LETTER

ATP-dependent looping of DNA by ISWI

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Received 12 May 2008, revised 4 July 2008, accepted 9 July 2008
Published online 6 August 2008

Key words: tethered particle motion, ISWI, chromatin remodeling, atomic force microscopy, DNA looping, DNA supercoiling

Supporting Material is available online under www.biophotonics-journal.org (abstract link of the article).

Snf2 related chromatin remodelling enzymes possess an ATPase subunit similar to that of the SF-II helicases which hydrolyzes ATP to track along DNA. Translocation and any resulting torque in the DNA could drive chromatin remodeling. To determine whether the ISWI protein can translocate and generate torque, tethered particle motion experiments and atomic force microscopy have been performed using recombinant ISWI expressed in E. coli. In the absence of ATP, ISWI bound to and wrapped DNA thereby shortening the overall contour length measured in atomic force micrographs. Although naked DNA only weakly stimulates ATP hydrolysis by ISWI, both atomic force microscopy and tethered particle motion data indicate that the protein generated loops in the presence of ATP. The duration of the looped state of the DNA measured using tethered particle motion was ATP-dependent. Finally, ISWI relaxed positively supercoiled plasmids visualized by atomic force microscopy. While other chromatin remodeling ATPases catalyze either DNA wrapping or looping, both are catalyzed by ISWI.

1. Introduction

Chromatin remodelling proteins are a burgeoning group of ATP-hydrolyzing machines that are critical for embryogenesis and development [1]. These proteins direct access to DNA by regulating chromatin structure on several levels [2]. Their trademark biochemical functions are the mobilization or modification of nucleosomes to expose appropriate DNA sequences. These proteins have been grouped into four functionally/structurally similar families: SWI/SNF, ISWI, NURD/Mi-2/CHD, and INO80 and

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SWR1 [3]. The SWI/SNF and ISWI families have been best characterized and although they both utilize ATP to power DNA translocation, they remodel chromatin in distinct ways. While “many ISWI-containing” complexes reposition nucleosomes to create ordered arrays (an exception is the ISWI containing NURF complex which generates disorder) [4], SWI/SNF related complexes act to disrupt nucleosome structure [5]. A number of SWI/SNF-type chromatin remodelling proteins have been well characterized using single molecule assays. For example the RSC protein was shown to translocate DNA at up to 200 base pairs/s to create a negatively supercoiled loops an average of 450 base pairs (bp) long [6]. Similar translocation on nucleosomal substrates slowed to 13 bp/s for 100 bp distances but only stalled for forces above 12 pN [7]. RSC is a large complex and the structure of the yeast homolog includes a large cavity in which extensive contact with the nucleosomal DNA most likely identifies the translocase domain [8, 9].

Although equivalent structural detail has yet to become available for ISWI family members, careful footprinting assays have shown regions of contact with nucleosomes to suggest a pumping mechanism that drives a small loop of DNA around the histone octamer [10]. This data and photochemical crosslinking of ISW2 [11] indicate a translocase domain positioned near the dyad of the histone octamer with nucleosomes to suggest a pumping mechanism for ISWI although nucleosomal substrates were excluded by judging the symmetry of the scatter of coordinates [17, 18].

Experimental

Preparation of the flow chamber for tethered particle motion

The flow chambers for TPM measurements were similar to those described by Finzi and Dunlap [15]. A flow chamber was incubated for 30 minutes with 20 µg/ml anti-digoxigenin (Sigma, St. Louis, MO) in PBS. After washing the flow chamber with 800 µl of ISWI buffer (50 mM KCl, 10 mM HEPES (pH 7.8), 3 mM MgCl₂, 0.1 mM DTT and 60 µM BSA) a 900 bp biotin- and digoxigenin-labelled DNA amplified from pUC19 was introduced and incubated for 1 hour before washing out unbound DNA with 800 µl ISWI buffer. An excess of 430 nm diameter, streptavidin-coated beads (Indicia, Oullins, France) in ISWI buffer was then introduced and incubated for 30 minutes. Unattached beads were then flushed from the chamber with ISWI buffer. Experiments were carried out in ISWI buffer and 20 nM concentrations of protein. Recombinant Drosophila ISWI was purified as described previously [16].

Tethered particle motion (TPM) measurement

Video–enhanced, differential interference contrast micrographs of the tethered beads were recorded with a CCD camera (JAI A60, Copenhagen, Denmark) at 25 frames/second on a SuperVHS recorder. Images were digitized during playback with a PCI-1409 frame grabber (National Instruments, Austin, Texas, USA) and analyzed using custom software to determine the x and y coordinates of the bead versus time. A 100 point (4 second) moving average was used to determine the drift-corrected x and y coordinates of the anchor point of the DNA tether, and the Brownian motion of the bead was then calculated as: $\rho = \sqrt{(x-x_0)^2 + (y-y_0)^2}$, with $\rho_{m}$ Conditions were chosen to produce widely spaced (non-interfering) beads on the glass and multiple tethers were excluded by judging the symmetry of the scatter of xy coordinates [17, 18].

AFM imaging

DNA was synthesized by PCR amplification of an 891 bp segment of the plasmid pUC19 and purified with a PCR purification kit (Qiagen, Hilden, Germany). About 100 ng of this DNA was incubated for 8 minutes with equimolar amounts of ISWI in 500 µl ISWI buffer (without BSA and with or without ATP (25 µM)). After incubation, 5–10 µl were deposited at room temperature for 2–3 min on freshly cleaved mica (Ted Pella Inc., Redding, CA) coated with poly-L-ornithine (MW 35,000 Sigma). The mica was then gently washed with 1 ml of HPLC grade H₂O (Sigma) and subsequently dried with a stream of nitrogen gas. The sample was imaged in air with a NanoScope IV atomic force microscope (Digital Instruments, Santa Barbara, CA).
operating in tapping mode. The AFM images were analyzed with the open source program ImageJ (NIH, Bethesda, MD) to determine the contour lengths of single DNA molecules. Single-lobed but not multi-lobed blobs were included in the contour length analysis to avoid skewing the results with DNA bearing multiple proteins. The program WSxM (Nanotech, Inc., Madrid, Spain) was used to determine the height and diameter of the proteins alone and bound to DNA.

Supercoiled plasmid analysis

Plasmid pUC19 was positively supercoiled by adding chloroquine to nicked plasmid and ligating with T4 ligase (NEB) followed by purification with a PCR purification kit (Qiagen). Plasmids were reacted with ISWI at an equimolar ratio for 5 minutes in the presence of 25 μM ATP. This solution was used to prepare specimens for AFM as described above. Images were analyzed by counting the number of intersections between segments in the supercoiled plasmids. In plectonemic sections, the length of the plectoneme was divided by the dimension of a single intersection to determine the number of crossovers.

Results and discussion

ISWI binding to DNA

Some chromatin remodeling proteins have been shown to induce supercoils in DNA substrates [6, 19] that could mobilize nucleosomes or provoke unwrapping [20]. The idea that the generation of torsion may catalyze nucleosome repositioning [21] as suggested by the early work of Havas et al. [13] and Gavin et al. [22] has fallen out of favor, because nicked substrates often remodel as efficiently as substrates without nicks. One exception to this is a localized need for torsion in remodeling near the nucleosome pseudodyad (superhelix location 2) for the yeast ISW2 complex [23]. In addition, recent single molecule experiments have shown that in the SWI/SNF family the generation of torsion is coupled to DNA loop formation [6, 7, 19]. Although ISWI is a similar DNA translocase [12] which is hypothesized to push loops of DNA onto and around nucleosomes

![AFM data on the ISWI/DNA complex](online colour at: www.biophotonics-journal.org)

Figure 1 (online colour at: www.biophotonics-journal.org) AFM data on the ISWI/DNA complex. a) AFM image of bare, linear DNA molecules and unbound ISWI. b) AFM image of an ISWI/DNA complex in the absence of ATP. c) AFM image of two ISWI/DNA complexes in presence of 25 μM ATP, one located at the middle (lower) and another at the end (upper) of the molecule. d) Histograms of the contour length of DNA in AFM images such as those in a, b and e. 202 molecules without protein averaged 306.7 +/− 1.0 nm in length; 109 molecules in the presence of 20 nM ISWI averaged 268.7 +/− 2.1 nm in length; 91 molecules in the presence of 20 nM ISWI and 25 μM ATP averaged 199.9 +/− 6.1 nm in length. e) Histogram of the angle (diagrammed in inset of b) between segments of DNA entering and exiting the complex in the absence of ATP.
To study the form of the ISWI/DNA complex (i.e. to determine whether the DNA is wrapped on the enzyme or gathered into a supercoiled loop), an AFM was used to image the protein bound to linear, 900 bp DNA in the absence of ATP. ISWI proteins do not require ATP for binding to DNA or chromatin [12, 14]. For individual proteins bound to DNA shown in Figure 1b, the average angle between the DNA segments entering and exiting the complexes was about 120° (Figure 1c). In addition, the apparent contour length of the DNA was an average of 38 nm (113 bp) shorter in presence of ISWI (Figure 1c), suggesting that the DNA was actually wrapped about the enzyme. Indeed, no DNA loop emanates from the bound protein although the DNA is frequently sharply bent by the enzyme. A looped segment of the same size would be expected to have a 5 nm radius and exhibit a central opening. The slightly bigger loop in the lower left panel of figure 4b is only 58 nm in circumference with a radius of 7.5 nm but the central opening is clearly visible.

If the DNA were wrapped around two thirds of the protein, then the length of DNA should be slightly greater than a two-thirds circumferential arc around the protein. Individual protein molecules were measured in AFM images both associated with DNA and isolated on the poly-L-ornithine-coated mica surface (Figure 1). The radius of the protein increased from 7.8 to 11.2 nm upon association with DNA which could be due to the DNA encircling the protein (Figure 2). Using a simple geometrical model of a filament wrapping two-thirds of a disc and assuming that the wrap has a radius of 9.6 nm (radius of ISWI alone, 7.8 nm, plus one-half of the difference with and without protein, 1.8 nm), one can estimate that approximately 40 nm of DNA would be required. This value is in rough agreement with the DNA contour length reduction observed in presence of ISWI. The CSB protein, which is a member of the SWI2/SNF2 family of ATP-dependent chromatin remodeling factors, has also been shown to wrap DNA in the absence of ATP. It was hypothesized that CSB functions as a dimer which has also been suggested for ISWI [5]. However, for AFM imaging, ISWI and DNA were mixed in equimolar amounts (see methods) and no clustering of protein was observed that would suggest high cooperativity. In addition, the dimensions of ISWI bound to DNA measured in these AFM images averaged only 7.8 nm in diameter and 1 nm in height to give a cylindrical volume of 198 nm³. Considering that proteins generally have a partial specific volume of 0.74 cm³/g and that ISWI has a molecular weight of 140 kDa, the protein should occupy 260 nm³. Therefore ISWI visualized with AFM most likely bound DNA as a monomer. In contrast to ISWI and CSB, DNA bound to another chromatin remodeler, the RSC complex, did not show any change in the length of the DNA molecule [6]. Thus different chromatin remodeling proteins bind DNA in different ways that presumably correlate with their specific functions.

**DNA translocation by ISWI**

While relaxed DNA generally lies on the substrate in open forms, equivalently sized segments of supercoiled DNA have writhe that gathers the DNA into contorted shapes in which the path of the duplex is obscured. In presence of 25 μM ATP, the complex was often found near the end of a DNA molecule associated with an amorphous coil of DNA (78% of...
This could result if the enzyme were to move along the DNA without dissociating rapidly from the ends. When the protein was found in the middle of the molecule, it was often associated with a hairpin capping a plectonemic region of tightly wound DNA helices with single duplex tails emerging on the other end (Figure 1c lower). These results contrast starkly with images recorded in absence of ATP in which only 14% of 109 protein-bearing DNA molecules were bound at extremities. In the remaining 86% of ISWI-bound DNA molecules, individual proteins were distributed randomly along the DNA with no evidence of looping or supercoiling. The average contour length of the DNA fragment with ISWI bound in the presence of ATP was 106 nm shorter than the naked DNA. It is difficult to imagine that a single enzyme can spool 300 bp of DNA onto itself and seems more likely that it must create a loop in the DNA. It has been shown, using single molecule assays, for the RSC chromatin remodelling complex [6, 7]. These new AFM data support the idea that ISWI binds and hydrolyzes ATP to execute a cycle that translocates DNA [14].

To corroborate the AFM experiments, the extension of single DNA molecules exposed to ISWI with and without ATP was monitored using tethered particle motion assays [15, 25] (Figure 3a). In the absence of protein, a microsphere tethered to a glass slide through a single DNA molecule exhibits Brownian motion limited by the length of the tether. The RMS deviation of the microsphere about the anchor point is an observable indication of the tether length that may change as DNA binding proteins modify topology [17].

Figure 3b is a plot of the two-dimensionally projected distances of the microsphere (bead) from the anchor point versus time, which in the absence of ISWI are broadly scattered. The 4 second moving average of this signal is a stable trace at about 125 nm RMS deviation (red solid line). This was among the higher levels measured after correcting for drift using a 4 second moving average of the position of the bead under analysis. This time window is a compromise to eliminate low frequency drift without excessively attenuating the higher frequency bead mo-

Figure 3 (online colour at: www.biophotonics-journal.org) Loops detected with tethered particle motion: a) A schematic diagram shows how 430 nm diameter microspheres were tethered to a glass surface. b) Tethered particle motion without protein exhibits a broad scatter (green) of tether lengths that does not change during the course of the experiment. The 4-second moving average of this projected distance between the microsphere and the anchor point for a representative DNA tether without protein equals approximately 125 nm. c) Tethered particle motion with 60 µM ATP. Intervals exhibiting the broad scatter of the extended DNA alternate with intervals of considerably shorter projected tether lengths. d) Tethered particle motion with 250 µM ATP. The durations of short and long tether lengths were measured as \( t_{on} \) and \( t_{off} \). e) The average duration of the shorter tether length as a function of ATP concentration. The red curve is a fitted Michaelis-Menten function with \( K_m = 15.1 \pm 2.9 \) µM and \( t_{on, max} = 87.7 \pm 3.8 \) seconds. f) The average duration of the longer tether length linearly decreased as ATP concentration increased.
tion [17]. Nonetheless, the RMS deviation observed for control (no ISWI) beads was below the 200 nm that would be expected for 900 bp of DNA, and therefore loop lengths derived from the TPM data could not be readily interpreted.

Instead, a lifetime analysis of the bistable time-series recorded after adding ISWI produced meaningful information. 20 nM ISWI with 60 μM ATP caused abrupt, reproducible transitions between states with 125 and 50 nm RMS deviations (Figure 3c). Since the amplitudes of these states are constant and even the lower RMS deviation is distinct from that of a bead stuck on the surface (see supplementary information figure S3), the observed transitions were identified as ISWI/ATP-hydrolysis induced contour length changes in the DNA. Figure 3d shows a representative trace with transitions observed using 250 μM ATP in which intervals of the long and short tether states were also observed. For a series of ATP concentrations, the lifetimes of the two states were found to be exponentially distributed (see supplementary information figure S2) and constants representing the characteristic lifetimes for both states were determined by curve fitting. These constants are plotted versus ATP concentration in Figures 3e and f. The lifetime of the long tether state progressively diminished as the ATP concentration was increased. However the characteristic lifetimes of the short tether fell along a Michaelis-Menten curve with $K_m = 15.1 \pm 2.9 \mu M$ and $t_{\text{max}} = 87.7 \pm 3.8$ s. Such a $K_m$ is consistent with micromolar values determined for other ATPases [26]. This behavior might suggest that ATP stabilizes ISWI binding to DNA, but high affinity is observed even without ATP in 1:1, nanomolar concentrations of protein and DNA (Figure 1A). Alternatively, ISWI might hydrolyze ATP to maintain the looped or wrapped DNA state. Preliminary data collected using magnetic tweezers to gently stretch nicked DNA in the presence of ISWI and ATP shows variable degrees of tether length contractions [27]. This variability is more compatible with looping rather than wrapping, which is constrained by the available surface area of the protein.

**Plasmid relaxation**

Whether by wrapping or looping, ISWI has previously been shown to alter the supercoiling of chromatin templates [13]. As seen in Figure 1, ISWI in the presence of ATP gathered almost 300 bp of DNA near the bound protein. To further investigate this activity, positively supercoiled plasmids were generated, incubated with ISWI and ATP, and imaged using atomic force microscopy. Figure 4a shows representative supercoiled plasmids with loops separated by single nodes (intersections between DNA segments) or series of nodes in a tightly wound plectoneme. After incubation with ISWI and ATP, most plasmids had fewer loops, and nodes and plectonemic stubs were often near the protein as shown for the plasmids in Figure 4b. Ensembles of plasmids were imaged and nodes were counted. Histograms of this data show that incubation with ISWI greatly reduced the number of nodes. This topological change results when ISWI creates a positively supercoiled loop which is compensated by the introduction of negative supercoils that relax the remainder of the plasmid. Thus fewer nodes are visible and the writhe distortions in the plasmids are concentrated in the loop maintained by ISWI.
Conclusion

ISWI has been proposed to translocate on DNA by pulling a loop of extranucleosomal DNA onto the surface of the nucleosome [24]. The loop might then be pumped across the nucleosome by the translocase domain which makes contact near the dyad [11]. Similarly to ISW2 [14], ISWI bound randomly to linear DNA without ATP. Substantial shortening of the DNA indicated that about ten helical turns of DNA were wrapped around the monomeric protein in a manner reminiscent of that observed for the SWI/SNF family protein CB [19]. As has been shown for the SWI/SNF family archetype RSC [6], when ATP was available, ISWI shifted toward the ends of the fragment and produced supercoiled loops. This was likely the result of ATP-driven translocation on the DNA, since the duration of protein-induced transitions in tethered particle motion assays was ATP-dependent. Recombinant *Drosophila* ISWI appears to generate supercoiled, looped DNA templates by initially wrapping DNA and then positively supercoiling it during translocation. Whether or not this behaviour persists when ISWI is assembled with its usual subunit partners, remains to be investigated. Generally speaking, loop formation coupled to DNA translocation appears to be a common mechanism among chromatin remodelling enzymes.

Acknowledgements This work was supported by the Human Frontier Science Program (LF), Italian Funding of Basic Research (DD, LF), and the Wellcome Trust (T.O-H).

References