DNA “breathing” is a thermally driven process in which base-paired DNA sequences transiently adopt local conformations that depart from their most stable structures. Polymerases and other proteins of genome expression require access to single-stranded DNA coding templates located in the double-stranded DNA “interior,” and it is likely that fluctuations of the sugar–phosphate backbones of dsDNA that result in mechanically useful local base pair opening reactions can be exploited by such DNA regulatory proteins. Such motions are difficult to observe in bulk measurements, both because they are infrequent and because they often occur on microsecond time scales that are not easy to access experimentally. We report single-molecule fluorescence experiments with polarized light, in which tens-of-microseconds rotational motions of internally labeled iCy3/iCy5 donor–acceptor Förster resonance energy transfer fluorophore pairs that have been rigidly inserted into the backbones of replication fork constructs are simultaneously detected using single-molecule Förster resonance energy transfer and single-molecule fluorescence-detected linear dichroism signals. Our results reveal significant local motions in the ~100-μs range, a reasonable time scale for DNA breathing fluctuations of potential relevance for DNA–protein interactions. Moreover, we show that both the magnitudes and the relaxation times of these backbone breathing fluctuations are significantly perturbed by interactions of the fork construct with a nonprocessive, weakly binding bacteriophage T4-coded helicase hexamer initiation complex, suggesting that these motions may play a fundamental role in the initial binding, assembly, and function of the processive helicase–primase (primosome) component of the bacteriophage T4-coded DNA replication complex.

Significance

Unique single-molecule fluorescence techniques were used to monitor DNA “breathing” at and near the junctions of model DNA replication forks on biologically relevant microsecond-to-millisecond time scales. Experiments performed in the absence and presence of helicase complexes addressed the role of these fluctuations in helicase function during DNA replication. These studies simultaneously monitored single-molecule Förster resonance energy transfer and single-molecule fluorescence linear dichroism of “internal” Cy3/Cy5 labels placed rigidly into the DNA backbones at positions near the fork junction. Our results showed significant breathing at the fork junction that was greatly augmented by the presence of weakly bound helicase, followed by still larger fluctuations and strand separation after full duplex DNA unwinding by the complete tightly bound and processive helicase complex.
junction be transiently exposed from behind the sugar–phosphate backbones that shield them from the solvent environment. As shown in Fig. 1A–C, the T4 gp41 helicase hexamer binds weakly to the replication fork junction, and is more likely to dissociate from the fork rather than to engage in processive dsDNA unwinding (13, 14). In contrast, the T4 primosome helicase, which consists of a gp41 hexamer and a gp61 monomer, binds tightly at the replication fork junction and manifests very processive unwinding activity in the presence of NTP. The breathing fluctuations of base pairs located in the immediate vicinity of a replication fork or a p/t junction are much more extensive than those of base pairs located deep within a dsDNA region (6, 13). A hypothetical free-energy surface (FES) describing base-pair dynamics near the fork junction is portrayed in Fig. 1D. In this model, base pairs can thermally fluctuate into the open state, which is separated from the native DNA structure by a free-energy barrier. The height of this barrier is likely dependent on several variables, such as salt concentration, pH, base composition, and base pair sequence. By observing the fluctuations of dsDNA constructs within regions that have been labeled with fluorescent dyes, it should be possible to obtain information about the FESs to which defined segments of DNA bases and sugar–phosphate backbones are subject, and also to determine how these surfaces are affected by the presence of the T4 helicase.

We here perform single-molecule experiments on DNA replication fork constructs that have been “internally” labeled with the Förster resonance energy transfer (FRET) donor–acceptor chromophores iCy3 and iCy5 (14). These cyanine dyes were rigidly incorporated into the sugar–phosphate backbone using phosphoramidite chemistry, with each fluorophore replacing an opposed DNA base at each label position (Fig. 2A). In some experiments, the fluorophores were positioned close to the replication fork junction; in others, deep within the DNA duplex. Our measurements use a polarized excitation scheme that simultaneously monitors, on submillisecond time scales, the smFRET between the iCy3/iCy5 donor–acceptor chromophore pair and the fluorescence-detected linear dichroism (smFLD) of the iCy3 donor chromophore. The principle of these measurements is based on the idea that a single-oriented DNA replication fork construct will absorb light with different probabilities, depending on the polarization of the laser field. By rapidly modulating the laser polarization, and recording the phase of the modulation for every detected signal photon, we were able to monitor the time-dependent projections of the absorption transition dipole moment onto arbitrarily defined laboratory axes. Whereas the smFRET signal is sensitive to the relative separation and orientation of the iCy3/iCy5 chromophore labels, the smFLD signal is sensitive to the iCy3 orientation within the laboratory frame. DNA breathing and helicase-facilitated duplex unwinding are expected to influence both types of signals.

Single-molecule FRET experiments have previously been used to examine helicase binding at replication fork junctions (15). Moreover, polarization-resolved single-molecule experiments have previously been used to study the orientations of chromophores embedded in complex systems, including glasses (16, 17), biological macromolecules (18–20), conjugated polymers (21), and light-harvesting complexes (22). The majority of these methods are capable of sampling a single-molecule signal with time resolution on the order of tens of milliseconds or longer. To observe DNA breathing, it was necessary to record single-molecule signals with submillisecond time resolution.

**smFLD and smFRET**

We performed control experiments on a model DNA replication fork construct that had been internally labeled using the donor–acceptor FRET chromophore pair iCy3/iCy5 as shown in Fig. 2A. In this way, both smFLD and smFRET signals could be simultaneously monitored to provide information about DNA backbone rigidity and the relative displacement of the conjugate strands at the level of the iCy3/iCy5 probes. Ensemble linear dichroism (LD) measurements are often used to determine the orientations of absorption transition dipole moments of chromophores embedded in uniaxially strained polymer films (23, 24). The LD of such samples

![Fig. 1.](image)

**Fig. 1.** (A) The isolated hexameric gp41 helicase (blue spheres), the subunits of which are bound together by intersubunit (and in this case non-hydrolyzable) NTP ligands (not shown for clarity), binds weakly to the ss-dsDNA junction of a replication fork, favoring dissociation over binding, as shown in B. Introduction of gp61 primase (orange ellipsoid), in a 1:6 gp41:gp61 subunit ratio, causes the resulting T4 primosome helicase complex to bind strongly to the replication fork, as shown in C. (D) A hypothetical FES describing local dsDNA fluctuations near the fork junction in the absence of helicase.
is defined as the difference between the absorption of light polarized parallel and perpendicular to the strain axis.

$$LD = A_1 - A_\perp \quad [1]$$

In FLD, the relative population of molecules excited in the two polarization directions is determined by measuring the difference between the corresponding fluorescence intensities. In our smFLD experiments, the DNA replication fork substrates were chemically attached to the surface of a glass microscope slide using biotin/NeutrAvidin linkages (Fig. 2A and Sample Preparation Methods). The DNA fork construct, with its rigidly attached iCy3 donor chromophore, was thus oriented with respect to the laboratory frame. A total internal reflection fluorescence (TIRF) microscope was used to illuminate the sample with an evanescent field, as shown in Fig. 2B. The polarization of the incident laser beam was rapidly modulated (at 1 MHz) so that the sample was alternately excited using plane polarizations in the directions $-45^\circ$ (designated as $\parallel$) and $+45^\circ$ (designated as $\perp$) relative to the surface normal (Fig. 2C; see instrument details provided in SI Text, Instrumentation for Single-Molecule Fluorescence-Detected Linear Dichroism and Single-Molecule Förster Resonance Energy Transfer). Because the absorption probability depends on the square projection of the laser spect to the laboratory frame. A total internal reflection fluorescence (TIRF) microscope was used to illuminate the sample with an evanescent field, as shown in Fig. 2B. The polarization of the incident laser beam was rapidly modulated (at 1 MHz) so that the sample was alternately excited using plane polarizations in the directions $-45^\circ$ (designated as $\parallel$) and $+45^\circ$ (designated as $\perp$) relative to the surface normal (Fig. 2C; see instrument details provided in SI Text, Instrumentation for Single-Molecule Fluorescence-Detected Linear Dichroism and Single-Molecule Förster Resonance Energy Transfer). The 1-MHz polarization modulation of the excitation beam was implemented using acousto-optic Bragg cells placed in the beam path of the laser (SI Text, Instrumentation for Single-Molecule Fluorescence-Detected Linear Dichroism and Single-Molecule Förster Resonance Energy Transfer). The sample image was raster-scanned using a computer-controlled x-y piezo-scanning microscope stage (NPS-XY-100A; Quantengate), and the stage position was held fixed when the fluorescence from a single molecule was detected. Donor and acceptor fluorescence from a single molecule was masked using a pinhole, directed along separate paths using a dichroic beam-splitter, and separately detected using two fast photon-counting detectors (SPCM-AOR-16, 175-μm active area; Perkin-Elmer Optoelectronics). The detection times of individual photoelectron counts were stored on a computer and additionally assigned values of the laser polarization phase $\phi$, which was subdivided into 64-bin increments. We thus recorded time- and $\phi$-dependent trajectories of the donor (acceptor) signals, $I_D(\tau, \phi) = \sum_{n=1}^{N_{\text{fl}}} \delta(t - t_n^D)(\exp(i\phi))$, where $t_n^D$ and $\phi^D$ are the time and phase of the $n$th donor (acceptor) signal count, respectively, and $N_{\text{fl}} = N_D + N_A$ is the total number of signal counts. In principle, single-molecule kinetic information could be extracted from these data with a time resolution of $\sim\text{1 μs}$ and a polarization phase resolution of $\sim\text{2α/64} \approx 0.1 \text{ rad}$. From the signal trajectories $I_D(\tau, \phi)$, we constructed the cumulative donor-acceptor signal $I_T = I_D + I_A$ and the FRET efficiency parameter, defined as $I_A/I_T$. We note that the presence of the FRET acceptor chromophore does not alter the meaning of the FLD measurement because the acceptor signal depends on the excitation of the donor chromophore, which was excited with polarization specified by $\phi$. Two separate approaches were taken to process these data. The first approach was used to visualize the smFLD and smFRET trajectories on millisecond time-scales, and the second served to resolve the tens-of-microseconds dynamics of the DNA sugar-phosphate backbone.

Because the fluorescence intensity from single iCy3/iCy5-labeled bases was on the order of $\sim\text{25,000 s}^{-1}$, we integrated the cumulative signal $I_T$ over $\sim\text{800 modulation cycles}$ to obtain a $\phi$-dependent probability distribution from which the smFLD signal could be determined. We extracted the smFLD signal through a post data-acquisition procedure that was operationally similar to the algorithm used by a lock-in amplifier (25). For a single molecule of fixed orientation, the integrated histogram of photoelectron counts vs. polarization phase $\phi$ approximates a smoothly varying sinusoidal function $f(\phi) = A + C \cos(\phi + \phi_0)$, where $C$ is the modulation amplitude (equal to the FLD signal), $A$ is a constant offset, and $\phi_0$ is a known phase determined by the modulation electronics. We numerically calculated the smFLD signal by multiplying the function $f(\phi)$ by $\cos(\phi + \phi_{\text{ref}})$, where we set $\phi_{\text{ref}} = \phi_0$, and subsequently performing an average over the phase angle $\phi$:

$$\langle \cos(\phi + \phi_{\text{ref}}) f(\phi) \rangle_\phi = \frac{C}{2} \text{FLD}$$

We define the reduced FLD (FLD) $= \text{FLD}/N_{\text{hist}}$, where $N_{\text{hist}}$ is the number of photoelectron counts contributing to the integrated histogram. The quantity FLD is independent of signal intensity, and thus depends only on the orientation of the molecular transition dipole moment. We found that accurate results could be obtained with as few counts as $N_{\text{hist}} = 20$. Thus, a smFLD signal trajectory could be obtained from a molecule with an emission count rate of $\sim\text{25,000 s}^{-1}$ and a time resolution of $\sim\text{800 μs}$.

The second method we used to process our trajectories is based on an analysis of time correlation functions (TCFs). We focus on the fluctuating smFRET and smFLD signals, $\text{FRET}(\tau) = \langle R(\tau) \rangle$ and $\text{FRET}(\tau) = \langle R(\tau) \rangle$, respectively, which were recorded using a DNA replication fork construct at equilibrium. We define the TCFs $G_{\text{FRET}}(\tau) = \langle R(\tau) \rangle$ and $G_{\text{FRET}}(\tau) = \langle R(\tau) \rangle$, which describe the decay of correlations of the fluctuating smFRET and smFLD signals, respectively, with increasing time interval $\tau$. The angle brackets indicate an average over time according to

$$G_{\text{FRET/FLD}}(\tau) = \int_{-\infty}^{\infty} S(t) S^* (t + \tau) dt.$$

In Eq. 3, $S(\tau)$ represents the fluctuating smFRET or smFLD signal. The TCFs, so defined, are expected to decay on a time scale associated with the same underlying microscopic processes that give rise to spontaneous fluctuations of the system away from its stable equilibrium configuration (26). We note that the short time limit of $G(0) = \langle S^2 \rangle$ represents the mean square magnitude of the fluctuating signal, whereas the long time limit $G(\infty) = \langle S^2 \rangle$ represents the square mean signal. The TCF analysis provides access to time scales much faster than the histogram integration method described previously, because the latter relies on multiple detection events to reconstruct a sinusoidal waveform. Because the TCF method correlates the properties of individual photoelectron signal counts, we were able to extract kinetic information from single-molecule trajectories with $\sim\text{20 μs}$ time resolution.

We performed control experiments to calibrate the smFLD and smFRET signals from known test samples, and to ensure that our measurements were not influenced by the dynamics of laser-induced excited triplet states (SI Text, Sample Preparation and Model DNA Replication Fork Constructs).

Results

We investigated the dynamics of the two iCy3/iCy5-labeled DNA replication fork constructs (SI Text, Sample Preparation and Model DNA Replication Fork Constructs). For both types of DNA constructs, the iCy3/iCy5 chromophores replaced nucleotide bases on opposite strands directly across from one another. The “duplex-labeled” construct had the iCy3/iCy5 pair placed deep in the dsDNA region of the fork construct, whereas the “fork-labeled” construct contained the iCy3/iCy5-labeled probe pair at the ss–dsDNA junction. Using these DNA constructs, we were able to simultaneously measure the smFRET and smFLD signals. We initially examined the duplex-labeled DNA construct

and its unwinding by the T4 primosome helicase complex. In the absence of any helicase, we observed that smFRET signals were constant over time, with an average FRET efficiency $I_d/I_T \approx 0.7$. Photobleaching from these samples occurred only occasionally, presumably because the fluorophores were protected from the solvent environment by the sugar-phosphate backbones of the dsDNA (14). In the absence of unwinding proteins, the smFLD signals also remained at a constant value, which could lie anywhere within the range $\sim-1$ to 1. Example control data sets for duplex-labeled constructs are presented in SI Text, Control Experiments.

Upon introducing the T4 hexameric helicase and 6 μM ATP into the sample chamber, DNA-unwinding events could be observed that were characterized by a sudden drop in the iCy5 fluorescence intensity and a simultaneous increase in the iCy3 fluorescence intensity, defined as a drop in the FRET efficiency. Similar helicase-unwinding FRET-conversion events have been observed previously with this system (14). smFLD, traces were also significantly affected by these events. In Fig. 3 A–C, we present simultaneously measured single-molecule iCy3/iCy5 intensities, representing smFRET and smFLD, trajectories, respectively, of the duplex-labeled DNA replication fork construct in the presence of the processively unwinding T4 helicase–primase system (300 nM gp41 and 50 nM gp61) and 6 μM ATP. Before the unwinding event at the approximate tick mark, the iCy5 intensity remained high and constant, resulting in a FRET efficiency of over 0.7. During this time, the smFLD signal fluctuated near a nonzero constant value. Immediately after the unwinding event at $\sim 17.5$ s, characterized by a drop in the smFRET signal, the smFLD signal fluctuated broadly around zero because the iCy3-labeled strand had been freed from the tethered dsDNA construct by the helicase. In Fig. 3 Right, we also show selected polarization $\phi$-dependent signal distributions, which were used to determine the smFLD trajectory shown in Fig. 3B.

Having established that our experiment was able to simultaneously monitor the smFRET and smFLD trajectories, we next sought to observe the breathing dynamics of the system using model replication fork constructs with the iCy3/iCy5 probes placed at either the ss–dsDNA fork junction or deep within the duplex region. Our initial studies focused on observing dynamics revealed by calculating two-point TCFs for the smFRET and smFLD, trajectories, with the goal of observing DNA breathing fluctuations at equilibrium. The TCFs should reveal thermally activated backbone fluctuations as the iCy3/iCy5 probes sample the FES shown hypothetically in the simplified diagram of Fig. 1C. The decays of the TCF report on the timescale of these equilibrium fluctuations, and the functional form of the decay, can provide information about the nature of the fluctuations.

In Fig. 4, we show examples of TCFs calculated from simultaneously recorded smFRET and smFLD trajectories. Though the paired TCFs derived from smFRET and smFLD signal traces decayed on time scales of the order of $\sim 100$ μs, they generally appeared to be nonidentical, reflecting the distinct origins of the two types of signals. The decays of the TCFs should be sensitive to the motions of the rigidly integrated probes, and thus of the DNA sugar–phosphate backbones, and hence likely reflect important aspects of DNA breathing. To ensure that potential “flickering” dynamics of long-lived excited triplet states did not significantly affect our results (27), we performed control studies to check that the observed TCFs were independent of the excitation intensity of the laser (SI Text, Control Experiments). In Fig. 4, we show results from the fork-labeled iCy3/iCy5 construct in the presence of helicase proteins. Additional examples of data sets are presented in SI Text, Control Experiments, and show those of the same DNA construct in the presence of gp41 helicase hexamers that had been assembled using nonhydrolyzable GTPγS as the NTP ligand. These helicase hexamers, assembled with nonhydrolyzable NTPs in the absence of primase, bind weakly to the ss–dsDNA junction of the replication fork constructs, but cannot catalyze duplex unwinding (5, 13, 14).

Our experimentally derived TCFs are consistent with earlier reports from ensemble fluorescence correlation spectroscopy (FCS) experiments performed by Altan-Bonnet et al. (7), in which DNA breathing dynamics were observed in the 30- to 100-μs range. These authors proposed a model to explain the functional form of the TCFs measured in their solution phase experiments that was based on a rate equation in which a DNA breathing “bubble” can open or close by 1 bp, with the closing rate much faster than the rate of opening. The TCFs produced using this model have a functional form

$$G(\tau) \propto \left(1 + \frac{\tau}{2\tau_c}\right) \text{erfc} \left(\sqrt{\frac{\tau}{4\tau_c}}\right) - \sqrt{\frac{\tau}{\tau_c}} e^{-\tau/4\tau_c}. \tag{4}$$

We have used Eq. 4 to fit the TCFs calculated from our single-molecule experiments, including those shown in Fig. 4, and in many cases we found that this model fit our data very well. Approximately 50% of the molecules we observed in the absence of unwinding proteins fit this model precisely, whereas $\sim 25\%$ of the molecules we observed in the presence of unwinding proteins also fit the model. For those molecules in which there is a discrepancy, the model generally does not match the fastest time components of the decays, and therefore a refinement to the model may be necessary to capture the shortest time dynamics to which our experiments are sensitive. Because the closing rate of the DNA bubble should be faster than the opening rate, the relaxation times reported in ref. 7 and in our data can be considered to represent the lifetime of the open state of the bubble. The moderate agreement we observe between this model and our experimental data suggests the possibility that the chromophore-labeled region of the DNA construct may experience multiple bubbles of various sizes over the course of the observation time window. We note that the average lifetime we observed in our experiments $\sim 4–5$ times larger than that observed in ref. 7, which is likely due to the different labeling schemes used in the two studies. The internally labeled iCy3 and iCy5 chromophores replace native bases in our DNA strands, and this relatively rigid configuration might extend the lifetime of spontaneously formed bubbles in the local dsDNA sequence surrounding the probes. In

Fig. 3. (A) Single-molecule iCy3/iCy5 signals were recorded during a T4 primosome–helicase unwinding experiment. An unwinding event occurred at the 17.5 s tick mark. (B) The simultaneously recorded smFLD signal exhibits a change in behavior coincident with the unwinding event defined by the smFRET conversion efficiency shown in C. Horizontal dashed lines (red/green) indicate the magnitude of the fluctuations immediately before and after the unwinding event. (Right) Laser polarization $\phi$-dependent signal distributions that were used to determine the FLD signal shown in B. The donor–acceptor chromophore labels are placed deep within the double-stranded region of the DNA replication fork construct (SI Text, Sample Preparation and Model DNA Replication Fork Constructs).
contrast, the chromophores used in ref. 7 were attached using flexible linkers outside of the DNA, leaving native bases intact, so that the dynamics observed are likely perturbed by the enhanced mobility of the probe labels.

To examine the effects of the presence and initial binding of the helicase protein on DNA breathing, we defined the relaxation time $\tau_e$ as the time required for the TCF to decay to a factor $1/e$ of its initial value. In Fig. 5A, we show histograms of the relaxation times ($\tau_e$) obtained from the TCFs of the smFRET data using DNA fork constructs that had been labeled either deep in the duplex region (blue) or at the replication fork junction (red). We characterized these data using the gamma distribution function

$$f(\tau_e) = \left[ e^{-\beta(\tau_e - \alpha)}/\beta \Gamma(\alpha) \right]^{\gamma/\alpha} \left( \tau_e - \alpha \right)^{\gamma-1} e^{-(\tau_e - \alpha)/\beta},$$

which is an appropriate distribution for the time domain. Here, the parameters $\alpha$ and $\beta$ describe the degree of skewness and the width of the distribution, respectively, and $\Gamma(z) = \int_0^\infty e^{-z} e^{-z} dz$ is the gamma function. We also present the corresponding rate domain ($k_i = 1/\tau_e$) histograms, which are fit to Gaussian distributions in SI Text, Control Experiments. In the absence of unwinding proteins (Fig. 5A), the duplex-labeled construct exhibits a slightly faster average decay time (blue, 201 ms) in comparison with the fork construct (red, 289 ms). The faster decay time for the duplex sample suggests that the closing rate of a spontaneously formed bubble in the duplex region of dsDNA is faster than that of a similarly formed unstable conformation near the ss–dsDNA fork junction. DNA breathing fluctuations have been shown to occur much more frequently at replication fork junctions than at sequences positioned well within duplex DNA regions (5, 6). The relative magnitudes of the lifetimes we have observed for the fork- and duplex-labeled DNA constructs are consistent with previous observations (7).

The relaxation times of equilibrium fluctuations observed in our data can be dramatically lengthened by the introduction of the GTPγS-stabilized hexameric gp41 helicase, which binds weakly to replication fork junctions. In this experiment we assembled the gp41 helicase (300 nM gp41, no gp61) using 6 μM nonhydrolyzable GTPγS rather than NTP. Under these conditions, the hexameric helicase forms and binds weakly at the fork junction, as depicted in Fig. 1A–C, but unwinds the dsDNA sequence by only one base pair (13, 14). As shown in Fig. 5B, we observed that the distribution of relaxation times for the DNA construct with chromosome labels placed near the replication fork junction were dramatically broadened (blue), with an average decay time of 650 μs. Clearly, the presence of the gp41 helicase at the iCy3/iCy5-labeled replication fork junction extended the lifetimes of open (unpaired) DNA bubbles formed in that region, and broadened their distribution. Furthermore, both the width and the average magnitude of fluctuations at the replication fork increased in the presence of the T4 helicase. In Fig. 5C, we plot the distribution of the magnitude of the relative fluctuations of the smFLD, signal ($S^2 - S^2$), which can be used to characterize the width of the distribution of open DNA fork conformations. Though both the duplex- and fork-labeled DNA constructs exhibit a relatively narrow distribution of open conformations (Fig. 5C, Left and Center), the average magnitude and width of the fluctuations at the replication fork junction increases in the presence of the T4 helicase (Fig. 5C, Right). The combination of these results for the helicase-dependent distributions of relaxation times and fluctuation magnitudes is consistent with the notion that microsecond time-scale breathing fluctuations at and near the fork junction likely play a significant role in helicase-binding mechanisms at this locus.

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**Fig. 4.** Examples of single-molecule FRET and FLD, TCFs. TCFs determined from (A) FRET and (B) FLD, trajectories obtained from a single DNA model fork construct in the absence of helicase proteins. Red lines represent optimized fits to Eq. 4, where the fit parameter $S^2$ is the mean square magnitude of the signal fluctuations, $\langle S^2 \rangle^2$ is square average signal, and $\tau_e$ is the correlation time. The donor–acceptor chromophore labels are placed at the ss–ds junction of the DNA construct (SI Text, Sample Preparation and Model DNA Replication Fork Constructs).

**Fig. 5.** Dynamics of breathing fluctuations at the replication fork in the absence and presence of helicase. (A) Histograms of relaxation times ($\tau_e$) obtained from the analysis of smFRET/smFLD trajectories for DNA fork constructs, which were labeled with iCy3/iCy5 placed deep in a duplex region (blue) or at the replication fork junction (red) in the absence of helicase protein. (B) A comparison is shown for a fork-labeled construct in the absence (red) and presence (blue) of the “frozen” hexameric helicase (gp41 ∙ GTPγS) composed of 300 nM gp41 and 6 μM GTPγS. (C) A comparison is shown of histograms of the relative magnitudes ($S^2 - S^2$) of the fluctuating smFLD, signal for duplex-labeled DNA (Left), fork-labeled DNA (Center), and fork-labeled DNA + gp41 ∙ GTPγS (Right). Histograms of the relaxation times were characterized using the gamma distribution function, given in the text, with skewness and width parameters $\alpha$ (dimensionless) and $\beta$ (in units of milliseconds), respectively.
Discussion

Observations of the local conformations of macromolecules through smFRET and smFLD should provide insights into the functional mechanisms of the “protein machines” that manipulate DNA. As a helicase processively unwinds a DNA duplex, the local conformation and flexibility of the opposing DNA strands must vary considerably. Such motions will be reflected by fluctuations in the orientation and separation of fluorophores that substitute for nucleotides within the sugar–phosphate backbone, and these motions can be simultaneously monitored using smFLD and smFRET.

The smFLD method is a useful complement to smFRET because it can help to avoid misinterpretation of false smFRET signals. For example, if the acceptor fluorophore of a coupled donor–acceptor FRET pair was to undergo a photobleach event (permanent or temporary), there would be a drop in acceptor intensity coincident with a rise in donor intensity. An acceptor photobleach event could thus be misinterpreted as a sudden separation between the donor–acceptor pair. This ambiguity can be addressed by simultaneously monitoring the smFLD signal, because a FRET conversion event due to donor–acceptor pair separation will be accompanied by a significant change in the smFLD signal, whereas an acceptor photobleach event will not.

Our finding that the presence of the gp41 hexameric helicase greatly enhances the widths and average values of both the lifetime and the magnitude distributions of “open” conformations at the fork junction implies that these thermally populated minority states of the DNA substrate play a role in the helicase-binding mechanism. The significance of our results lies in the context of understanding the energetic factors that contribute to the functional assembly of biological macromolecular machines. An open question is whether the initial steps of helicase–primase (primosome) assembly occur at the replication fork junction through a cooperative mechanism in which the binding of the gp41 hexameric helicase fundamentally alters the nature of DNA fluctuations at the fork junction and thereby facilitates the subsequent binding of the gp61 primase during initial primosome helicase assembly, as well as during the subsequent unwinding reaction. Alternatively, DNA breathing and helicase binding might not influence one another, in which case the formation of the primosome–DNA complex must reflect a random coincidence of events. Though the latter scenario can describe the chemical kinetics of small molecular systems, which may be characterized by a stationary potential energy landscape, our current results appear to support the more complex former situation, in which a highly mutable time-evolving energy landscape can respond to locally changing environments, including here the presence of the weakly bound helicase complex at the DNA replication fork junction.

Methods

Instrumentation for smFLD and smFRET. A smFRET instrument was designed to use rapid modulation of the excitation beam. The detection system involved custom electronics and analysis to perform single photon-counting phase-sensitive techniques. Instrument details and theoretical considerations to perform these experiments are provided in SI Text, Instrumentation for Single-Molecule Fluorescence-Detected Linear Dichroism and Single-Molecule Förster Resonance Energy Transfer.

Sample Preparation and Model DNA Replication Fork Constructs. Model DNA replication fork constructs, which were labeled with the iCy3/iCy5 FRET chromophore pairs, were purchased from Integrated DNA Technologies. Model DNA sample chambers were constructed from microscope slides and coverslips. Details of the sample chamber construction, cleaning procedures, and reagents used are given in SI Text, Sample Preparation and Model DNA Replication Fork Constructs.

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