFDuet-1 MSC2 (Novagen). The recombinant *Mj*Spt4 contained a His_6-tag at its N-terminus.

The transformed *Escherichia coli* strain BL21 (DE3) (Stratagene) cells were grown in LB medium with 50 mg/L kanamycin, and induced with 1 mM IPTG for 4 h at 310 K after growing to OD_{600} = 0.6. Cells were harvested by centrifugation (8000 rpm for 5 min at 277 K) and suspended in lysis buffer [20 mM Tris–HCl (pH 8.0 at 298 K), 200 mM NaCl]. Suspended cells were lysed by sonication followed by heat treatment at 328 K for 15 min. After centrifugation (12,000 rpm for 25 min at 277 K), the supernatant was loaded onto a nickel-chelating column (GE Healthcare) and washed with 20 column volumes of wash buffer [20 mM Tris–HCl (pH 8 at 298 K), 200 mM NaCl, and 40 mM imidazole]. The Spt4:Spt5 protein was eluted using elution buffer [20 mM Tris–HCl (pH 8.0 at 298 K), 200 mM NaCl, and 500 mM imidazole], concentrated, and chromatographed on a size-exclusion column (Superdex 75 16/60, GE Healthcare) pre-equilibrated with 20 mM Tris–HCl at pH 8.0, and 50 mM NaCl. The peak corresponding to the Spt4:Spt5 heterodimer was collected and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), concentrated to 20 mg/mL, and stored at 193 K. Site-directed mutagenesis of Spt4:NGN was performed using the MutanBEST kit (Takara) following the manufacturer's instructions. Spt4:NGN proteins were expressed and purified by the same protocol as that used for Spt4:Spt5.

2.2. CD spectroscopy

CD spectra of Spt4:Spt5, Spt4:NGN, and Spt4:NGN mutants were recorded using a Jasco-810 spectropolarimeter at 298 K. Proteins were diluted to 100 μg/mL in 50 mM phosphate-buffered saline, pH 8.0, and recorded at wavelengths between 190 and 240 nm using a cell with path length of 0.1 cm. Buffer without added protein was used as blank. The molar ellipticities [θ] were plotted against wavelength, and the reference curve was subtracted from each curve before plotting.

2.3. Analysis of Spt4:Spt5 interactions by electrophoretic mobility shift assay (EMSA)

A 15-bp dsDNA oligonucleotide was produced by annealing single-stranded DNA (ssDNA) (5′-TCCAGTGCCGGTGTC-3′) with its complementary strand. Various concentrations of purified Spt4:Spt5 protein were incubated with 0.2 μM of the 15-bp dsDNA oligonucleotide at room temperature for 30 min, and analyzed by 8% native PAGE in 1 × TBE buffer at 4 °C. The gel was harvested and soaked in staining solution (GelRed™ 2× solution) after electrophoresis, agitated gently for 45 min, and then viewed and photographed under UV light.

2.4. NMR HSQC titration

HSQC spectra were recorded on a Bruker DMX 600 spectrometer equipped with a cryoprobe, at 315 K. The ^{15}N-labeled Spt4:NGN sample was diluted to a final concentration of 0.1 mM in 0.5 mL of buffer containing 100 mM Na_2HPO_4/NaH_2PO_4, pH 8.0, and 10% D_2O, and titrated using the 15-bp dsDNA oligonucleotide, at a molar ratio of protein:DNA of 1:0.2 and 1:0.4, respectively. In order to avoid significant dilution of the solution containing ^{15}N-labeled Spt4:NGN, the total volume of dsDNA solution added did not exceed 550 μL. The HSQC spectrum of each sample was recorded. Then, the NMR data were processed using NMRPipe and NMRDraw software (Delaglio et al., 1995) and analyzed using Sparky software.

2.5. Crystallography

Spt4:Spt5 crystals were grown using the vapor-diffusion method. Hanging drops containing 0.5 μL protein (15 mg/mL) and 0.5 μL reservoir solution were equilibrated with 200 μL reservoir solution at 295 K. Two types of crystals were observed after 5 days of growth under two different crystallization conditions: the first utilizing 5% PEG 5000 MME, 6% propanol, 100 mM MES, pH 6.5, and 10 mM sarcosine, and the second utilizing 15% PEG 6000, 5% MPD and 100 mM MES at pH 6.5. All crystals were cryoprotected by transition of the crystals into reservoir solution supplemented with 25% glycerol, followed by flash freezing with liquid nitrogen. Diffraction data of the first crystal were collected at BSRF (Beijing Synchrotron Radiation Facility) beamline 3W1A at a wavelength of 1.000 Å. The second crystal was diffracted at SSRF (Shanghai Synchrotron Radiation Facility) beamline 17U at a wavelength of 0.97915 Å. Data were processed, integrated, and scaled using the HKL2000 software package (Otwinowski and Minor, 1997).