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Soaking of DNA into crystals of archaeal RNA polymerase achieved by desalting in droplets

Transcription is a fundamental process across the three domains of life and is carried out by multi-subunit enzymatic DNA-directed RNA polymerases (RNAPs). The interaction of RNAP with nucleic acids is tightly controlled for precise and processive RNA synthesis. Whilst a wealth of structural information has been gathered on the eukaryotic Pol II in complex with DNA/RNA, no information exists on its ancestral counterpart archaeal RNAP. Thus, in order to extend knowledge of the archaeal transcriptional apparatus, crystallization of *Sulfolobus shibatae* RNAP (molecular mass of ~400 kDa) with DNA fragments was pursued. To achieve this goal, crystal growth was first optimized using a nanoseeding technique. An *ad hoc* soaking protocol was then put into place, which consisted of gently exchanging the high-salt buffer used for apo-RNAP crystal growth into a low-salt buffer necessary for DNA binding to RNAP. Of the various crystals screened, one diffracted to 4.3 Å resolution and structural analysis showed the presence of bound DNA [Wojtas *et al.* (2012). *Nucleic Acids Res.* **40**, doi:10.1093/nar/gks692].

1. Introduction

RNA polymerase (RNAP) enzymes are complex multi-subunit machines that are found in all three domains of life: Eukarya, Bacteria and Archaea.

During the past two decades, RNAPs from Bacteria and Eukarya have been intensively investigated at the structural level (Campbell *et al.*, 2002; Murakami *et al.*, 2002; Cramer *et al.*, 2008; Cheung *et al.*, 2011). Initial studies of bacterial RNAPs (Campbell *et al.*, 2002; Vassilyev *et al.*, 2002, 2007) were rapidly followed by structural elucidation of the more complex eukaryotic RNAPs (Cramer *et al.*, 2001; Kettenberger *et al.*, 2003; Westover *et al.*, 2004b; Brueckner *et al.*, 2009; Chen *et al.*, 2010). The structure of the complete 13-subunit archaeal RNAP from the archaeon *Sulfolobus shibatae* (*Ssh*RNAP; Korkhin *et al.*, 2009) was then determined and showed striking structural similarities to the eukaryotic counterpart Pol II (Werner & Grohmann, 2011). Indeed, the complete *Ssh*RNAP structure revealed the presence of two additional subunits: Rpo8 and Rpo13. Whilst Rpo8 had been predicted to exist in the Crenarchaeota phylum from genetic studies (Kwapisz *et al.*, 2008; Koonin *et al.*, 2007), the existence of Rpo13 had only been noted by Zillig and coworkers during biochemical studies of *S. acidocaldarius* (Lanzendorfer & Langer, 1994). The original *Ssh*RNAP crystal structure showed Rpo13 to be located in close proximity to the Rpo5 subunit and downstream of the DNA-entry cleft (Korkhin *et al.*, 2009).

In order to extend our knowledge of archaeal transcription, its ancestral evolutionary relationship to Pol II and the role played by the Rpo13 subunit, we embarked on the crystallization of *Ssh*RNAP in a binary complex with DNA. Common practices in crystallization of DNA–protein complexes are (i) to mix the protein and DNA in binding buffer solution prior to crystallization and/or (ii) to soak the DNA into pre-formed crystals (Hassell *et al.*, 2007; Kettenberger *et al.*, 2003; Westover *et al.*, 2004a). In our case both protocols were initially followed, but a lack of success obliged us to adopt a procedure for desalting pre-formed apo-RNAP crystals owing to their growth in high-salt buffer conditions. This strategy of desalting

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followed by DNA soaking led to successful data collection from an *Ssh*RNAP–DNA crystal complex. This structure mimics either an open complex-like state or an elongation complex without an RNA primer in the transcription bubble (Wojtas *et al.*, 2012).

Despite the fact that desalting of crystals in crystallization drops was proposed some time ago (Ray *et al.*, 1991; Zhang *et al.*, 1994; Schreuder, 1988), to our knowledge this is the first example of a desalting procedure *in situ* of pre-formed crystals of a large and multi-component macromolecule (~400 kDa) and is thus a proof-of-principle that could be of inspiration for other similar cases.

2. Materials and methods

2.1. RNAP and DNA sample preparation

RNAP from *S. shibatae* was purified as described elsewhere (Korkhin *et al.*, 2001). Several DNA fragments differing in length and with a pre-formed bubble were designed based on the *Sulfolobus* spindle virus 1 (SSV1) T6 promoter, for which the archaeal RNAP shows high binding efficiency *in vitro* (Qureshi *et al.*, 1997).

HPLC-purified DNA template and nontemplate strands (Sigma–Aldrich) were mixed in a stoichiometric 1:1 ratio and the double-stranded (ds) DNAs were prepared by denaturing DNA at 368 K for 10 min on a PCR thermocycler and were then cooled to room temperature on the bench. The same set of DNA fragments were

used for soaking and for RNAP–DNA binding experiments in solution. Details of the DNA sequence that led to the successful data collection of the RNAP–DNA binary complex are described in Wojtas *et al.* (2012).

Figure 1 Optimized crystals of *S. shibatae* RNAP. (a) Front view of the largest dimensions of the crystal; (b) side view of a different crystal showing its thickness; both (a) and (b) are to scale. Drops were set up by a Mosquito robot and were visualized using a Nikon SMZ 1500 microscope. These crystals appeared after 3 d and were imaged 7 d after their appearance.

Figure 2 *Ssh*RNAP–DNA binding assay using size-exclusion gel chromatography. (a) RNA polymerase alone in a high-salt buffer condition (11.2 ml elution volume). (b) RNAP mixed with dsDNA in high-salt buffer (11.2 ml, RNAP; 15.2 ml, DNA). (c) Elution profiles of RNAP mixed with dsDNA in low-salt buffer (11.4 ml, RNAP plus DNA; 14.67 ml, DNA alone). Black and grey lines correspond to readings at 280 and 260 nm, respectively.

2.2. Crystal optimization and RNAP–DNA binding assays in solution

Crystallization drops on a nanolitre scale (400 nl) were dispensed using a Mosquito robot (TTP LabTech) by the sitting-drop vapour-diffusion method into 96-well crystallization plates (Innovaplate SD-2). Improved crystals of apo *Ssh*RNA polymerase grew in the presence of a high salt concentration by mixing 200 nl RNAP at $\sim 7 \text{ mg ml}^{-1}$ in storage buffer (50 mM Tris pH 7.8, 500 mM KCl, 25 mM MgCl_2 , 10 mM β -mercaptoethanol, 10% glycerol) with 200 nl buffer consisting of 100 mM sodium cacodylate pH 6.5, 100 mM SrCl_2 (in some cases 1 mM ZnCl_2 was also used) and 12% PEG MME 5K; the drops were then equilibrated against reservoir buffer (50 mM Tris pH 7.8, 100 mM sodium cacodylate pH 6.5, 500 mM KCl, 25 mM MgCl_2 , 100 mM SrCl_2 , 10 mM β -mercaptoethanol, 10% glycerol, 12% PEG MME 5K; Wojtas *et al.*, 2011, 2012; Korkhin *et al.*, 2009). The crystals were further optimized using the nanoseeding technique (Walter *et al.*, 2008). A seeding stock was prepared and consisted of 40% reservoir buffer, 50% water, 10% protein solution (7 mg ml^{-1}) and crystals. Three serial dilutions (1:100, 1:1000 and 1:2000) were prepared from this seeding stock using the reservoir buffer. Crystal optimization with the particular aim of improving the thickness of the crystals was carried out by dispensing 50 nl of each of the four seeding solutions onto the pre-dispensed crystallization drops (400 nl). In this experiment, the percentage of precipitant (PEG 5K MME; Hampton Research) in the mixing buffer was varied from 7 to 12% (and accordingly for the reservoir buffer). Thicker single crystals appeared after 3 d at 8 and 9% PEG (Fig. 1); some of these were also used for diffraction and one extended the resolution to $3.2 \text{ \AA}$ (Wojtas *et al.*, 2012), the highest resolution achieved to date for an archaeal RNAP.

Initial attempts to directly soak DNA fragments into these crystals never led to a crystal for which diffraction and structure solution demonstrated the presence of DNA bound to the RNAP enzyme. Similarly, adding DNA directly to the RNAP sample (in a 3:1 molar ratio) prior to crystallization never led to an RNAP–DNA crystal.

Thus, optimum buffer conditions for DNA binding were explored biochemically by gel-filtration chromatography on an ÄKTA purifier using a Superdex 200 10/300 GL column (GE Healthcare). DNA was mixed with purified RNAP in a 3:1 molar ratio and loaded onto the column. Three experiments were run: (i) polymerase alone in high-salt (500 mM KCl) and low-salt (50 mM) buffer conditions, (ii) RNAP–DNA binding in the buffer containing high salt and (iii) RNA polymerase with DNA in the presence of low-salt buffer (Fig. 2).

The high-salt buffer consisted of 500 mM KCl, 50 mM Tris pH 7.8, 25 mM MgCl_2 , 10 mM β -mercaptoethanol, whilst the low-salt buffer consisted of 50 mM KCl, 50 mM HEPES pH 7.5, 2.5 mM MgCl_2 , 2 mM DTT. Samples were prepared by mixing $10 \mu\text{l}$ 7 mg ml^{-1}

RNAP with the corresponding buffer and DNA in a $100 \mu\text{l}$ total volume and were incubated for 10 min on the bench prior to injection. Samples were injected in a $100 \mu\text{l}$ loop and the flow rate was set to 0.5 ml min^{-1} . The elution profile of RNAP alone in high-salt buffer (Fig. 2*a*) showed a symmetrical peak that differed from the streaked elution peak that was obtained in low-salt buffer and was suggestive of possible nonspecific interactions with the matrix column (data not shown). The above experiments were repeated in the presence of dsDNA. Fig. 2*b*) shows the elution profile of the high-salt buffer RNAP–DNA mixture with two independent peaks: one for RNAP and the other for dsDNA (11.2 and 15.2 ml, respectively). We monitored the absorbance at two wavelengths: 280 and 260 nm. The calculated ratio at 260/280 nm of 0.55 (Hassell *et al.*, 2007) did not suggest bound DNA in the elution peak of the RNA polymerase. In contrast, the elution profile of the RNAP–DNA mixture run in low-salt buffer shows a well behaved protein with a symmetrical peak with no major tail peak corresponding to the DNA alone and with a 260/280 nm ratio of 1.2 that indicates co-elution of the DNA with the RNAP peak (Fig. 2*c*). As a result, we set up crystallization screenings with the low-salt buffer RNAP–DNA sample. However, this strategy did not yield crystals. Therefore, we implemented a protocol that would decrease the salt concentration within the already formed crystals to favour the binding of DNA to RNAP upon DNA soaking (see below).

2.3. Crystal buffer-exchange technique prior to DNA soaking

Morphologically indistinguishable *Ssh*RNAP crystals have been shown to pack in three different space groups, $P2_12_12$ (one RNAP molecule per asymmetric unit), $P2_1$ and $P2_12_12_1$ (two RNAPs per asymmetric unit), and in the best cases diffracted to between 3.4 and $3.8 \text{ \AA}$ resolution (Wojtas *et al.*, 2011). Once suitable newly optimized apo *Ssh*RNAP crystals had been obtained, an *in situ* buffer-exchange protocol was implemented to enable the binding of DNA to RNAP molecules in soaking trials. Selected crystallization-seeded sitting drops ($0.45 \mu\text{l}$ total drop volume) with apo RNAP crystals were mixed by manually pipetting $0.5 \mu\text{l}$ low-salt buffer (50 mM HEPES pH 7.5, 50 mM KCl, 2.5 mM MgCl_2 , 100 mM sodium cacodylate, 100 mM SrCl_2 , 10% glycerol, 9% PEG 5K MME) on top of the drop. Concomitantly, the crystallization solution within the reservoir was exchanged for low-salt buffer. The drop was resealed and left to equilibrate overnight. After 16–20 h, the crystals were inspected for the presence of morphological defects or cracking possibly caused by osmotic shock (Ray *et al.*, 1991). The majority of the inspected crystals did not show any visual imperfection and three additional steps of buffer exchange were implemented to ensure a further

Figure 3

Schematic of the crystal-desalting protocol implemented *in situ*. Apo *Ssh*RNAP crystals were grown by vapour-diffusion and sitting-drop techniques using microplates and the microseeding method. The crystals were initially buffer-exchanged by adding $0.5 \mu\text{l}$ low-salt buffer to the crystallization drop together with complete buffer exchange of the reservoir and left to equilibrate for 16–20 h. The crystals were then washed twice by removing $0.5 \mu\text{l}$ of the solution and adding a fresh $0.5 \mu\text{l}$ of low-salt buffer. Finally, $0.5 \mu\text{l}$ was aspirated and exchanged for $0.5 \mu\text{l}$ of buffer containing dsDNA. Crystals were soaked for 4–14 h, which was followed by cross-linking using $0.5 \mu\text{l}$ 25% glutaraldehyde placed in the adjacent well.

lowering of the salt concentration. This was achieved by removing 0.5 µl of solution from the drops and adding a fresh 0.5 µl of low-salt buffer; the drops were allowed to mix for 30 min each time while visually inspecting the crystals. Finally, 0.5 µl of solution was removed from the drop and exchanged for 0.5 µl low-salt buffer containing DNA at a tenfold molar excess over the initial nominal RNAP concentration. The DNA was left to soak for 4–14 h (Fig. 3). The crystals were then cross-linked for 30 min by gentle diffusion of 0.5 µl 25% glutaraldehyde (Sigma–Aldrich) added to the well adjacent to the crystallization drop (Heras & Martin, 2005; Fig. 3). The successful diffracting crystal (see below) was cross-linked after ~7 h of DNA soaking (Lusty, 1999; Ray *et al.*, 1991; Wine *et al.*, 2007). All soaked crystals were flash-cooled in liquid nitrogen using a cryoprotectant composed of crystallization solution with 25% glycerol as well as DNA.

2.4. Data collection and crystallographic analysis

Diffraction data were collected from DNA-soaked RNAP crystals on beamline BM14 at the European Synchrotron Radiation Facility (ESRF), France. Of the several putative *Ssh*RNAP–DNA crystals tested, a crystal belonging to space group $P2_1$ with two molecules per asymmetric unit diffracted to a useful resolution of 4.3 Å. Data were processed using the *HKL* program suite (Otwinowski & Minor, 1997). The structure was solved by the molecular-replacement technique using *Phaser* from *CCP4* (Winn *et al.*, 2011) and refined with *PHENIX* (Adams *et al.*, 2002), and showed the presence of DNA bound to each of the two RNAP molecules in the asymmetric unit (for data-collection and refinement statistics, see Table 1 in Wojtas *et al.*, 2012).

3. Results and discussion

The archaeal RNAP from Sulfolobales and Desulfurococcales species (Crenarchaeota phylum) is a multi-subunit enzyme composed of 13 subunits with a total molecular mass of ~400 kDa. Its overall architecture, subunit composition and organization resemble those of the eukaryotic Pol II. Therefore, in order to further understand this ancestral relationship, we investigated *Ssh*RNAP in complex with DNA (Wojtas *et al.*, 2012), but routine complexation strategies, including soaking techniques, failed owing to the incompatibility of the crystal nucleation/growth buffer and the DNA-binding buffer. Therefore, we explored a protocol of crystal desalting inspired by previous work on relatively small proteins (from 50 to 60 kDa) in which crystal mother liquor containing a high concentration of ammonium sulfate (~2.3 M) was removed to favour the binding of a small ligand (Schreuder, 1988; Ray *et al.*, 1991; Zhang *et al.*, 1994). However, in contrast to the above studies, our targets for slow desalting were crystals of the archaeal RNAP, a large macromolecular machine of ~400 kDa composed of 13 distinct subunits, which was then soaked with dsDNA. Many of the structures of DNA–protein complexes solved to date have used an *ab initio* cocrystallization strategy (Luscombe *et al.*, 2000; Tan & Davey, 2011), including the eukaryal Pol II–DNA complexes, crystals of which have been obtained by mixing the enzyme with dsDNA (and RNA) in the presence of 40 mM KCl (Gnatt *et al.*, 2001; Kettenberger *et al.*, 2004; Sydow *et al.*, 2009). The bacterial holoenzyme was also cocrystallized with DNA (a 35 bp longer strand) but in the presence of 1.6–1.8 M ammonium sulfate and the crystals diffracted to 6.5 Å resolution (Murakami *et al.*, 2002). Interestingly, a rapid desalting step in the presence of DNA has proven to be successful in a recent study of the DnaA–ssDNA complex, although this case involved an ~30 kDa

protein forming a tetramer binding to one ssDNA per asymmetric unit (Duderstadt *et al.*, 2011).

In conclusion, we succeeded in buffer-exchanging crystals of the archaeal *Ssh*RNAP from a nominal concentration of ~500 mM KCl to ~60 mM KCl with no visible damage and subsequently soaking them with dsDNA. Thus, we obtained an *Ssh*RNAP–DNA complex crystal that diffracted to 4.3 Å resolution and belonged to space group $P2_1$ with a total of ~0.8 MDa protein in the asymmetric unit.

This achievement could in principle encourage the application of such a protocol to crystals of other large macromolecular assemblies where the crystal-growth conditions and standard soaking techniques are incompatible with the binding of ligands.

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