Hexapeptides That Inhibit Processing of Branched DNA Structures Induce a Dynamic Ensemble of Holliday Junction Conformations*

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**Background:** Anti-microbial hexapeptides trap Holliday junctions and inhibit junction-processing enzymes. 

**Results:** Hexapeptides induce multiple conformations and dynamic fluctuations of two Holliday junctions that differ in core sequence. 

**Conclusion:** Destabilization of the functional junction conformation likely contributes to inhibition of enzymes that process Holliday junctions. 

**Significance:** Ligand-induced conformational dynamics may contribute generally to the action of anti-microbial agents that target specialized DNA structures.

Holliday junctions are critical intermediates in DNA recombination, repair, and restart of blocked replication. Hexapeptides have been identified that bind to junctions and inhibit various junction-processing enzymes, and these peptides confer anti-microbial and anti-tumor properties. Earlier studies suggested that inhibition results from stabilization of peptide-bound Holliday junctions in the square planar conformation. Here, we use single molecule fluorescence resonance energy transfer (smFRET) and two model junctions, which are AT- or GC-rich at the branch points, to show that binding of the peptide KWWCRW induces a dynamic ensemble of junction conformations that differs from both the square planar and stacked X conformations. The specific features of the conformational distributions differ for the two peptide-bound junctions, but both junctions display greatly decreased Mg$^{2+}$ dependence and increased conformational fluctuations. The smFRET results, complemented by gel mobility shift and small angle x-ray scattering analyses, reveal structural effects of peptides and highlight the sensitivity of smFRET for analyzing complex mixtures of DNA structures. The peptide-induced conformational dynamics suggest multiple stacking arrangements of aromatic amino acids with the nucleobases at the junction core. This conformational heterogeneity may inhibit DNA processing by increasing the population of inactive junction conformations, thereby preventing the binding of processing enzymes and/or resulting in their premature dissociation.

The Holliday junction is a four-way branched DNA structure that is a key intermediate in fundamental biological processes including homologous recombination, DNA damage repair, and restart of blocked replication forks (1–5). Holliday junctions are also produced during DNA exchange mediated by conservative site-specific recombinases of the tyrosine family (6–8). In recombination by these enzymes, cleavage and exchange of the first pair of strands result in a Holliday junction intermediate, which then isomerizes to trigger the second strand cleavage exchange step, resolving the junction into recombinant products.

The conformations of Holliday junctions are exquisitely sensitive to their ionic environments. At low ionic strength, repulsion of the negative charges at the core and along the DNA backbone causes the junction to adopt a nearly square planar form, with the extended arms arranged in a cruciform-like shape (9, 10). In high monovalent ion concentrations or modest concentrations of divalent cations such as Mg$^{2+}$, the arms stack co-axially to form an anti-parallel right-handed X-shaped structure (9, 11–13). Each arm may stack with either of two partners, generating two possible conformations between which the junction may oscillate (14, 15) (see Fig. 1A). The relative abundance of these conformations is determined by sequence-specific interactions at the branch point and is unaffected by the Mg$^{2+}$ concentration (16, 17). The transition rates between the conformations decrease with increasing Mg$^{2+}$, most likely because transit through obligatory square planar or tetrahedral intermediates depends on a loss of Mg$^{2+}$ ions from the junction (16, 18–20). Although the canonical stacked X and square planar conformations have provided a very useful framework for understanding junction structures and dynam-
ics, recent studies have shown that a broader range of conformations is accessible as transient intermediates or even stable states in isolation or when bound by a protein (19–22) (see “Discussion”).

In principle, molecules that interfere with or destabilize the functional conformations of Holliday junctions can be deployed to inhibit biological processes that generate these junctions as reaction intermediates. Such molecules could potentially serve as effective anti-microbial agents. Indeed, the peptides are rich in aromatic amino acids and are thought to derive binding energy from stacking interactions with solutions of one of the most potent peptides, KWWCRW (27, 29, 33), with two Holliday junctions that differ in the sequences surrounding their branch points (AT-rich and GC-rich; see Fig. 1B). We perform single molecule fluorescence resonance energy transfer (smFRET) analysis, along with native gel shift and small angle x-ray scattering (SAXS) assays, of the peptide-free and peptide-bound junctions. All three methods indicate that peptide binding changes the conformations of the DNA arms. The gel mobilities of the peptide-bound junctions do not conform to canonical square planar or stacked X conformations, whereas the SAXS data suggest junction conformations that are more open than stacked X conformations. The higher resolution of smFRET reveals additional detail, indicating an ensemble of conformations that fluctuate rapidly, with substantial effects of junction sequence on the dominant conformations. Together, the findings from the three approaches suggest a potential mechanism for peptide action in which inhibition of junction processing results from a decreased population of the biologically active state of the junction and a concomitant increase in multiple intermediates that are in rapid dynamic exchange.

### Experimental Procedures

**Hexapeptide Synthesis**—Hexapeptides H$_2$N-KWWCRW and H$_2$N-WWYCR were synthesized on a Symphony Peptide Synthesizer (Protein Technologies, Inc., Tucson, AZ) through the Protein and Metabolite Analysis Facility at the Institute for Cellular and Molecular Biology (University of Texas at Austin). The final purity of each peptide was 90–95%, as determined by MALDI mass spectrometry (data not shown).

### Table 1

**Oligonucleotides used for Holliday junctions**

The 78- and 22-mer sets were used to construct junctions with 39- and 11-bp long arms, respectively. The components of the AT or GC junction are identified by the prefixes AT and GC, respectively. The large and small junctions were employed for gel mobility and FRET analyses, respectively. The 11-bp arm sequences proximal to the branch point are identical between a large junction and its truncated counterpart. Each DNA arm is named for the restriction enzyme cleavage site that it contains in the large junction: BamHI (B), HindIII (H), EcoRI (R), or XbaI (X).

| Name      | Sequence                                                                 |
|-----------|---------------------------------------------------------------------------|
| AT-R-39   | 5’-AGG CTC ACC CAA GGC AGC CAA GGA ATT CCC CAC CCC CTA GCA ATT CCA CGG CTC ACC CCA GAA GCT CCA GGT CTA GGG TGT ACC CCA GGA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA ATT CCC CAC CCC CTA CCA GCA AT...
Conformational Dynamics of Peptide-bound Holliday Junctions

Preparation of DNA Junctions for smFRET Experiments—The sequences of the junction oligonucleotides are listed in Table 1. The GC junction was assembled as described previously (16). The AT junction did not assemble efficiently using this procedure, and it was therefore assembled with a modified procedure. The four stands (R, X, H, and B; 3 μM each) were heated to 90 °C for 2 min. The mixture was transferred to a water bath at 70 °C, which was cooled slowly to 50 °C on the bench top. Solution conditions were 25 mM Tris-Cl (pH 7.4), 25 mM NaCl for both junctions. The assembled junctions were placed on ice.

Preparation of DNA Junctions for Gel Mobility Assays—In the junctions used for the gel mobility shift assays, each arm contained a cleavage site for the restriction enzymes BamHI, HindIII, EcoRI, or XbaI. A series of six two-long-arm (39 bp), two-short-arm (11 bp) constructs was produced by digestion with pairwise combinations of these enzymes. The names of the junctions correspond to the two undigested arms.

smFRET Measurements—Junctions (40 nm) were incubated with peptide (100 μM) on ice for 30 min in 10 mM Tris-Cl (pH 7.4), 25 mM NaCl and visualized using an experimental setup that has been described previously (42–45). Peptide-junction complexes were formed in the absence of Mg²⁺ and applied at a concentration of 20–50 pM to a quartz slide (G. Finkenbeiner Inc., Waltham, MA) passivated with a 99:1 methoxy polyethylene glycol-biotinylated PEG mixture (Laysan Bio Inc., Arab, AL). Prior to addition of the biotinylated complexes, 0.1 μg/ml streptavidin was applied to the slide so that the biotinylated junction complexes could be specifically immobilized by binding the streptavidin. Buffer solution with 100 μM KWWCRW and with or without Mg²⁺ was flowed over the slide, and junctions were imaged by prism-type total internal reflection microscopy with an Olympus IX-71 microscope upon excitation with a 532-nm laser (Crystalaser, Reno, NV). A 637-nm laser (Coherent, Santa Clara, CA) was used to confirm the presence of Cy5 for each junction. Images were acquired at 10–25 Hz with a cooled I-PentaMAX IIC CCD (Princeton Instruments, Trenton, NJ). A deoxygenating imaging buffer, used to remove stray excitation light was removed using notch filters. Donor and acceptor emissions were matched for each molecule using custom software by plotting the FRET value of frame n versus the FRET value of frame n + 7 for the entire length of the movie. Frame n + 7 was selected to eliminate any bias from averaging. Increasing this gap to frame n + 13 did not change the results significantly (data not shown). For each molecule, the average FRET values and standard deviations were calculated from the entire time trace until data collection ended or photobleaching occurred.

Simulations of smFRET Data—To evaluate the effects of limited sampling time on the distribution of average FRET values for individual junction molecules, we simulated smFRET data to correspond to the experimental data sets. Monte Carlo simulations were performed for 200 molecules with Gaussian noise levels (σ_app) that corresponded to the experimental data sets and were introduced randomly on a frame by frame basis for 300 frames. The average FRET value was then calculated for each simulated molecule, and the distribution was compared with the corresponding experimental distribution.

Polyacrylamide Gel Electrophoresis—The peptide-free junctions were analyzed by electrophoresis in 8% polyacrylamide (acylamide to bis-acrylamide, 19:1). Electrophoresis was performed at 4 °C in TBE buffer (50 mM Tris, 41 mM boric acid, 0.5 mM EDTA) for 6 h at 6.6 V/cm. Electrophoresis of the peptide-bound junctions was performed in 6% polyacrylamide gels (19:1) in TBE buffer with 0.1% DMSO at 4 °C for 48 h at 3.3 V/cm. The gel solution contained 10 μM peptide prior to polymerization. In addition, 10 μM peptide was included in the running buffer, which was refreshed in the anodic and cathodic chambers at 12-h intervals.

Small Angle X-ray Scattering—SAXS data were collected at the Advanced Photon Source (Beamline 12-ID-C, 12 keV, 2-m sample-detector distance). DNA junction concentrations were 25–50 μM, and peptide concentrations were 100–200 μM. Solution conditions were 30 mM Tris-Cl (pH 8.0), 10 mM NaCl, and 0.1% DMSO with 0–10 mM Mg²⁺. To ensure that samples were maintained at 0.5 mM free Mg²⁺ when desired, despite the high concentrations of nucleic acid, buffer exchange was performed using Amicon Ultra filters (EMD Millipore, 10-KDa cutoff) prior to adding peptide. An independent blank was used for each sample. The data were analyzed with IgorPro (WaveMetrics) and ATSAS. Kraty plots were normalized by the forward scattering intensity, I(0). The Rg values were determined from the interatomic distance distribution functions, P(r), calculated by AUTOGNOM (46) and verified by Guinier analysis. The data at very small angles that were affected by aggregation were excluded from Rg analysis.
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Results

smFRET Characterization of Holliday Junctions Harboring AT- or GC-rich Branch Points—We used immobile Holliday junctions with 11-bp arms, designated B, H, R, and X, following previous nomenclature (9) (Fig. 1A). The 5’ ends of the B and H arms were labeled with Cy5 (red circle) and Cy3 (green circle) dyes, respectively. The R arm included a 5’ biotin (brown circle) for immobilizing the junctions to streptavidin-coated slides. The junction can fold into two possible anti-parallel conformations, Iso1 and Iso2, which give distinct FRET signals because of the relative positions of the dyes. B, the nucleotide sequences surrounding the immobile branch point for the AT and GC junctions are shown. The two junctions are identical in the rest of the arm sequences, which are not shown.

In the absence of Mg2+, the AT junction gave time traces centered on a low FRET value (0.2), without detectable excursions to other FRET states (Fig. 2A). The corresponding histogram from all molecules revealed a unimodal distribution centered at 0.22. This FRET value is in the range expected for the square planar conformation based on ensemble FRET measurements (10, 13). Although the dyes may stack on the helix ends, the FRET value is also in good agreement with a calculated value (0.27) for the square planar conformation using arm lengths of 11 bp and assuming free rotation of the dyes. The GC junction displayed similar behavior (data not shown).

Upon addition of Mg2+, changes were visible in the AT junction, both in the dominant structures and the frequency of transitions between them. With low Mg2+ concentrations, the average FRET value increased with increasing Mg2+ concentration, plateauing at ~0.43 at 2 mM Mg2+ (Fig. 2B and D). The FRET distribution remained unimodal but broadened (Fig. 2B). We infer that Mg2+ destabilizes the square planar form and allows the junction to fold into the antiparallel stacked X isoforms, with the two isoforms exchanging fast enough that they are detected as a single distribution (tens of ms or faster). With higher Mg2+ concentrations, the FRET distribution was resolved into two well-formed peaks centered at ~0.05 and ~0.65, which correspond to the two stacked X isoforms (Fig. 2C) (16). Under these conditions, transitions between the two conformations were readily detectable, and the rate constants decreased for both states with increasing Mg2+ concentration (Fig. 2E). In contrast, the equilibrium between the conformations remained unchanged with Mg2+ concentration, with a mild but significant preference for Iso2 (Kiso2 = 1.8; Fig. 2E). The results for the GC junction were qualitatively analogous and consistent with previous findings, with an equilibrium value of 1.1 (data not shown) (16). As observed previously (16, 47), we found that a fraction of the molecules for both junctions displayed rate and equilibrium constants that were more divergent from the average behavior than expected by chance, indicating

FIGURE 1. Holliday junctions used in the smFRET experiments. A, the four 11-bp arms of the junctions (termed B, H, R, and X). The 5’ ends of the B and H arms were labeled with Cy5 (red circle) and Cy3 (green circle) dyes, respectively. The R arm included a 5’ biotin (brown circle) for immobilizing the junctions to streptavidin-coated slides. The junction can fold into two possible anti-parallel conformations, Iso1 and Iso2, which give distinct FRET signals because of the relative positions of the dyes. B, the nucleotide sequences surrounding the immobile branch point for the AT and GC junctions are shown. The two junctions are identical in the rest of the arm sequences, which are not shown.

FIGURE 2. smFRET measurements of the AT junction. A–C, representative time traces are shown for individual junctions (left panels) with the cumulative histograms from all observed molecules (right panels) in the absence of Mg2+ (A, n = 115) or with 2 mM (n = 104) or 50 mM (n = 139) Mg2+ (B and C, respectively). D, changes in the average FRET value as a function of Mg2+ concentration. These values were fit with a hyperbolic equation with a K1/2 value of 0.6 mM Mg2+. E, rate and equilibrium constants for transitions between Iso1 and Iso2. The rate constants were determined from single exponential fits to the cumulative dwell time histograms for each isoform. The equilibrium constants were calculated as the quotient of the rate constants. This method gave values that were the same within error as the quotient of the integrated peak volumes in the cumulative histograms (data not shown).
molecular heterogeneity. We did not determine whether this behavior arises from conformational or chemical differences between molecules or analyze the behavior further. Thus, in the absence of an interacting peptide, both junctions adopt the square planar form in the absence of Mg$^{2+}$. They switch to the two stacked X forms (IsoI and IsoII) in the presence of Mg$^{2+}$, with a detectable bias for IsoII (~2-fold) by the AT junction. The distributions and lifetimes of these conformations provide a reference for interrogating potential peptide-induced alterations in the structural and dynamic properties of each junction.

Conformational Changes in Peptide-bound Holliday Junctions Revealed by smFRET—We next used smFRET to follow changes in the conformations and dynamics of the AT and GC junctions upon peptide binding. Previous work showed that KWWCRW interacts strongly with Holliday junctions at low ionic strength, binding to free junctions and inhibiting recombination with an equilibrium constant in the range of 10–25 nM (24, 27, 33). To ensure full binding at all Mg$^{2+}$ concentrations, we preincubated junctions with 100 μM KWWCRW and the desired Mg$^{2+}$ concentration.

In the presence of KWWCRW and without Mg$^{2+}$, the AT junction gave a pronounced upward shift and broadening of the FRET value relative to the peptide-free junction (Fig. 3, A and C). These effects indicate that peptide binding does not simply stabilize the square planar conformation under these conditions but instead leads to the formation of a new conformation or conformations. A similar broad distribution of FRET values was induced by the peptide in 50 mM Mg$^{2+}$ (Fig. 3, B and D), replacing the two X-form peaks observed in its absence (dashed curve in Fig. 3D), and similar results were obtained at lower Mg$^{2+}$ concentrations (0.5–10 mM Mg$^{2+}$; data not shown). The broadening of the FRET distribution is consistent with formation of multiple conformations. The FRET histograms did not provide evidence for discrete conformational states because of a lack of well resolved peaks. However, this simple analysis may not resolve states that give similar FRET values or detect states that are sparsely populated.

To probe more incisively whether bound KWWCRW produces discrete junction conformations and transitions between them, we generated transition density (TD) plots from the smFRET data (Fig. 4). TD plots have been used as a way of illustrating the frequency of transitions that were identified using thresholds or Hidden Markov modeling (48). Here, in the absence of any clearly defined states identifiable from the histograms, we generated the TD plots directly from the data. In these plots, density along the line $y = x$ corresponds to an unchanged FRET value from one time bin to the next (500 ms), and transitions between conformations with different FRET values are represented by off-axis peaks (Fig. 4, A–D). In the presence of peptide, significant density is present away from $y = x$ (Fig. 4, C and D), indicating extensive transitions on the time scale of 0.5 s or faster. However, this density is not present in discrete peaks, suggesting a lack of dominant conformations. Together, these data indicate that upon binding of KWWCRW in the absence or presence of Mg$^{2+}$, the AT junction populates a broad ensemble of conformations that exchange rapidly with each other.
Analogous experiments with the GC junction revealed broadly similar behavior but with some clear differences (Fig. 5, A–D). In the absence of Mg\(^{2+}\), the FRET value of the GC junction increased upon binding KWWCRW, indicating a conformational change away from the square planar form (Fig. 5, A and C). However, the extent of broadening of the FRET histogram was smaller than for the AT junction (compare Fig. 5C with Fig. 3C), suggesting that the peptide-GC junction complex samples a more restricted region of conformational space. With 50 mM Mg\(^{2+}\), bound peptide also yielded a single peak of FRET values, closer to the \(\text{Isoll}\) peak (Fig. 5D) and narrower than that for the AT junction (compare Fig. 5D with Fig. 3D). Furthermore, comparison of the TD plots for the peptide-free and peptide-bound states of the GC junction (Fig. 6, A–D) reveals a narrow range of FRET values for conformational fluctuations, which neighbors \(\text{Isoll}\) in its mean FRET value, without evidence for discrete states. As a further test of the generality of peptide-induced conformational changes, we incubated the GC junction with the peptide WRWYCR, which also inhibits Holliday junction resolution (29, 31, 33). The effects of WRWYCR on GC junction behavior were analogous to those of KWWCRW (data not shown).

The results above showed that the peptides induce structural changes in Holliday junctions, resulting in conformations that can exchange rapidly. To probe whether there are also conformations that exchange slowly, appearing as static heterogeneity in our experiments, we plotted the standard deviations (\(\sigma_{\text{FRET}}\)) versus the average FRET value for each junction molecule in the presence and absence of KWWCRW (Fig. 7). Static heterogeneity would be expected to give a broader range of average FRET values for individual junction molecules, whereas increased dynamics would give increases in \(\sigma_{\text{FRET}}\) without a broadening of the average value beyond that which would reflect the limits of the finite sampling time. The peptide-bound AT junction clearly displayed a significant increase in \(\sigma_{\text{FRET}}\) in both the absence and presence of Mg\(^{2+}\), as well as broadening of the average FRET values (Fig. 7, A, B, E, and F). In contrast, the GC junction showed increased \(\sigma_{\text{FRET}}\) values with bound peptide but displayed minimal change in the width of the average FRET values (Fig. 7, C, D, G, and H).

To test whether the broadening of the average FRET values for the AT junction was sufficient to indicate static heterogeneity, we performed Monte Carlo simulations to generate average FRET values of individual junction molecules with and without bound peptide (Fig. 8). These simulations showed that
the broadening of the average FRET values upon peptide binding was larger than that expected from the increased standard deviation and the experimental sampling time, both in the presence and absence of Mg$^{2+}$. The distributions of the experimental FRET values for individual molecules are shown by gray bars. The corresponding simulated distributions (dashed curves) were generated from 300-frame time traces for 200 individual molecules with Gaussian noise levels that correspond to the experimental data sets. This analysis did not include simulations of junctions in the presence of Mg$^{2+}$ and the absence of peptide because the reversible transitions between Isol and Isol make the average FRET value for each molecule dependent on the frequency of the transitions rather than a simple dynamic variability in FRET value from frame to frame.

FIGURE 8. Comparison of the average FRET distributions from smFRET experiments and Monte Carlo simulations. Distributions of individual molecules are shown for the AT (A–C) and GC (D–F) junctions. Simulations were performed as described under “Experimental Procedures” for the free junctions in the absence of Mg$^{2+}$ (A and D) and for the peptide-bound junctions in the absence (B and E) and presence (C and F) of 50 mM Mg$^{2+}$. The distributions of the experimental FRET values for individual molecules are shown by gray bars. The corresponding simulated distributions (dashed curves) were generated from 300-frame time traces for 200 individual molecules with Gaussian noise levels that correspond to the experimental data sets. This analysis did not include simulations of junctions in the presence of Mg$^{2+}$ and the absence of peptide because the reversible transitions between Isol and Isol make the average FRET value for each molecule dependent on the frequency of the transitions rather than a simple dynamic variability in FRET value from frame to frame.

values (Fig. 8, A and D), reflecting a population of junctions with long-lived conformational or chemical differences relative to the main populations. Together, the data indicate that upon binding KWWCRW, both junctions populate an ensemble of rapidly exchanging conformations, and the AT junction is also partitioned into long-lived families of conformations.

Together, the smFRET results suggest that these inhibitory hexapeptides strongly influence the arrangements of the junction arms in the presence and absence of Mg$^{2+}$. Peptide binding increases the range of accessible conformations, especially for the AT junction, as revealed by the increased breadth of the histograms. The Mg$^{2+}$ concentration dependences are greatly reduced, perhaps because the peptide shields or occupies Mg$^{2+}$ sites within the junction core. Aromatic amino acids of the peptide probably form stacking interactions with the nucleobases surrounding the junction, increasing the conformational range and dynamics of the junction by allowing these bases to be less dependent on stacking with their neighbors. This reduced dependence generates a population of conformations that are neither square planar nor stacked X conformations. Nevertheless, the identities of the bases at the core contribute to the conformational properties of the junction even with bound peptide, because the range of accessible conformations is larger for the AT junction than the GC junction.

**Lifetimes of Peptide-Junction Complexes**—The junction dynamics induced by the hexapeptides could result from short- or long-lived complexes. We used the change in junction behavior revealed by smFRET to monitor the lifetimes of KWWCRW with each junction. After immobilizing the peptide-junction complexes on a microscope slide, we washed out the peptide and monitored the FRET distributions at various times thereafter. In the absence of Mg$^{2+}$, the AT and GC junctions retained their peptide-bound FRET states for more than an hour after removal of the excess peptide (Fig. 9, A and D). A previous analysis using a gel shift assay indicated a half-life for WYWYCR of approximately 1 h at 4 °C (33). In the presence of 50 mM Mg$^{2+}$, dissociation of KWWCRW was accelerated (Fig. 9, B and E). For the AT junction, complete dissociation occurred within 20 min and proceeded through an intermediate state with a FRET value of ~0.05 (Fig. 9, B and C). For the GC junction, dissociation occurred on the same time scale as for the AT junction but with no evidence of the intermediate, signifying either an alternative pathway or a lack of sufficient accumulation of the intermediate for it to be detected (Fig. 9, E and F). Nevertheless, under all of the conditions tested, the complex persisted on the time scale of minutes, orders of magnitude longer than the lifetimes of the conformational states detected by smFRET. Thus, the conformational fluctuations described in the sections above occur, whereas the peptide remains bound to the junction.

**Hexapeptide-induced Changes in Junction Conformations Revealed by Gel Mobility Shifts**—The square planar and stacked X conformations give characteristic electrophoretic gel mobilities, because the lengths of the DNA arms in conjunction with the angles subtended between adjacent arms determine the migration velocities (49). To complement the smFRET analysis of junction conformations and to relate the current work to

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**Conformational Dynamics of Peptide-bound Holliday Junctions**

**FIGURE 8. Comparison of the average FRET distributions from smFRET experiments and Monte Carlo simulations. Distributions of individual molecules are shown for the AT (A–C) and GC (D–F) junctions. Simulations were performed as described under “Experimental Procedures” for the free junctions in the absence of Mg$^{2+}$ (A and D) and for the peptide-bound junctions in the absence (B and E) and presence (C and F) of 50 mM Mg$^{2+}$. The distributions of the experimental FRET values for individual molecules are shown by gray bars. The corresponding simulated distributions (dashed curves) were generated from 300-frame time traces for 200 individual molecules with Gaussian noise levels that correspond to the experimental data sets. This analysis did not include simulations of junctions in the presence of Mg$^{2+}$ and the absence of peptide because the reversible transitions between Isol and Isol make the average FRET value for each molecule dependent on the frequency of the transitions rather than a simple dynamic variability in FRET value from frame to frame.**
previous analyses, we performed gel mobility shift analyses on the peptide-free and peptide-bound junctions.

We performed pairwise enzyme digestion on junction constructs harboring specific endonuclease cleavage sites to yield six junction derivatives, each with two long (39 bp) and two short (16 bp) arms (Fig. 10A) (9). In the absence of Mg\(^{2+}\), both the AT and GC junctions migrated in the typical “4-2” migration pattern expected for the square planar conformation (Fig. 10, B and C). Upon incubation with KWWCRW, both junctions gave migration patterns distinct from those of the peptide-free junctions (Fig. 10, D and E). In these gels, the lower and upper sets of sextet bands were interpreted as the free and bound forms of the junctions, respectively. Consistent with this assignment, the lower band set followed the 4-2 pattern for both junctions (labeled Peptide-Free in Fig. 10, D and E). For the AT junction, the peptide-bound forms migrated as diffuse bands, consistent with an ensemble of junction conformations. The peptide-induced upward shift was more prominent for one of the faster migrating bands (BR) than its partner band (HX) (Fig. 10D). The slower migrating quartet also displayed unequal upward shifts among individual bands following peptide binding. As a result, the band pattern did not conform to the canonical 4-2 pattern of the square planar form or to the 2-2-2 pattern of the stacked X forms (15). The BR and HX bands of the GC junction were also strongly retarded by peptide binding, BR more so than HX (Fig. 10E). Five of the six bands of the bound

FIGURE 9. Dissociation kinetics of KWWCRW from the AT and GC junctions. Peptide dissociation was followed in the absence (A and D) or presence (B and F) of 50 mM Mg\(^{2+}\) by monitoring smFRET after washing out peptide from solution. C and F, the fractions of the peptide-bound (open circles), peptide-free (filled circles), and intermediate (open squares) states of the junctions are plotted as a function of time. The data for the AT junction, analyzed by a best fit model with two irreversible transitions, yield rate constants of 0.14 min\(^{-1}\) for the formation of the intermediate and 0.60 min\(^{-1}\) for the transition from the intermediate to the peptide-free junction. The rate constant for peptide dissociation from the GC junction, based on a single exponential fit, is 0.14 min\(^{-1}\). The relative fractions of each species were determined by fitting the FRET distribution at each time point with the FRET distributions of the peptide-free and peptide-bound junctions through a least squares minimization procedure. The data for each time point were collected from a different field of molecules to minimize photobleaching effects.
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Gave large changes in overall size, as indicated by the radius of gyration ($R_g$) values (Table 2), which is not surprising because $R_g$ is dominated by the lengths of the helix arms (50).

As a more sensitive probe of junction conformation, we used Kratyk analysis (Fig. 11). Consistent with CRYSTAL simulations of a hexapeptide with 11-bp arms (Fig. 11, A and B) (46), Kratyk plots from both junctions include two peaks (Fig. 11, C–F). For the AT junction in the absence of Mg$^{2+}$, comparison of the relative heights of these two peaks with those in the simulations suggests that the square planar form is dominant, as expected (Fig. 11C). The relative prominence of the peak at lower $q$ values for the GC junction may indicate a detectable population of less open conformations with stacked X character even in the absence of Mg$^{2+}$ (Fig. 11D). The addition of KWWRCW in the absence of Mg$^{2+}$ did not affect the relative peak heights for either junction (Fig. 11, C and D). The addition of 0.5 mM Mg$^{2+}$ in the absence of peptide increased the prominence of the low $q$ peak, indicating a shift that favors stacked X conformations (Fig. 11, E and F). Higher Mg$^{2+}$ concentrations produced little further change, indicating that both junctions adopt largely folded conformations with 0.5 mM Mg$^{2+}$ (data not shown). Peptide addition in the presence of 0.5 mM Mg$^{2+}$ decreased the prominence of the low $q$ peak for both junctions (Fig. 11, E and F), suggesting that peptide binding reshapes the stacked X forms into more open forms.

Because the SANS data reveal an effect of peptide binding only in the absence of Mg$^{2+}$, a simple interpretation would be that peptide binding favors the square planar conformation. However, this interpretation is inconsistent with the smFRET and gel shift results. Evidently, the average global shape of the peptide-bound junction resembles that of the square planar form at the resolution afforded by SANS, but the gel shift analysis shows that the peptide-induced conformations are distinct from the square planar form, and the higher resolving power of smFRET is able to reveal the complex and dynamic set of conformations induced by the peptide.

**Discussion**

The mechanisms by which anti-microbial hexapeptides interact with three- and four-way DNA junctions and inhibit DNA unwinding, branch migration, or junction resolution are not well understood. Based on gel mobility shift, fluorescence quenching, and structural data, combined with modeling, it has been suggested that an inhibitory peptide dimer competes with processing enzymes for binding branched DNA structures.
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accelerates the dissociation of bound enzymes, and/or induces a ternary complex conformation in which catalysis is deterred (29, 32–34). Here, we used smFRET, gel mobility shift assay, and SAXS to probe the effects of one of these peptides on the global conformations of two Holliday junctions. Our results indicate an additional, unrecognized effect of the peptide. We find that peptide binding increases the range and dynamics of junction conformations and greatly reduces their dependence on interactions with Mg\textsuperscript{2+} ion. The formation of these rapidly exchanging conformations upon peptide binding, which by definition will include conformations that are not productive for enzyme binding or function, may be a key factor in the inhibition of Holliday junction processing enzymes.

**Peptide Binding to the Holliday Junction Core Generates Conformational Diversity and a Flattening of the Energy Landscape**—The previously uncharacterized AT junction follows the general behavior of other synthetic junctions in assuming the square planar conformation in the absence of Mg\textsuperscript{2+} and the IsoI and IsoII stacked X conformations in its presence. As observed for the GC junction (16), the rate constants for transitions of the AT junction between IsoI and IsoII decrease with increased Mg\textsuperscript{2+} concentration with no change in the equilibrium value, reflecting a requirement for transient unstacking of the bases at the junction core during exchange of the two conformations. The detectable preference for IsoII must arise from sequence differences relative to the GC junction, which populates IsoI and IsoII equally, but the specific origins of the differences are not clear. Previous work has shown that conformer preferences can be influenced by the branch point nucleotides (15, 16), their nearest neighbors (51), and even the third base pairs from the junction (15), all of which include differences between the AT and GC junctions.

Upon binding KWWCRWR, the conformational properties of both junctions are altered dramatically. In the absence of Mg\textsuperscript{2+}, both junctions display FRET histograms with increased average values and broadened distributions, indicating the formation of conformational ensembles distinct from the square planar form. With bound peptide, the FRET distributions of both junctions are relatively insensitive to Mg\textsuperscript{2+} and have average values between those of the two stacked X conformations. The simplest interpretation of these results is that the conformational ensembles also include open conformations, distinct from the stacked X conformations. The TD and $\sigma_{\text{FRET}}$ analyses show that the broadening of the FRET distributions is present on a molecule by molecule basis, particularly for the AT junction, indicating dynamic transitions between conformations.

We considered whether the changes in FRET behavior might arise from an artificial effect of the peptide on one or both dyes rather than an effect on the junction conformations, but we rejected this hypothesis because: (i) the AT and GC junctions respond differently to the presence of peptide, whereas an effect on the dyes would be expected to be the same; (ii) the junction behaviors persisted for minutes after the peptide was washed out in dissociation experiments, ruling out the effect requiring high peptide concentrations as might be expected for a weak, nonspecific interaction with the dyes; (iii) the behaviors of the junctions ultimately returned to their intrinsic behaviors after peptide washout, ruling out irreversible effects; and (iv) the total intensity distribution (Cy3 + Cy5) was unchanged by the peptide (data not shown), ruling out quenching by the peptide.

Although an alternative interpretation of the smFRET results would be that the junctions transition between stacked X conformations fast enough that they cannot be resolved in our measurements, this interpretation is inconsistent with the SAXS results, which show that peptide binding increases the population of open conformations at low Mg\textsuperscript{2+} concentration. The gel mobility shifts are also consistent with the FRET data, indicating nonstandard junction conformations spawned by peptide binding, without conveying the diversity of these conformations or fluctuations within them.

At a structural level, the effect of the peptides most likely arises because the positively charged amino acids promote DNA binding, reducing the dependence on Mg\textsuperscript{2+} concentration, and the hydrophobic amino acids can stack in various
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arrangements with the nucleobases at the junction core (29, 32–34), resulting in an array of conformations with various angles between the helical arms. In the parlance of an energy landscape representing the conformational space available to the junction, the peptide induces landscape flattening such that multiple conformations are approximately equivalent in free energy and therefore populated. As suggested by the SAXS analysis, the peptide-induced ensembles are dominated by conformations that are more open than the stacked X conformations. Nevertheless, the peak FRET values are distinct from those observed under the low ionic strength conditions that favor the square planar conformations, indicating a set of distinct conformations. Although the canonical model involving stacked X and square planar conformations has provided an important foundation for understanding junction conformations and dynamics, there is increasing evidence for additional, noncanonical conformations along the pathways for exchange between stacked X conformations (19, 20), as stable ground states for mobile junctions (22), or in complexes with junction-binding proteins (21). It is possible that the peptide-induced conformational ensembles observed here resemble one or more of these noncanonical junction conformations.

In addition to equalizing the valley depths, the peptide decreases the peak barriers between them, facilitating conformational exchanges. This effect is most obvious with high Mg$^{2+}$ concentrations, where junction transitions in the absence of peptide are slow enough to be measured by smFRET. The rapid structural transitions coupled with the long lifetime for peptide binding to the junction imply that the patterns of stacking interactions between the amino acids and the nucleobases at the junction can fluctuate readily and repeatedly while the peptide remains bound to the junction.

Differences in Peptide Effects on the AT and GC Junctions—Although the two junctions share some properties upon binding peptide, there are also notable differences. Most prominently, the GC junction occupies a more closely nested set of conformations, with FRET values that more closely resemble the high FRET IsoII than the IsoI or square planar forms. Nevertheless, it is possible that the same underlying physical model accounts for the peptide effects on the two junctions. For the GC junction, peptide binding may similarly induce conformations that include stacking between amino acid side chains and the junction nucleobases, replacing base-base stacking, but with an energetic preference for conformations with helical arm arrangements similar to the IsoII conformation. Even a modest energetic preference of 1–2 kcal/mol would produce the observed narrowing of the FRET distribution.

Inhibition of Enzyme Activity—Hexapeptides such as KWWCRW have been shown to inhibit a broad group of enzymes that catalyze reactions involving branched DNA structures as substrates or intermediates (24, 27–29, 32, 33). Although the mechanisms of inhibition have not been established and may vary among proteins, there are several reasons to think that the ability of bound peptide to shift the conformational distribution away from square planar and increase dynamics may contribute to enzyme inhibition.

First, a common theme of the diverse group of inhibited enzymes is that they act on junctions in a square planar-like conformation, suggesting that a shift away from this conformation could result in inhibition. Second, peptide binding has been shown to accelerate dissociation of at least one protein (RecG), ruling out a model involving strictly competitive inhibition in which the peptide simply occupies the same site as the protein (33). In addition, x-ray crystallography shows a peptide ternary complex with a recombine-bound DNA junction in which the peptide is bound away from the protein-binding site at the center of the junction (32), consistent with global rather than local effects. Finally, the observations of inhibition of multiple enzymes by multiple peptides suggest a generalized mode of action such as that observed here. Peptide-induced effects on the conformational ensemble and dynamics of junctions can be imagined to accelerate protein dissociation, slow binding, and/or inhibit catalytic steps by increasing the population of nonproductive conformations. Although there may also be sources of inhibition that are local and specific to certain enzymes and junctions, it seems likely that the peptide-induced increases in junction conformations and dynamics observed here are linked to inhibition. If the increased range of conformations is important, a simple prediction is that junctions with sequences corresponding to the AT junction will be more strongly inhibited than those corresponding to the GC junction.

Conclusions—The application of smFRET to synthetically assembled nucleic acid structures that model their biologically relevant counterparts is uniquely suited for exploring the role of ligand-mediated conformational dynamics and/or diversity in DNA transactions. Our results demonstrate that a hexapeptide broadens the conformational distribution of DNA junctions. The principle of blocking DNA transactions by enhancing, rather than constraining conformational freedom, may be more broadly applicable to a variety of peptide and non-peptide ligands that recognize specific DNA structures. A subset of such agents may engender desirable anti-microbial activities.

Author Contributions—A. H. K., B. C., M. J., and R. R. designed the study. A. H. K. and B. C. prepared the junction molecules. B. C. performed and analyzed the smFRET experiments. A. H. K. and B. C. performed the gel shift experiments. I. J. performed the SAXS experiments. B. C., A. H. K., M. J., and R. R. wrote the manuscript. All authors analyzed results and approved the final version of the manuscript.

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