Single-molecule level structural dynamics of DNA unwinding by human mitochondrial Twinkle helicase

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Running title: Single-molecule dynamics of Twinkle DNA helicase

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Keywords: atomic force microscopy, DNA binding protein, DNA Helicase, DNA replication, mitochondrial disease, mitochondrial DNA (mtDNA), single molecule biophysics, Twinkle, structural dynamics, ssDNA-binding protein (SSB)

ABSTRACT

Knowledge of the molecular events in mitochondrial DNA (mtDNA) replication is crucial to understanding the origins of human disorders arising from mitochondrial dysfunction. Twinkle helicase is an essential component of mtDNA replication. Here, we employed atomic force microscopy (AFM) imaging in air and liquids to visualize ring assembly, DNA binding, and unwinding activity of individual Twinkle hexamers at the single-molecule level. We observed that the Twinkle subunits self-assemble into hexamers and higher-order complexes that can switch between open and closed-ring configurations in the absence of DNA. Our analyses helped visualize Twinkle loading onto and unloading from DNA in an open-ringed configuration. They also revealed that closed-ring conformers bind and unwind several 100 base pairs of duplex DNA at an average rate of ~240 bp/min. We found that addition of mitochondrial ssDNA-binding protein (mtSSB) both influences the ways Twinkle loads onto defined DNA substrates and stabilizes the unwound ssDNA product, resulting in a ~5-fold stimulation of the apparent DNA-unwinding rate. mtSSB also increased the estimated translocation processivity from 1750 bp to >9000 bp before helicase disassociation, suggesting that more than half of the mitochondrial genome could be unwound by Twinkle during a single DNA-binding event. The strategies used in this work provide a new platform to examine Twinkle disease variants and the core mtDNA replication machinery. They also offer an enhanced framework to investigate molecular mechanisms underlying deletion and depletion of the mitochondrial genome as observed in mitochondrial diseases.

Mitochondria produce >90% of cellular ATP through the process of electron transport and oxidative phosphorylation (1). The mitochondrial genome encodes thirteen subunits of the electron transport chain and is essential for cellular respiration (2). Human mitochondrial DNA (mtDNA) is a double-stranded, circular, 16569 base pair genome with a purine-rich heavy (H-) strand
and a pyrimidine-rich light (L-) strand. Depletion of mtDNA or accumulation of point mutations and deletions in mtDNA directly leads to mitochondrial dysfunction. Inherited defects in mtDNA maintenance result in degenerative and neuromuscular diseases and are a major driving factor in the aging process (3-6).

The mitochondrial genome is replicated by a unique set of nuclear-encoded enzymes that is distinct from the nuclear DNA replication machinery (7-9). A minimal DNA replication apparatus capable of synthesising ~16 kb of DNA in vitro is formed by combining mitochondrial DNA polymerase γ (Pol γ encoded by POLG and POLG2) with Twinkle helicase (encoded by TWNK) and the mitochondrial single-stranded DNA binding protein (mtSSB encoded by SSBP1) (10). Human Pol γ is a heterotrimer consisting of a monomeric catalytic subunit POLG and a homodimeric processivity subunit POLG2 (11-13). POLG (140 kDa) catalyzes 5’-3’ DNA polymerase, 3’-5’ exonuclease, and 5’ dRP lyase activities (14,15), whereas POLG2 (55 kDa) enhances DNA binding and processivity of the Pol γ holoenzyme (11,16). Twinkle is homologous to bacteriophage T7 gene 4 helicase-primase (17) and is able to assemble onto a closed circular ssDNA template in the absence of nucleotide cofactors or a specialized loader protein (18). Twinkle unwinds dsDNA with 5’-3’ directionality (17,19), and functional interaction between the Pol γ catalytic subunit and Twinkle facilitates efficient DNA replication (10). Human mtSSB enhances primer recognition and processivity of Pol γ (20-22) and specifically stimulates polymerase γ activity by organizing the DNA template and eliminating secondary structure (23,24). DNA unwinding by Twinkle is specifically stimulated by the mtSSB protein (19,20,25), and ChIP-seq experiments indicate mtSSB binds and stabilizes the displaced parental H-strand during mtDNA replication in vitro (26).

Twinkle is essential for maintenance of mtDNA, as knockdown of TWNK (previously designated C10orf2) expression by RNAi in cultured human cells reduces mtDNA copy number (27), and conditional knockout of TWNK in mice results in severe and rapid mtDNA depletion in targeted tissues and embryonic lethality (28). Transgenic mice expressing an autosomal dominant progressive external ophthalmoplegia (PEO) allele of TWNK accumulate multiple mtDNA deletions during aging and develop progressive respiratory dysfunction (29) through a mechanism driven by stalled mtDNA replication (30). Disease alleles of the human TWNK gene are associated with improper mtDNA maintenance and cosegregate with severe heritable neuromuscular disorders, including infantile onset spinocerebellar ataxia, PEO with multiple mtDNA deletions, hepatocerebral mtDNA depletion syndrome, and Perrault syndrome (17,31). Genetic analysis of affected patients has identified at least 49 missense mutations in TWNK that conspicuously cluster in the linker region and C-terminal helicase domain of Twinkle (31). Biochemical analyses of many of these disease variant proteins indicate diminished DNA unwinding activity and altered capacity to form stable hexameric conformations (32-35), consistent with expectations from structural models that predict the C-terminal domain is essential for hexamerization (36,37).

Knowledge of the molecular mechanisms governing mtDNA replication is crucial to understanding the origins of human disorders known collectively as “mitochondrial diseases”. Although proteomic analysis of mitochondrial nucleoids and biochemical studies with purified proteins have identified core protein components of the mtDNA replication fork (38), specific information on the composition, stoichiometry, conformation, and dynamics of protein-protein and protein-DNA interactions remains less well defined. We wanted to assess the mechanism of Twinkle helicase binding and unwinding DNA in vitro. We were concerned that the varied stability of wild type Twinkle helicase and certain disease variants would limit the utility of bulk biochemical assays due to ensemble averaging effects, so we selected a single-molecule technique. Atomic force microscopy (AFM) is a single-molecule technique that does not require labeling or chemical modifications of biomolecules including proteins and DNA (39-42). AFM is particularly advantageous to examine Twinkle function on long DNA substrates while accounting for structural heterogeneity. Accordingly, we employed direct imaging by AFM in air and liquids to investigate the structure and dynamics of assembly, DNA
binding, and unwinding activity of individual Twinkle hexamers.

Here, we found that Twinkle exists as a heterogeneous population of oligomeric states that self-assemble into hexamers capable of switching between open- and closed-ring configurations in the absence of DNA. Twinkle appears to load onto and to unload from circular gapped plasmid DNA in the open-ring configuration. Closed-ring conformers unwind several hundred base pairs of duplex DNA when ATP is present, and Twinkle disassociates from the DNA upon completion of unwinding. Displaced ssDNA products fold into higher-order structures at extended reaction times. Addition of mtSSB stimulates DNA unwinding by Twinkle by modulating the ways Twinkle loads onto DNA and by stabilizing the unwound ssDNA product. This study provides a new platform to examine Twinkle disease variants and the core mtDNA replication machinery. Our single-molecule approach will facilitate investigation of molecular mechanisms underlying deletion and depletion of the mitochondrial genome as seen in mitochondrial diseases.

Results
AFM imaging in air reveals the heterogeneity of Twinkle oligomers

Hydrodynamic analysis indicates that, like other members of the Superfamily 4 DNA helicases, purified recombinant human Twinkle exists principally as a hexamer in solutions with moderate ionic strength (34,43). Low salt conditions promote disassociation of oligomeric structures and protein aggregation, but addition of divalent metal ions, nucleotides, and nonionic detergents facilitate assembly and stabilization of higher order oligomers as large as heptamers (34,44). Examination of oligomeric structure by two dimensional electron microscopy of adsorbed Twinkle complexes showed coexistence of hexamer, heptamer, and open-ring structures and suggested a dynamic interconversion between the three forms (44). A recent analysis of Twinkle in solution by small angle X-ray scattering also revealed a mixture of hexameric and heptameric conformations, and construction of a 3D-averaged molecular model from cryo-electron microscopy images of glutaraldehyde cross-linked Twinkle hexamers revealed two distinctly stacked layers with 6-fold rotational symmetry (37). Recombinant human Twinkle helicase was purified to >95% homogeneity and its robust DNA helicase activity in vitro was validated using a forked oligonucleotide substrate (Supplementary Figure S1), as described previously (34) and in Experimental Procedures. We then utilized AFM to assess the volume distribution of individual Twinkle complexes in buffer containing 0.4 M NaCl without chemical cross-linking or prior selection for the hexameric subpopulation. The average AFM volume (552 ±347 nm3) confirmed that the majority of Twinkle complexes occupied higher oligomeric states (volume > 450 nm3) at this salt concentration in the absence of DNA (Supplementary Figure S2).

We also applied AFM imaging to assess conformational heterogeneity of Twinkle oligomers at 0.1 M NaCl to test conditions more favorable to DNA binding and enzymatic activity. When Twinkle helicase (100 nM) was deposited onto a mica surface in the presence of ATP (4.5 mM) and imaged using the AC AFM imaging mode in air, Twinkle oligomers were identified as spatially resolved particles in both AFM topographic and phase images, with phase images providing superior contrast (Figure 1). Various oligomers with clearly identifiable subunits were visually classified as monomeric (26%), dimeric (19%), trimeric (20%), tetrameric (9%), pentameric (12%), and hexameric (14%) complexes (N=162). AFM volumes of complexes were measured (Supplementary Figure S3A) and compared to globular proteins with known molecular weights (Supplementary Figure S3 B). AFM volumes of globular proteins increase linearly with their molecular weights (39,45), and this trend holds for the various Twinkle oligomers (Supplementary Figure S3B), although AFM volumes increase more steeply due to extra space between Twinkle subunits (Figure 1) relative to globular reference proteins of matched molecular weights (45). To control for image selection bias, the AFM volumes of all Twinkle complexes were also plotted. The data revealed that the majority (82.0%) of Twinkle complexes were dimers, trimers or tetramers (<450 nm3), whereas a minority (18.0%) occupied higher oligomeric states such as pentamers, hexamers or heptamers (volume > 450 nm3) in the absence of
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DNA (Supplementary Figure S3C). Among hexameric Twinkle complexes (N=83), 35% existed in the closed-ring configuration (Figure 1F) and 65% existed as open rings (Figure 1G). Collectively, these results indicate that Twinkle exists in solution as a heterogeneous mixture of oligomers in the absence of DNA.

**Twinkle oligomers self-assemble on DNA**

Twinkle is able to bind and unwind a closed circular DNA template in vitro, indicating that neither a free DNA end nor a specialized helicase loader are required for the helicase to function (18). Structural studies indicate that ring helicases load onto such substrates either by assembling from monomers into a ring around the DNA or by cracking open a pre-assembled ring to allow entry of ssDNA (46). Twinkle may behave like other hexameric helicases and adopt a non-planar conformation during translocation or convert to a hexameric right-handed helix when loading onto ssDNA (47,48). The observed distribution of the Twinkle oligomer population suggests active Twinkle hexamers may assemble from lower order oligomers such as dimers and trimers, as proposed for *E. coli* DnaB or bacteriophage T7 gene product 4 (49,50). Preincubation of Twinkle with ssDNA oligonucleotides (108 nt) prior to AFM imaging (ratio = 6 subunits/oligonucleotide) shifted the fraction of higher order Twinkle oligomers (AFM volume > 450 nm$^3$) from 18.0% to 33.3% (Supplementary Figure S3C), suggesting the assembly of Twinkle hexamers is facilitated by the presence of ssDNA. To begin to decipher the mechanism by which Twinkle helicase loads onto DNA, we pre-incubated Twinkle with a circular DNA substrate containing site-specific 37 nt ssDNA gaps (Figure 2A). Two ssDNA gaps were generated by removing short ssDNA segments from one DNA strand between closely-spaced nick sites (51), and the presence of the ssDNA gaps was validated by restriction digestion (see Experimental Procedures). Individual protein-DNA complexes were examined by AFM imaging in air. Various lower order Twinkle oligomers were observed to bind the DNA in the absence of ATP (Supplementary Figure S4A-D), although the specificity for single-stranded gapped regions cannot be determined because the circular substrate lacks a DNA end to be used as a reference point. Higher order Twinkle oligomers also bind the DNA, and a subpopulation of Twinkle oligomers bind in an open-ring configuration with DNA clearly visible (Supplementary Figure S4E). Taken together, our observations support a model in which Twinkle monomers spontaneously assemble into higher order structures that admit DNA through an opening in the helicase ring.

**Twinkle binds ssDNA gaps with high specificity**

Twinkle helicase binds single-stranded, double-stranded, and forked DNA substrates with high affinity ($K_{d(DNA)}$ is 5-10 nM) as determined by electrophoretic mobility shift assay and fluorescence anisotropy (34,52). However, these methods cannot distinguish the binding position of Twinkle on DNA ends or on ssDNA regions of artificial replication forks, nor can they identify the specific oligomeric structure of Twinkle bound to DNA. To refine our understanding of Twinkle binding to more complex DNA structures, we also produced a linear DNA substrate containing a site-specific 37 nt ssDNA gap flanked by double-stranded DNA arms (see Experimental Procedures). AFM imaging shows a contour length of 663.8 ±19.4 nm for this substrate (Figure 2A). The ssDNA gap is located 470 bp (23%, ~160 nm assuming 0.34 nm/bp) from one blunt end of the 2030 bp DNA fragment. Specific binding of mtSSB confirms the position and single-stranded nature of this gapped region (Figures 2B and 2D). The DNA-binding specificity of Twinkle helicase was determined by AFM analysis of Twinkle bound to the linear gapped DNA substrate in the absence of ATP (Figure 2C). The position distribution of bound Twinkle indicated preferential binding to a position 20.7% ±4.9% from the end, consistent with the position of the ssDNA gap (Figure 2D). Although some nonspecific DNA binding was observed on this gapped substrate, bound Twinkle was distributed randomly along the non-gapped linear DNA (Figure 2D). Analysis of the fractional occupancies (39) of Twinkle along the linear gapped DNA establishes that Twinkle has a high binding specificity ($S=K_{SP}/K_{NSP}$, ~2.5 x 10$^3$) for the ssDNA gap. In comparison, mtSSB displays a higher binding specificity ($S=K_{SP}/K_{NSP}$ = 1.45 x 10$^3$) determined by the same method (53). Highly specific binding of Twinkle to the ssDNA gap suggested this defined DNA substrate is ideally
suited to assess DNA unwinding at the single-molecule level.

**Twinkle unwinds DNA with 5'-3' polarity in a closed-ring configuration**

To evaluate DNA unwinding by Twinkle, we imaged the linear gapped DNA substrate (663.8 ±19.4 nm) following incubation with Twinkle in the presence of ATP. Inclusion of ATP caused the helicase to move away from the position of the gap, and structures resembling short segments of ssDNA were visible adjacent to Twinkle-DNA complexes in AFM images (Supplementary Figure S5A). The complexity of these structures prevents accurate assessment of the length of the unwound ssDNA segments, so instead we determined the contour length of the remaining duplex DNA following unwinding reactions. The average length of the remaining linear dsDNA (486 ±84 nm) was 73.2% ±12.8% of the total length of the original linear gapped DNA substrate (Supplementary Figure S5B), strongly suggesting Twinkle unwinds the short arm of this linear gapped DNA substrate.

Consistent with the strand-specific structure of the ssDNA gap (Figure 2A) and the obligate 5'-3' polarity of translocation for Superfamily 4 DNA helicases, we infer that Twinkle binds the substrate at the position of the ssDNA gap and translocates along the intact 2030 nt DNA strand to unwind the shorter DNA arm. Loss of the displaced DNA strand and compaction of ssDNA segments after unwinding complicates the analysis of ssDNA structures on this linear DNA substrate. Accordingly, we shifted our focus to a circular gapped DNA substrate to assess the configurations and actions of Twinkle helicase.

Although Superfamily 4 helicases may load and slide along DNA as open-ringed hexamers (46), the active form of the Twinkle is predicted to be a closed-ring hexameric configuration (17,36). AFM imaging shows Twinkle in the closed-ring configuration (N=101) bound to the circular gapped DNA substrate (Figure 3A) in the presence of ATP. In some cases, AFM phase images clearly reveal DNA passing through the center channel of the Twinkle ring (right panels, Figure 3A). Inclusion of ATP in the DNA binding reactions permits direct visualization of individual Twinkle complexes unwinding DNA, as unwound ssDNA products exhibit an AFM height (200 ±104 pm, Gaussian peak) notably shorter than that of the dsDNA (371 ±116 pm, Gaussian peak) substrate (Figures 3B and 3C). Control reactions with supercoiled or nicked circular DNA produced no detectable DNA unwinding events.

Importantly, the capacity of AFM imaging to unambiguously identify the ssDNA product has permitted us to discern the functional configuration of Twinkle during DNA unwinding. The circumference of active Twinkle complexes in our AFM images was 79.7 ±12.8 nm (Gaussian peak ±S.D.), which corresponds to a ring with a diameter of 25.4 ±4.1 nm (Figure 3D). This value agrees with the previously reported 24.0 nm maximum particle dimension for Twinkle in solution that was calculated from the small angle x-ray scattering pattern (37). Taken together, AFM imaging directly shows that Twinkle unwinds DNA in a closed-ring hexameric conformation.

**Quantifying DNA unwinding by Twinkle at the single-molecule level**

Typical DNA substrates utilized to assess DNA unwinding by Twinkle in vitro contain forked structures with short duplex regions and are constructed from synthetic oligonucleotides or by annealing oligonucleotides (≤ 60 nt) to single-stranded M13 DNA (19,34,52). Biochemical assay of Twinkle DNA helicase activity on such substrates bearing free 5'-tails (Supplementary Figure S1B) provided an average steady state unwinding rate of 2.16 ±0.19 bp/min for Twinkle hexamers (34). Since this value is normalized to total protein, the true unwinding rate is likely much higher because not all Twinkle monomers are assembled into higher order oligomers and bound to the DNA substrate in an active conformation. Accordingly, we sought to minimize ensemble averaging effects by quantifying the DNA unwinding activity of Twinkle at the single-molecule level. Unwinding reactions were assembled as before with the circular gapped DNA substrate, and regions of unwound single-stranded DNA were identified by their AFM height (as in Figures 3B and 3C). The length of ssDNA product generated in a 1 minute incubation was 121 ±98 nm (Gaussian peak ±S.D., N=221). Assuming one nucleotide of ssDNA spans 0.5 nm (54), the average rate of individual unwinding events is 242 ±195 bp of dsDNA unwound per minute (Figures 4A and 4C).
We note that the pSCW01 plasmid utilized to construct the DNA helicase substrate is a tandem duplication of 2030 bp (Experimental Procedures), so circular gapped substrates derived from pSCW01 possess two independent unwinding sites adjacent to ~2kb segments of duplex DNA available for unwinding. Helicase action on circular DNA molecules (N=76) displayed either single (80%) or double (20%) unwinding events (Figure 4A). Calculated rates of unwinding were not affected by having one or two independent unwinding events on the same DNA molecule. The DNA unwinding rate determined for single molecules correlates well with the steady state rate of ATP hydrolysis for Twinkle in the presence of calf thymus DNA (349 ±32 ATP hydrolyzed/minute per Twinkle hexamer), as previously reported for bulk biochemical reactions (34). The similar rates are broadly consistent with the notion that processive translocation is closely coupled to ATP hydrolysis in our single-molecule Twinkle reactions, as seen earlier for the homologous T7gp4 helicase (55-57).

Longer incubation times were expected to cause proportionately longer regions of the DNA substrate to be unwound, however the length of ssDNA products reached an apparent limit in longer reactions (Figures 4B and 4C). Regions of ssDNA generated in 20-minute reactions displayed a length of 30.8 ±26.8 nm, or 61.7 ±53.7 nt (Gaussian peak ±S.D., N=101), which reduced the apparent unwinding rate to only 3.08 ±2.68 bp/min. As this value is approaching the unwinding rate observed in ensemble reactions, we searched for barriers to unwinding at extended reaction times. Twinkle is both an unwinding and an annealing helicase, and transient stalling and reannealing of complementary ssDNA directly interferes with efficient translocation (58,59). Interestingly, longer incubation times (20 minutes) for Twinkle on the circular gapped DNA substrate also converted a significant fraction of DNA unwinding events (95% of N=53 circular DNA molecules) into extended DNA tracts with unexpectedly taller AFM heights (Figure 4B). These structures exhibited an average AFM height of 664 ±177 pm (Figure 3C) and an average length of 47.8 ±35.5 nm (N=37). We interpret these structures as extended segments of folded DNA, although the mechanism of forming these higher-order secondary structures in open circular DNA are unclear.

Furthermore, because intact Twinkle hexamers are not always visible at the ssDNA-dsDNA junctions in unwound circular DNA molecules (Figures 4A and 4B), we suspected that stalling of translocation promotes disassociation of protein-DNA complexes. To address this question, we examined AFM images to assess the fraction of DNA unwinding events with Twinkle remaining bound at increasing times of incubation. Although Twinkle subunits were retained at the majority of unwinding events (92.8%, N=125) after 1 minute of incubation, Twinkle binding at unwinding sites was reduced to 56.7%, 43.0%, and 23.4% after 5 (N=141), 10 (N=100), and 20 minute (N=149) incubations, respectively (Figure 4D). Retention of protein complexes smaller than hexamers also suggested partial disassembly of Twinkle hexamers over time. This decrease in Twinkle occupancy fits well to a function of single exponential decay, suggesting that disassociation/disassembly of Twinkle occurs as a distinct transition. Treating these events as one population (N=515) permits calculation of a disassociation rate constant of 0.138 ± 0.029 events/min (Figure 4D). Similarly, combining the rates of DNA unwinding (242 ±195 bp/min) and disassociation (0.138 ±0.029 events/min) established by our single-molecule method provides an estimation of translocation processivity (1750 ±1480 bp/disassociation) for isolated Twinkle helicase on the circular gapped DNA substrate.

**mtSSB stimulates DNA unwinding by Twinkle**

The ability of mtSSB to stimulate the unwinding activity of Twinkle helicase in ensemble reactions *in vitro* has been documented with a number of DNA substrates (19,20,23,25). The means and specificity of this stimulation are debated, and proposed mechanisms underlying stimulation by mtSSB include binding the displaced DNA strand to suppress reannealing of unwound ssDNA, direct stabilizing interaction with the helicase, and binding the DNA substrate in a manner that facilitates loading of Twinkle. To further understand the role of mtSSB protein in DNA unwinding by Twinkle at the single-molecule level, we repeated unwinding reactions with the circular gapped DNA substrate in the presence of...
AFM imaging in air allowed us to visualize extensive DNA unwinding products as longer ssDNA regions both within the closed circular structure and as frayed ssDNA “tails” (Figure 5A). Such ssDNA structures would be generated simultaneously by initiation of a single unwinding event at the position of the gap, followed by strand separation during translocation of the helicase. Images also revealed increased lengths of DNA unwinding products for one minute incubations containing 71 nM Twinkle with 35 nM mtSSB (Figure 5A). The stimulatory effects of mtSSB were assessed by calculating rates of unwinding for separate reactions with a fixed concentration of Twinkle and varied concentrations of mtSSB. Whereas the unwinding rate in the absence of mtSSB was 242 ±195 bp/min, reactions containing 17.8, 35.0, 54.0 or 142 nM mtSSB tetramers exhibited unwinding rates of 287 ±226, 466 ±453, 1145 ±539, and 1265 ±541 bp/min (Gaussian peaks ±S.D.), respectively (Figure 5D). Stimulation of unwinding was generally proportional to added mtSSB, and maximal stimulation reached a plateau with apparent unwinding rates about 5-fold higher than observed for reactions lacking mtSSB. Control samples showed no observable unwinding of the gapped DNA substrate upon binding mtSSB in the absence of Twinkle helicase (Figures 2B and 2D), although passive unwinding of a few nucleotides may be inferred (see Discussion).

Analysis of single-molecule unwinding reactions offers hints at the mechanisms by which mtSSB could stimulate DNA unwinding by Twinkle. ChIP-seq experiments have shown that mtSSB binds the displaced H-strand of mtDNA replication intermediates in vivo (26). Binding of SSB to freshly separated ssDNA strands would suppress reannealing and appear to stimulate the helicase in vitro, both in ensemble and single-molecule reactions. Previous work has demonstrated that both human and Drosophila mtSSB, as well as the heterologous T7 gp2.5 SSB, can stimulate Twinkle helicase activity on DNA substrates containing short duplex regions in vitro (23,58). The lack of species specificity suggests non-specific stimulation due to stabilization of ssDNA products (analogous to the inclusion of excess complementary oligonucleotide to trap a displaced DNA strand). Specific stimulation of helicase activity would be expected for binding factors that interact with the helicase to diminish pausing or disassociation. The time scale required to monitor helicase pausing is not readily accessible through AFM. However, the presence of mtSSB substantially increased the average length of DNA unwinding products in our single-molecule images, which is consistent with the hypothesis that mtSSB reduces pausing or disassociation of the helicase. Twinkle preferentially binds ssDNA with high affinity (34), and the helicase loads onto DNA by assembling a hexameric structure around a covalently closed circular ssDNA substrate or by threading onto the single-stranded 5'-tail of a forked DNA substrate (18). Utilizing a plasmid-based DNA substrate with strand-specific, site-specific 37 nt single-stranded DNA gaps, we have shown that Twinkle binds at the position of a gap and initiates unwinding of the duplex portion of the substrate (Figures 2-5).

Although this circular gapped DNA substrate has two unwinding sites, and the majority of DNA molecules imaged by AFM exhibit single unwinding events (Figures 4A, 5A). Whether Twinkle initiates translocation on the covalently closed circular DNA strand or at a 5'-end of the gapped DNA strand in the presence of mtSSB is not clear. However, inspection of AFM images offers useful insight, because the obligate 5'-3' polarity of the helicase constrains double unwinding events to certain predictable structures. For example, unwinding initiated at both gaps by Twinkle translocating on the covalently closed strand would generate two expanding single-stranded regions with complementary free 3'-tails at the expense of two shrinking double-stranded regions on opposite sides of the DNA circle. Initiation of unwinding at both 5'-ends of the gapped DNA strand in the presence of mtSSB is not clear. Nonetheless, these general configurations were not observed in AFM images of more than 500 DNA molecules. Similarly, one single-stranded region flanked by both 5' and 3'-tails would be an indication of two Twinkle complexes initiating divergent unwinding at just one gap site, but this configuration also was not observed. In contrast, Twinkle hexamers initiating translocation on the covalently closed strand at one gap and on the 5'-end at the opposite gap would converge. Incomplete unwinding would generate two single-stranded regions between the gap sites and both 5'-
and 3’-tails on the same half of the DNA substrate, and continued unwinding would result in complete removal of an open strand between the two gap sites. Structures matching these descriptions were readily apparent in AFM images (Figures 5B and 5C, respectively, and as depicted in Supplementary Table S1). Also, the measured length of the double-stranded DNA region in the unaffected half of the DNA substrate remained essentially constant for all double unwinding configurations (Supplementary Table S1). This length (594 ±63 nm, N=174) was consistent with the distance between the two ssDNA gaps (2030 bp) on the substrate, lending further support to the existence of converging double unwinding events on this DNA substrate.

**Real-time imaging of the dynamics of Twinkle assembly and DNA binding**

Although AFM imaging in air creates remarkable static images of protein-DNA complexes, dynamic interactions such as conformational changes and DNA binding are more difficult to interpret. Therefore, we also utilized AFM imaging in liquids, which permits serial scanning of individual complexes at high resolution (Figure 6). First, we monitored conformation dynamics of Twinkle applied to an APS-treated mica surface in the presence of ATP. Successive images display the apparent sequential recruitment of Twinkle subunits to form higher-order Twinkle oligomers (Figure 6A, N=28). A separate series of images show closed-ring Twinkle hexamers appearing to switch spontaneously to open-ring configurations (Figure 6B, N=27 observations). When circular gapped DNA was deposited onto APS-treated mica (Figure 6C) and Twinkle was introduced directly to the solution, interaction of Twinkle with the DNA substrate could be observed over time. In a representative example (Figure 6D), opening of the Twinkle ring (green arrow becomes hollow arrow) coincides with disappearance of the DNA molecule (red arrow) from the mica surface, which implies opening of the ring structure correlates with disassociation of Twinkle from the DNA substrate. In summary, time-lapse AFM imaging in liquids enables near real-time assessment of several dynamic behaviors of Twinkle helicase, such as ring assembly, conformational changes, and disassociation from DNA.

**Discussion**

AFM imaging in air has demonstrated that Twinkle exists in solution as a heterogeneous mixture of varying oligomeric states, and that ssDNA facilitates self-assembly of higher order Twinkle oligomers. AFM imaging in liquids suggests Twinkle hexamers can be completed by the sequential recruitment of subunits, and that Twinkle spontaneously switches between open- and closed-ring hexameric configurations. Collectively, our imaging results support a model in which Twinkle loads onto DNA in an open-ring configuration, unwinds DNA as a closed-ring structure, and unloads from DNA upon re-opening of the ring structure. Quantifying DNA unwinding activity in such a complex system required a single molecule approach to avoid underestimation caused by ensemble averaging. Direct visualization of DNA unwinding products by AFM imaging showed that Twinkle is capable of unwinding several hundred base pairs of duplex DNA in a single unwinding event. When we restricted reactions times to one minute to minimize ssDNA product reannealing and enzyme disassociation, the average rate for individual Twinkle unwinding events was ~240 bp per minute. Dividing this rate by the calculated disassociation rate (0.138 min⁻¹) allowed us to estimate the average translocation processivity for Twinkle to be 1750 bp per disassociation event, which is approximately 10% of the length of mtDNA (16569 bp). However, titration of mtSSB into Twinkle reactions greatly increased lengths of DNA unwinding products, thereby raising the apparent unwinding rate by a factor of 5. Utilizing the disassociation rate determined for isolated Twinkle, we estimate a translocation processivity for Twinkle helicase in the presence of mtSSB to be 9150 ±4360 bp per disassociation event. Even though helicase products can exceed one kilobase, processivity may be undervalued because the calculation relies on rates derived from one minute unwinding reactions. Any reduction of the Twinkle disassociation rate elicited by mtSSB would further enhance helicase processivity. Nevertheless, this first report of Twinkle helicase processivity suggests over half the mitochondrial genome could be translocated during a single Twinkle binding event under optimal conditions in vitro. Similarly, Twinkle helicase
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does not function alone at the mtDNA replication fork in vivo. The natural abundance of mtSSB in cultured HeLa cells is sufficient to bind the entire mitochondrial genome (26,60), suggesting that helicase processivity may approach the unit length of the mitochondrial genome in vivo.

Several processes likely contribute to the ability of mtSSB to enhance both the apparent efficiency and processivity of DNA unwinding by Twinkle helicase. Binding the displaced DNA strand to suppress product reannealing has been shown to stimulate helicase activity in ensemble reactions in vitro. In one study, mtSSB enhanced Twinkle unwinding activity more than 2-fold, although mtSSB sufficient to saturate both strands in the M13 ssDNA-based substrate was utilized (19). In the current study, mtSSB also stimulated unwinding of the circular gapped plasmid-based DNA substrate, and initial stimulation was roughly proportional to added mtSSB. Previously, we utilized AFM to examine the binding modes of E. coli SSB and human mtSSB to ssDNA. Unlike E. coli SSB, mtSSB does not form nucleoprotein filaments that are a hallmark of cooperative DNA binding but instead binds non-cooperatively to extended ssDNA structures at physiological concentrations of salt (53). The current study utilizes similar solution conditions, and nucleoprotein filaments were not observed in any of our AFM images. This absence is noteworthy and implies mtSSB binds non-cooperatively to freshly generated ssDNA when Twinkle helicase unwinds the circular gapped DNA substrate. We observed maximal stimulation of Twinkle helicase activity at a mtSSB concentration far below the quantity needed to saturate unwound ssDNA products (Figure 5D), which is consistent with stabilization of the unwound DNA product by non-cooperative binding. Protein binding partners that reduce pausing of the helicase would serve to stimulate Twinkle activity. As mentioned above, including mtSSB in unwinding reactions increased the length of ssDNA products in our single molecule images, suggesting mtSSB may facilitate an increased rate of translocation or a decreased rate of disassociation for Twinkle. However, quantifying the relative contributions of these possible effects is not feasible, because the inability to easily differentiate mtSSB tetramers (61.3 kDa) and Twinkle monomers (73.5 kDa) by AFM prevents determination of the Twinkle disassociation rate in the presence of mtSSB.

An additional important scenario is the possibility that mtSSB contacts Twinkle or modifies the DNA substrate in a manner that helps to load the enzyme onto the gapped substrate in an active conformation. Knowledge of Twinkle's substrate specificity informs this discussion. Twinkle prefers forked DNA substrates with free 5'-tails, and the enzyme cannot efficiently unwind fully hybridized DNA substrates or duplex substrates with blunt or recessed 5'-ends (19,59). However, Twinkle actively unwinds both 5'-tailed and 3'-tailed oligonucleotides hybridized to M13 ssDNA in vitro, albeit with different efficiencies (18,58). This paradoxical result stems from Twinkle utilizing two modes for loading onto DNA substrates. Intact Twinkle hexamers are able to thread directly onto the free end of the 5'-tailed substrate, whereas hexamers must assemble around the covalently closed circular strand of the 3'-tailed substrate. The reduced efficiency of unwinding the 3'-tailed substrate can be explained by the additional steps required to assemble the active enzyme (18). The circular gapped DNA substrate used in the current study has one covalently closed circular strand, and the complementary open strand bears two 37 nt gaps in symmetrical positions on opposite sides of the plasmid (Figure 2). Twinkle complexes bind and initiate unwinding from one or both of the ssDNA gaps. As described above, the pattern of utilizing the two gaps changes when mtSSB is included in unwinding reactions. In the absence of mtSSB, 80% of circular gapped DNA substrate molecules unwound by Twinkle were initiated at a single gap site (Figure 4A). In the presence of mtSSB this fraction was reduced to 44%, and remaining molecules appeared to utilize two initiation sites in double unwinding events (Figure 5 and Supplementary Table S1). The ability of mtSSB to stimulate the apparent rate of translocation (Figure 5D) is inadequate to explain this significant increase in double unwinding structures when mtSSB is included in unwinding reactions. The critical point is that converging double unwinding events require the Twinkle complexes to employ different modes for loading onto each of the two ssDNA gaps. It is tempting to speculate that the presence of mtSSB at the single-stranded gaps modulates assembly of Twinkle
around the covalently closed circular DNA strand. Alternatively, mtSSB may partially fray the fully duplex 5’-ends of the open DNA strands to facilitate loading of Twinkle over a freshly exposed free 5’-tail. Either or both options could enhance the opportunity to form the double unwinding events seen on circular DNA substrates in our AFM images. In summary, mtSSB appears to stimulate Twinkle helicase unwinding the circular gapped DNA substrate by a combination of effects, including stabilization of the unwound ssDNA product and changing the way Twinkle loads onto DNA.

The bacteriophage T7 DNA replisome is an extensively studied model system for strand displacement DNA replication (61). Rapid quench kinetics measurements indicate that the T7 DNA helicase unwinds duplex DNA slower than the rate of DNA synthesis catalyzed by the DNA polymerase, making DNA synthesis the driving force behind progression of the replication fork (62). Although the T7 DNA polymerase and DNA helicase work on separate DNA strands, an overall effect is that the enzymes remain functionally and physically coupled at the DNA replication fork (48,63). Mitochondrial DNA replication also follows a strand displacement model, in which Twinkle helicase translocates on the displaced parental mtDNA H-strand and DNA Pol γ utilizes the parental L-strand as the DNA template for synthesis of the nascent H-strand. Pre-steady state kinetics measurements show that the reconstituted Pol γ holoenzyme can synthesize DNA at ~45 nt/sec under optimal conditions in vitro (16), which predicts one strand of the 16,569 bp mitochondrial genome could be replicated in just over 6 minutes. Our AFM study suggests the maximal rate of DNA unwinding by Twinkle in the presence of mtSSB is ~21 bp/sec. Taken together, these values suggest wild type Pol γ and Twinkle also remain coupled at the mtDNA replication fork. Missense disease variants of TWNK and POLG disrupt the function and stability of the enzymes, which interferes with coordinated action of the enzymes and likely promotes the formation of mtDNA deletions during replication and repair of mtDNA in vivo (35,64,65). For example, persistent pausing of the helicase, such as at DNA secondary structures, would stall the mtDNA replication fork and promote disassociation of Twinkle from the unwinding site.

Similarly, stalling of the polymerase due to enzyme dysfunction or miscoding lesions in the DNA template could uncouple the replisome and prompt Twinkle to advance ahead of the polymerase and generate unusually long regions of single-stranded DNA. We have indirect evidence for excess ssDNA in the mitochondrial genome of Saccharomyces cerevisiae strains bearing replication-defective, disease-associated missense mutations in mip1, the yeast homolog of human POLG. When we exposed cells to the exogenous base-alkylating agent methyl methanesulfonate (MMS), mutant strains exhibited a 30-fold increase in mutagenesis of their mtDNA relative to similarly treated wild type strains. Because base excision repair cannot remove MMS damaged bases from ssDNA, the increased vulnerability to mutagenesis suggested irreparable damage to mtDNA that occurred during DNA replication (66).

In summary, we utilized AFM imaging in air and real-time imaging in liquids to observe and report the configurations and dynamic steps of Twinkle oligomer assembly, DNA binding and unwinding. Our findings address fundamental questions regarding the ways Twinkle loads onto DNA and the mechanisms underlying stimulation of unwinding by mtSSB. This work provides a new framework to study the mtDNA replication machinery, the molecular defects of protein variants identified in heritable mitochondrial genome maintenance disorders, and the impact of mtDNA lesions on mtDNA replication.

**Experimental procedures**

**Proteins** – Recombinant human Twinkle helicase bearing a C-terminal His6-affinity tag (73.5 kDa monomer form) was overproduced in E. coli BL21 (DE3) CodonPlus-RIPL (Stratagene) and purified to >95% homogeneity by sequential Nickel-affinity, anion exchange, and heparin-affinity chromatography (Supplementary Figure S1A), as described previously (34). Twinkle protein concentration was determined by digital imaging of Coomassie stained SDS-PAGE gels with BSA as a standard. The human SSBP1 cDNA encoding mtSSB was overexpressed in E. coli JM105(DE3), and recombinant human mtSSB was purified to homogeneity as described previously (67). Human mtSSB (15.3 kDa monomer form) was dialyzed into buffer containing 30 mM
HEPES-KOH (pH 7.6), 1 mM dithiothreitol, 0.25 mM EDTA, 0.25% (w/v) myoinositol, 0.01% (v/v) NP-40, 0.1 mM PMSF, 0.25 M KCl and 25% glycerol. MtSSB protein was quantified by UV absorbance at 280 nm utilizing an extinction coefficient (19060 M⁻¹cm⁻¹ for monomers) calculated from the primary amino acid sequence (68,69), and stored at -20°C. 

**DNA substrates** – Synthetic oligodeoxyribonucleotides were obtained from Integrated DNA Technologies (IDT). Linear double-stranded DNA containing a 37 nt ssDNA gap was prepared by treating pSCW01 plasmid DNA (duplication of 2030 bp) provided by Peggy Hsieh (NIDDK) (51) with Nt.BstNBI to introduce two clusters of four closely-spaced nicks in one strand, incubating with complementary oligonucleotides (oligos:plasmid ratio = 10:1) at 68°C for 30 min followed by slow cooling to room temperature, and removing excess unbound oligos and short duplex DNA using 100K MW Amicon Ultra filtration (51,70). Circular gapped DNA was linearized by digestion with Scal in Buffer 3.1 (NEB), which positioned the 37 nt ssDNA gap 470 bp (23%) from one end of the blunt-ended 2030 bp linear DNA fragment. Diagnostic restriction digestion of the gapped region indicated typical DNA gapping efficiencies of 85 to 95% (53,71). Linear dsDNA lacking a ssDNA gap was prepared by directly linearizing pSCW01 plasmid DNA with Scal restriction endonuclease. A forked DNA helicase substrate was assembled by hybridizing two 40 nt synthetic oligonucleotides (5’-ATGCTAGCTTGCTGTAGCTTTAAAACCTGTCGTCCAGCT-3’ and 5’-AGCTGTGACGACGGTTTCCCCACTGGAAAGCGGCGAGTG-3’) to form a structure with 18 complementary base pairs and two 22 nt single-stranded tails. This substrate was utilized in ensemble biochemical reactions to assess and validate the DNA helicase activity of purified Twinkle in vitro (Supplementary Figure S1B), as described previously (34).

**AFM sample preparation** – To assess the oligomeric states of Twinkle helicase, protein samples were diluted in Helicase Buffer containing 20 mM HEPES (pH 7.5), 100 mM NaCl, 7.5 mM MgCl₂, 7% glycerol, 0.2% NP-40, and 4.5 mM ATP to a final concentration of 100 nM hexamers. Samples were immediately deposited onto freshly cleaved mica surfaces at room temperature, followed by washing with deionized water and drying with streams of nitrogen gas prior to AFM imaging. Twinkle complexes in Helicase Buffer containing 0.4 M NaCl were also examined by AFM, as indicated. To study the effects of ssDNA on the oligomeric state of Twinkle, 100 nM Twinkle hexamers were premixed with 100 nM single-stranded 108 nt oligonucleotides in Helicase Buffer and incubated at room temperature for 10 min prior to deposition for AFM imaging. The DNA binding specificity of mtSSB was determined by incubating 73.5 nM mtSSB (tetramers) with 3.6 nM linear gapped DNA. The DNA binding specificity of Twinkle was determined by incubating 71.2 nM Twinkle hexamers with 3.6 nM linear gapped DNA in Helicase Buffer without ATP. DNA unwinding was examined in reactions containing Helicase Buffer, 3.6 nM circular gapped DNA, 71.2 nM Twinkle helicase (hexamers), and 0-142 nM mtSSB (tetramers, as indicated). Samples were incubated at room temperature for 1, 5, 10, or 20 minutes (as indicated), further diluted in Helicase Buffer, and deposited onto a mica surface for AFM imaging as mentioned before. For real time imaging of Twinkle dynamics in liquids, 0.7 nM circular gapped DNA was deposited onto a freshly prepared aminopropyl silane (APS)-treated mica surface, incubated for 15 min, and washed with Helicase Buffer. Twinkle hexamers (71.2 nM) were incubated in Helicase Buffer for 5 min at room temperature and added to the DNA sample on the APS-mica surface with a pipette tip while scanning.

**AFM imaging and image analysis** – AFM imaging in air was carried out using the AC mode on a MFP-3D-Bio AFM (Asylum Research, Oxford Instruments) with Pointprobe® PPP-FMR probes (Nanosensors, spring constants at ~2.8 N/m). All images were captured at a scan size of 1-3 µm × 1-3 µm, a scan rate of 1–2 Hz, and a resolution of 512 × 512 pixels. AFM imaging in liquids was accomplished using a BL-AC40TS (BioLever Mini) probe (Asylum Research), and images were captured at a scan rate of 2-4 Hz with a resolution of 512 × 512 pixels. Lengths of dsDNA and ssDNA segments were measured with MFP-3D software (Asylum Research). AFM volumes of Twinkle oligomers in the absence and presence of DNA oligonucleotides were measured with Gwyddion.
software. Volume data are reported as mean ± standard deviation.

Data Availability – AFM data is available upon request to Parminder Kaur, North Carolina State University at pkaur3@ncsu.edu; or William Copeland, NIEHS at copelan1@niehs.nih.gov.
Acknowledgments: We wish to thank Drs. Carl Anderson and Leroy Worth at the NIEHS for critical evaluation of the manuscript.

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Author contributions: PK and MJL performed the experiments, and HP, WW, and PC assisted in the experiments. PK, MJL, HW, and WCC were involved in designing the study and writing the paper.
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**FOOTNOTES**

Funding was provided by the Extramural Research Program of the National Institutes of Health (R01GM107559, R21ES027641 and R01GM123246 to H.W.), the NCSU Center for Human Health and the Environment Pilot Project grant (P30ES025128 to P.K.), and the Intramural Research Program of the National Institutes of Health, National Institute of Environmental Health Sciences (ES065078 to W.C.C.).
Figure 1. AFM imaging reveals oligomeric states of Twinkle in the presence of ATP at 0.1 M NaCl.
AFM Topographic (top or left) and phase (bottom or right) images of Twinkle oligomers (N=162) show (A) monomeric (26%), (B) dimeric (19%), (C) trimeric (20%), (D) tetrameric (9%), (E) pentameric (12%), and (F and G) hexameric (14%) states. Hexameric Twinkle (N=83) exists both in (F) closed-ring (35%) and (G) open-ring (65%) configurations. XY scale bars = 25 nm.
Figure 2. Twinkle helicase binds specifically to ssDNA gaps in the absence of ATP. (A) Cartoon drawings and AFM images of the circular and linear gapped DNA substrates utilized in this study. Circular plasmid DNA with a 2030 bp head-to-tail duplication was used to generate the linear gapped DNA substrate. DNA binding reactions and AFM imaging were performed as described in Experimental Procedures. XY scale bars = 100 nm. (B) AFM images of mtSSB (arrows) binding to the linear gapped DNA. (C) AFM images of Twinkle helicase (arrows) binding to the linear gapped DNA in the absence of ATP. XY scale bars = 50 nm. (D) Position distributions of mtSSB (red, N=147) and Twinkle (green, N=55) bound to linear gapped DNA, and Twinkle (blue, N=94) on the non-gapped linear DNA substrates are shown. Gaussian fitting of position distributions centers mtSSB 24.2% (± 2.5%) and Twinkle helicase 20.7% (±4.9%) of the distance from the closer end of the linear gapped DNA. Boxes represent second and third quartiles. Error bars are standard deviation. Maximum (☐), 99% (⊞), mean (■), 1% (⊠), and minimum (☐) values are shown.
Figure 3. **AFM imaging in air reveals Twinkle hexamers binding and unwinding circular gapped DNA.** DNA binding and unwinding reactions were assembled and processed for AFM as described in Experimental Procedures. Reactions (one minute) included 3.6 nM circular gapped DNA, 71.2 nM Twinkle hexamers, and 4.5 mM ATP. (A) Representative pairs of AFM topography (left) and phase (right) images of Twinkle in the closed-ring form (arrows) on the circular gapped DNA substrate (N=101). Magnified regions (white inserts) show Twinkle helicase bound to DNA. XY scale bars = 100 nm. (B) Top panel: AFM topography (left) and amplitude (right) images of Twinkle in the closed-ring form at the site of DNA unwinding. Bottom panel: Cross-section analysis along paths drawn in the topography image show AFM heights for unwound ssDNA (green) and dsDNA (blue). (C) A combined data set from 1 and 20 min reactions shows AFM height distributions for the unwound ssDNA product (green, N=69, 200 ±104 pm), dsDNA (blue, N=70, 371 ±116 pm), and folded DNA (red, N=71, 664 ±177 pm). Boxes represent second and third quartiles. Error bars are standard deviation. Maximum (☐), 99% (⊞), mean (■), 1% (⊠), and minimum (☐) values are shown. (D) Circumferences of closed-ring Twinkle complexes unwinding circular gapped DNA in AFM phase images were estimated by MFP-3D software (Asylum Research). The insert shows an example of a Twinkle ring with a circle drawn at the center of the ring’s phase signal for the measurement of the circumference. The solid line in the histogram represents a Gaussian fit to the data (R²>0.94) with a peak centered at 79.7 ±12.8 nm (Gaussian peak ±S.D., N=86).
Figure 4. Quantifying DNA unwinding by Twinkle using AFM imaging in air. DNA unwinding reactions were assembled and processed for AFM as described in Experimental Procedures. Reactions included 3.6 nM circular gapped DNA, 71.2 nM Twinkle hexamers, and 4.5 mM ATP, and were incubated at room temperature for (A) 1 minute or (B) 20 minutes. White boxes mark the DNA unwinding sites (A) and extended tracts of folded DNA (B) in representative AFM topography images. Numbers within cartoon drawings are the distribution (%) of single and double unwinding configurations (N=76). XY scale bars = 100 nm. (C) Lengths of ssDNA regions produced in 1 and 20 minute reactions. Boxes represent second and third quartiles. Error bars are standard deviation. Maximum (☐), 99% (⊕), mean (■), 1% (⊗), and minimum (□) values are shown. (D) The fraction of DNA unwinding events (%) retaining bound Twinkle subunits following incubation for 1 min (N=125), 5 min (N=141), 10 min (N=100), or 20 min (N=149) are shown. Average values were fit to an equation for exponential decay (solid line, R²>0.985).
Figure 5. mtSSB stimulates unwinding activity of Twinkle on circular gapped DNA. DNA unwinding reactions were assembled and processed for AFM as described in Experimental Procedures. Reactions were incubated at room temperature for 1 minute and included 3.6 nM circular gapped DNA, 71.2 nM Twinkle hexamers, 35.8 nM mtSSB tetramers, and 4.5 mM ATP. Representative AFM topography images display (A) single unwinding events, (B) double unwinding events, and (C) complete removal of one open DNA strand. Green dotted lines trace the position of ssDNA identified by the AFM height as in Figure 3. Numbers within cartoon drawings are the observed distribution (%) of single, double, and complete unwinding configurations (Supplementary Table S1). XY scale bars=100 nm. (D) Lengths of ssDNA products (N=759) were determined from AFM topography images for separate unwinding reactions having final concentrations of 0 (red, N=211), 17.8 (green, N=100), 35.0 (blue, N=232), 54.0 (cyan, N=116) or 142 (magenta, N=100) nM mtSSB tetramers. Boxes represent second and third quartiles. Error bars are standard deviation. Maximum (☐), 99% (⊔), mean (■), 1% (⊤), and minimum (☐) values are shown.
Figure 6. Real-time AFM imaging in liquids shows dynamics of Twinkle assembly, conformational change, and DNA binding. Samples for AFM imaging in liquids were assembled and processed as described in Experimental Procedures. Serial images were scanned at the indicated times (min:sec). (A) Assembly of Twinkle oligomers through recruitment of additional subunits. XY scale bars = 50 nm. (B) Twinkle switching from a closed- to an open-ring configuration. XY scale bars = 50 nm. (C) AFM imaging in liquids of the circular gapped DNA affixed to APS-treated mica. XY scale bars = 400 nm. (D) Twinkle ring opening correlates with DNA disassociation. Note that the DNA fragment (red arrow) disappeared from the mica surface upon Twinkle ring opening (green arrow becomes hollow arrow). XY scale bars = 50 nm.
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J. Biol. Chem. published online March 25, 2020

Access the most updated version of this article at doi: 10.1074/jbc.RA120.012795

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