Cre recombinase is a prototypical member of the tyrosine recombinase family of site-specific recombinases. Members of this family of enzymes catalyze recombination between specific DNA sequences by cleaving and exchanging one pair of strands between the two substrate sites to form a 4-way Holliday junction (HJ) intermediate and then resolve the HJ intermediate to recombinant products by a second round of strand exchanges. Recently, hexapeptide inhibitors have been described that are capable of blocking the second strand exchange step in the tyrosine recombinase recombination pathway, leading to an accumulation of the HJ intermediate. These peptides are active in the λ-integrase, Cre recombinase, and Flp recombinase systems and are potentially important tools for both in vitro mechanistic studies and as in vivo probes of cellular function. Here we present biochemical and crystallographic data that support a model where the peptide inhibitor binds in the center of the recombinase-bound DNA junction and interacts with solvent-exposed bases near the junction branch point. Peptide binding induces large conformational changes in the DNA strands of the HJ intermediate, which affect the active site geometries in the recombinase subunits.

The tyrosine recombinases (formerly referred to as the λ-integrase family) include the well-studied bacteriophage λ-integrase, Cre recombinase from bacteriophage P1, Flp recombinase from the Saccharomyces cerevisiae 2-micron circle, the bacterial XerC/D recombinases, and well over 100 other proteins identified among bacteria and yeast (1, 2). These proteins mediate a variety of biological processes that involve large changes in the DNA strands of the HJ intermediate, which affect the active site geometries in the recombinase subunits.

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The atomic coordinates and structure factors (codes 1XNS and 1XO0) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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¶ The tyrosine recombinase mechanism of site-specific recombination is the generation of a 4-way Holliday junction (HJ) intermediate (3). The overall reaction pathway is shown schematically in Fig. 1A. Two recombination sites, each bound by two recombinase proteins, associate to form a synaptic complex at the start of the recombination process. One strand in each of the synapsed substrates is cleaved by a recombinase subunit to form a covalent 3'-phosphotyrosine linkage, liberating a free 5'-hydroxyl group. Strand swapping between the synapsed sites and subsequent intermolecular ligation between the 5'-hydroxyl group originating from one duplex substrate and the 3'-phosphotyrosine linkage of the other substrate completes the first reciprocal strand exchange and generates a 4-way DNA junction. Isomerization of the junction to an alternative, but structurally related conformer (11) allows cleavage and exchange of the second pair of strands between substrates using the same phosphoryl transfer chemistry and generation of recombinant duplex products. The individual steps in this process are highly reversible and a challenge in the mechanistic study of these systems has been to isolate intermediates that exist only transiently in the recombination pathway (12).

Segall and co-workers (13–15) have recently described an approach to identify small peptides capable of trapping intermediates in λ-integrase recombination pathways. By iterative screening of mixed pools of synthetic peptides for their ability to block specific recombination steps, several potent inhibitors have been identified for both the initial synaptic complex, where the initial strand exchange is blocked, and for the HJ intermediate, where resolution of the junction in either direction is blocked. In addition to their utility as mechanistic probes to dissect the complex mechanism of λ-integrase site-specific recombination, such inhibitors could have more general utility in other biological pathways, particularly those that generate HJ intermediates (16).

The hexapeptide WKHYNY, for example, was originally identified based on its ability to inhibit the λ-integrase bent-L pathway, where it caused an accumulation of HJ intermediates (13). This peptide specifically blocks the HJ resolution step of...
the reaction that would normally result in either exchange of the second pair of DNA strands to form recombinant products, or in reversal of the original strand exchange step to regenerate substrates. The same peptide does not inhibit the initial cleavage and strand exchange step of the pathway that generates the HJ intermediate, although closely related peptides (e.g. KWWCRW) have been identified that also inhibit the initial cleavage step at concentrations greater than 5 μM (14, 15). Interestingly, the WKHYNY peptide inhibitory activity (measured as IC₅₀ values) varies about 100-fold between the integrative, excisive, bent-L, and straight-L pathways for λ-integrase recombination (17). Presumably, subtle differences in the structure of the HJ intermediate in these pathways and corresponding differences in the interactions made with the peptide inhibitor are responsible for the range of activities observed.

The WKHYNY peptide also inhibits the Cre-loxP and Flp-flr recombination pathways, resulting in an accumulation of HJ intermediates in those systems (17). Given that λ-int, Cre, and Flp have very low sequence similarity outside of a small set of conserved catalytic residues, it seems unlikely that the peptide specifically targets a feature of the recombinase protein itself. Instead, it has been suggested that the primary target is the specific recombinase-HJ DNA assembly that is formed during the reaction, where both the recombinase and the HJ DNA are recognized (14). An alternative possibility is that the peptide specifically targets only the HJ DNA, which presents a surface of solvent-exposed bases and sugar phosphate backbone that is unique within the recombination pathway (11, 18). Indeed, related peptides have been identified that bind to bare HJ DNA with high affinity.² In principle, however, peptides such as WKHYNY could also bind, but not inhibit cleavage of, the initial synaptic complex formed between recombination sites at the start of the reaction. In both the synaptic complex and the HJ intermediate in the Cre-loxP system, a small number of DNA bases are exposed to solvent and could be available to interact with peptide or small molecule inhibitors (11, 19, 20).

To gain a deeper understanding of how peptide inhibitors block resolution of the HJ intermediate during site-specific recombination, we have applied spectroscopic and crystallographic methods to study the effects of the peptide inhibitor WKHYNY binding to the Cre-HJ intermediate. Although most studies thus far on related peptide inhibitors have focused on λ-integrase pathways (13, 14, 16, 17), the Cre system offers some experimental advantages. First, Cre is one of the simplest tyrosine recombinases, capable of recombining minimal 34-base pair loxP sites without accessory proteins or auxiliary DNA sequences (21). A second advantage is that crystal structures for each intermediate in the Cre-loxP recombination pathway are already available, which suggests that structural models of peptide inhibitors bound to the Cre-HJ complex may also be experimentally feasible.

Here we present biochemical and structural data that directly address the question of where peptide inhibitors bind to the Cre-HJ intermediate and how they block junction resolution. Using a fluorescence polarization assay with labeled hexapeptide, we show that the peptide binds specifically to the Cre-HJ intermediate, but not to the synaptic complex or to free HJ DNA. To determine whether the peptide contacts DNA bases at the center of the Cre-bound HJ, a synthetic 4-way junction corresponding to the loxP reaction intermediate was constructed with branch point adenine bases replaced by the fluorescent analog 2-aminoquinine (2AP). Peptide binding to this junction quenches the fluorescence of solvent-exposed 2AP bases at the center of the junction, supporting a model in which aromatic side chains from the peptide inhibitor interact with the central DNA bases.

The crystal structure of peptide WKHYNY bound to the Cre-loxPIPJ intermediate reveals the location of the peptide in the center of the junction where it is poorly ordered. As a result of peptide binding to the junction, both the crossing and continuous strands at the junction center undergo large conformational changes relative to structures of Cre-HJ complexes crystallized in the absence of peptide inhibitor. These changes are transmitted to the recombinative active sites, resulting in a rotation of the phosphodiester linkage that would normally be cleaved during resolution of the HJ intermediate. Surprisingly, the DNA junction conformation in the peptide-inhibited form of the Cre-HJ intermediate resembles that observed in the structure of His₆-tagged Cre bound to a similar loxP-derived junction (22).

MATERIALS AND METHODS

Protein and Oligonucleotide Purification—Expression, purification, and storage of Cre wild-type, R173K mutant, and K201A mutant proteins were performed as described (23). Oligonucleotides used to construct loxP-1 and HJ1 (Fig. 1B) were synthesized using standard phosphoramidite chemistry by the W. M. Keck Facility at Yale University and were purified by reverse-phase HPLC as described (23). DNA duplexes were formed by annealing the appropriate oligonucleotides at 1 mM concentration in 10 mM Tris-HCl, pH 8, 0.5 mM EDTA, 200 mM NaCl by heating to 70 °C in a thermal cycler and cooling the mixture to 4 °C with a linear gradient of 1 °C/min and were used without further purification. The junction HJ1 was prepared as described previously (11).

Oligonucleotides used to construct HJ3 (Fig. 1B) were synthesized by Integrated DNA Technologies, Coralville, IA and were purified on 6% denaturing polyacrylamide gels containing 7% urea. Purified single strands were annealed at 10 μM concentration in 10 mM Tris-HCl, pH 8, 0.5 mM EDTA, 200 mM NaCl buffer as described above. The HJ3 DNA obtained was then further purified under native conditions using a 6% polyacrylamide gel in 1× Tris borate/EDTA (TBE). Junction DNA was electroeluted at 4 °C in 1× TBE and concentrated with a Centricron YM-10 device (Millipore).

Fluorescence Polarization Measurements—5 μg of WKHYNY peptide was labeled at the N terminus with a 10-fold weight excess of fluorescein isothiocyanate by the W. M. Keck Facility at Yale University and purified. The labeled peptide was further purified on a C18 reverse phase column and desalted. Fluorescence polarization measurements were made at 18 °C using a Photon Technology Inc. Quartz master model C60/2000 L-format scanning spectrofluorometer. Emission intensities recorded at 390 nm were corrected for small dilution from 250 to 380 nm while monitoring emission at 390 nm. Acquisition of spectra. Preliminary excitation spectra were recorded from 250 to 380 nm while monitoring emission at 390 nm. Emission spectra were recorded by scanning from 340 to 500 nm with an excitation wavelength of 320 nm. Monochromator slit widths were fixed at 2 nm for all measurements. Samples contained 100 mM HJ DNA and 400 nM Cre K201A mutant in 10 mM Tris-HCl, pH 8, 10 mM MgCl₂, 150 mM NaCl, 1 mM dithiothreitol, and were titrated with increasing amounts of WKHYNY peptide in the same buffer. Parallel titrations were performed for HJ3 DNA containing 2AP residues (Fig. 1) and for HJ3 DNA containing wild-type Ade bases. The small signal from the non-fluorescent complex (< 1%) was subtracted as background. Fluorescence emission intensities recorded at 390 nm were corrected for small dilution.

² K. Kepple and A. M. Segall, unpublished data.
effects created by the titration and are plotted in Fig. 3. Error bars represent 2 S.D. based on sixty intensity measurements at each concentration, over two independent titrations.

**Cre-Holliday Junction-Peptide Complex**—Cre-loxPHJ-WKHYNY peptide crystals were formed from solutions initially containing 13 μM wild-type Cre, 19 μM loxP-1 DNA, and 13 μM peptide in 20 mM sodium acetate pH 5.6. Cre was added to the loxP-1 duplex and peptide that had been premixed in buffer, and the mixture was incubated at room temperature for 30 min. Calcium chloride was added to 20 mM and MPD to 25% (v/v). Hanging drops of 6-μl volume were then incubated against reservoirs containing 20 mM sodium acetate, pH 5.0, 20 mM CaCl₂, and 32–40% MPD at 18 °C. Plate-shaped crystals grew at 150 × 100 × 50 μm over a period of 4 weeks. These crystals diffracted to 2.8 Å at the Cornell High Energy Synchrotron Source (CHESS) A1 beamline. Diffraction data were measured using a Quantum4 CCD detector at 100 K and processed using the HKL suite (24). Data were scaled and merged using SCALA (25).

**Crystallographic Refinement**—The Cre-loxPHJ-peptide crystals have similar cell constants to those of the synaptic complex, the covalent Cre-DNA intermediate, and the Cre-HJ intermediate grown from similar conditions (11, 19, 26). The structure was therefore first optimized with rigid body refinement at 3 Å resolution, using the individual protein domains and the 13-bp inverted repeat DNA arms from 1CRX (26) as a starting model. The 8-bp spacer region encompassing positions 4 through 4 was omitted from the starting model and from refinements until the nucleotides could be unambiguously fit into unbiased electron density maps. Iterative cycles of positional refinement with REFMAC (25) and model building in O (27) were then performed at 3.0 and 2.8 Å. Subsequent σ_A-weighted 2F_o − F_c and F_o − F_c electron density maps showed clear density for the bases and for the backbone of the omitted central 8-base pairs that corresponded to a 4-way DNA junction. Electron density maps that would be diagnostic for a synaptic complex (i.e. two loxP-1 duplexes) was completely absent, confirming that the HJ intermediate had been trapped in the crystal lattice.

The central 8-bp of the HJ DNA strands were modeled as (dAdU)₄ until the final stages of refinement in order to avoid starting bias in the choice of sequence directionality (corresponding to which of two alternative conformers are present in the HJ intermediate). The (dAdU)₄ sequence is an excellent model for either direction of the alternating purine/pyrimidine sequence found in the crossover region of the loxP site (GCATACAT versus ATGTATGC) because the purine-N2 and pyrimidine-C5 groups required to distinguish Gua from Ade and Thy from Cyt are missing. The sequence assignment of the central DNA bases was made based on the presence or absence of difference map densities for Gua-N2 and Thy-C5. Electron density maps following refinement at 2.8 Å were consistent with only one direction for the loxP spacer, corresponding to a “top strand cleavage” configuration where the top strands of the loxP sites adopt the crossing configuration. This configuration was also observed in the structure of HisCre-loxPHJ (22) and Cre-loxPHJ (28), both of which contain the wild-type loxP crossover sequence. The basis for this isomeric preference is not yet known, but Cre has been shown to preferentially resolve synthetic HJC substrates to cleave the “top strands” (29), which corresponds to the expected resolution products of the conformer that is consistently observed.

Refinement of the complete model at 2.8 Å, including 296 solvent atoms and a correction for bulk solvent, converged at \( R_{	ext{free}} = 0.196 \) and \( R_{	ext{free}} = 0.265 \). Refinement results are summarized in Table I. Coordinates for the Cre-loxPHJ-peptide complex and for the unliganded Cre-loxPHJ complex (26) have been deposited in the Protein Data Bank, with accession codes 1XNS and 1XO0, respectively.

**RESULTS**

**Specific Interaction of WKHYNY with the Cre-loxPHJ Intermediate**—To determine whether the WKHYNY peptide binds specifically to the HJ intermediate or if it also binds to other intermediates and components of the Cre-loxP system, we used a fluorescence-based assay that can be performed in solution under equilibrium conditions. In this assay, peptide that has been labeled at its N terminus with fluorescein was held at a fixed concentration of 11 nM. The fluorescence polarization of fluorescein-labeled peptide was then monitored as Cre, DNA, or Cre-DNA complex was titrated over a range of concentrations. Two Cre-HJ complexes were tested for peptide binding. The first was formed with Cre R173K, an active site mutant that binds, but does not efficiently cleave the HJ intermediate (28), and an immobile 4-way junction (HJ1) based on the loxP sequence (Fig. 1B; Ref. 11). The second HJ complex was formed with wild-type Cre and loxP-containing DNA duplex (loxP-1), which form active recombination intermediates subject to binding and trapping by the labeled peptide during the experiment. As shown in Fig. 2 (upper curves), the labeled peptide binds with similar affinity to the preformed Cre R173K-HJ1 DNA complex and to Cre-HJ complexes formed by catalytic strand exchange.

In principle, the observed interaction of peptide with wild-type Cre-loxP-1 complex could also be explained by binding to the synaptic Cre-loxP intermediate prior to strand exchange (or following resolution of the HJ intermediate to form duplex products). To specifically test for binding to a synaptic Cre-loxP complex where cleavage and strand exchange has not occurred, we used the active site mutant Cre K201A bound to loxP-1 DNA. The Cre K201A mutant is unable to cleave the DNA substrate, but forms synaptic complexes with an affinity comparable to that observed for wt-Cre.³ Peptide binding to this synaptic complex is weak, and is similar to that observed in a titration with Cre alone (Fig. 2, lower curves). The labeled peptide also has no significant affinity for the free, immobile HJ DNA, which is not surprising given that in the buffer used for this experiment (which contains 10 mM CaCl₂), the HJ is expected to exist exclusively in the folded, stacked conformation (30, 31) with little resemblance to the open, planar form observed in the Cre- or Flp-bound structures (11, 18).

It has been suggested that hexapeptides such as WKHYNY inhibit HJ resolution of the tyrosine recombinases by binding specifically to the HJ intermediate as it is formed and blocking the cleavage and/or strand exchange process (14, 17). Indeed, the interaction of peptide inhibitors with λ-Int-HJ complexes has been demonstrated using a gel shift assay and the relative affinities of a series of inhibitors has been qualitatively compared by analyzing the stability of the complexes upon dilution (17). An alternative possibility, however, is that a peptide inhibitor such as WKHYNY binds with comparable affinity to both the synaptic and HJ intermediate complexes, but only inhibits cleavage and/or strand exchange of the HJ intermediate. In this alternative model, peptide binding would be necessary, but not sufficient for inhibition of a given reaction intermediate. The fluorescence polarization experiment shown in Fig. 2 strongly supports the former model. The WKHYNY peptide binds to and traps the HJ intermediate in the Cre-loxP system, but does not bind significantly to other intermediates in the pathway. Unfortunately, nonspecific binding observed between labeled peptide and both free Cre and the Cre K201A-loxP synaptic complex makes quantitative analysis using this assay difficult, because the effect becomes much more pronounced at higher concentrations of protein required to achieve binding saturation. This weak interaction most likely has a large electrostatic component, since fluorescein bears a negative charge at neutral pH and Cre is quite basic.

³ K. Ghosh, F. Guo, and G. Van Duyne, unpublished data.
bases at the branch point of the junction in a manner similar to the square-planar model for unbound junctions that is favored in the absence of divalent cations (30, 31). The Cre-bound HJ is distorted from perfect 4-fold symmetry, but otherwise shares many features of the square planar junction model, exposing all four of its A/T base-pairs at the branch point to solvent.

Part of the motivation for determining the crystal structure of a peptide-inhibited Cre-HJ intermediate was to identify the recognition elements between the peptide and the unique features of the DNA substrate. As described later, the crystal structure provides direct evidence that the peptide is bound in the center of the junction, near the exposed surfaces of the branch point bases. In an effort to obtain independent evidence for the nature of the peptide-HJ interaction, we reasoned that if the peptide interacts with the solvent-exposed bases at the branch point, then the fluorescence of 2AP in these positions should be quenched to some extent as a result of peptide binding. The fluorescence intensity of 2AP incorporated into oligonucleotides is normally highest in single-stranded DNA (ssDNA) and is quenched upon base stacking during formation of duplex DNA (31). This property of 2AP has been extensively used to study, for example, DNA and RNA polymerases (32), endonucleases (32), helicases (33), repair enzymes (34), and HJ-resolving enzymes (35). The fluorescence of 2AP bases at the branch point of a Cre-bound junction might be expected to have properties intermediate to those observed for ssDNA and dsDNA, since one surface of each base is stacked on a duplex junction arm and the other surface is solvent exposed. This was

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**Fig. 1.** A, schematic of the Cre-loxP site-specific recombination pathway. Recombinase subunits active for strand exchange catalysis are labeled A, B, sequences of duplex and HJ DNA substrates. LoxP-1 is the wild-type loxP sequence containing 5’-T overhangs to facilitate crystallization (40). Inverted repeats are underlined and arrows indicate the sites of cleavage during recombination. HJ1 is an immobile junction derived from loxP and is identical to HJ1 in Ref. 11. The loxPHJ junction is generated from loxP-1 duplexes by Cre-catalyzed strand exchange. HJ3 is a modified version of loxPHJ, where single base pair substitutions were made in the 13-bp Cre binding elements (as in HJ1) and 10-bp flanking sequences were added to the junction arms to allow formation of a partially mobile junction via annealing of the four component strands. Residues labeled X in HJ3 have either adenine or 2-aminopurine bases.
that multiple peptides bind to the HJ intermediate. The curve peptide is plotted as a function of concentration for Cre alone (intermediate. 

four junction arms, which are close mimics of the normal A/T 

fluorescent analog 2AP (junction HJ3 in Fig. 1 

sequence, but with the central adenine residues replaced by the 

HJ, we prepared a partially mobile junction based on the loxP 

To test this hypothesis experimentally and to provide additional evidence for peptide binding to bases at the center of the HJ, we prepared a partially mobile junction based on the loxP sequence, but with the central adenine residues replaced by the fluorescent analog 2AP (junction HJ3 in Fig. 1B). The resulting junction has a single 2AP/T base pair stacked on each of the four junction arms, which are close mimics of the normal A/T base pairs in terms of structure and energetics (37). The junction was bound by cleavage-defective Cre K201A, and held at a fixed concentration of 100 nM, while 2AP fluorescence was monitored as a function of peptide concentration. As a control for background fluorescence in this experiment that could in principle arise from Trp and Tyr protein residues (or very low signal from DNA bases), we monitored fluorescence in parallel from a Cre K201A-HJ complex in which the four branch point Ade residues were not replaced by 2AP. The contribution to fluorescence from protein and peptide residues would be expected to be quite low in this experiment, since the excitation (320 nm) and emission (390 nm) wavelengths used here for 2AP fluorescence are outside of the useful range for these intrinsic fluorophores. In practice, this was indeed the case; background fluorescence was less than <1% in all measurements.

As shown in Fig. 3, a decrease in fluorescence emission was observed with increasing addition of peptide inhibitor, indicating that 2-AP fluorescence is quenched upon peptide binding to the Cre-HJ complex. A plausible quenching mechanism involves stacking of aromatic side chains in the hexapeptide inhibitor (all isolated peptide inhibitors have so far been rich in Trp, Tyr, Phe, and His residues) with the branch point bases of the HJ. However, this experiment cannot rule out an indirect mechanism, where peptide binding triggers a conformational change in the bound protein or the junction DNA that in turn leads to quenching of 2AP fluorescence. As discussed below, the three-dimensional structure of the Cre-HJ-peptide complex argues in favor of a direct interaction mechanism.

The 2AP quenching experiment not only provides evidence for the mode of peptide binding to the junction, but the binding data can be fit by a Hill equation with n = 2, 3, or 4, indicating that multiple peptides bind to the HJ intermediate. The curve in Fig. 3 was fit with n = 4 to give an apparent peptide binding Kd = 1.3 ± 0.2 μM. The data cannot be described by a simple isotherm in which a single peptide binds to the junction or by a model in which more than one peptide binds independently to the junction. Because the quantitative relationship between the extent of quenching and the number of bound peptides is not yet known, we cannot establish the stoichiometry of binding from this experiment or establish a model for binding cooperativity. A two-site or four-site model is most consistent with the 2-fold-symmetric ligand density that we observe in the crystal structure of the complex, but additional experiments will be required to further address these questions.

Structure of a Cre-loxP-HJ-Peptide Inhibitor Complex—Since Cre recombinase is active in the buffers that we have used to crystallize Cre-DNA reaction intermediates, we reasoned that HJ intermediates could accumulate and crystallize during the incubation period of crystallization experiments when performed in the presence of a peptide inhibitor. The DNA duplex used for co-crystallization is a 34-bp loxP sequence flanked with 5’-overhanging Thy residues (loxP-1 in Fig. 1B). The 5’-T overhangs facilitate (but are not required for) crystallization of the complex under our buffer conditions and ensures that the same lattice packing will be present as for a series of previous Cre-DNA structures obtained from similar conditions. Crystals of the wild-type Cre-loxP-WKHYNY peptide complex could be grown reproducibly in this manner and diffracted at synchrotron sources to beyond 3 Å (Table I). The orthorhombic crystal form obtained is nearly isometric with crystal forms of the synaptic complex, the covalent intermediate, and the HJ intermediates described previously (11, 19, 22, 26). Interestingly, crystallization of wild-type Cre with loxP-1 DNA under these conditions is entirely dependent on the presence of hexapeptide inhibitor in our hands. This result differs from that of Baldwin and co-workers (22), who found His6-tagged Cre readily crystallizes with loxP DNA under similar conditions (but at higher protein/DNA concentrations) to form an HJ intermediate.

Molecular replacement and refinement of the Cre-loxPHJ-peptide structure was similar to the methods used for previous Cre-DNA complexes, with additional steps taken to determine if one or the other crossover isomer/conformer was best described by the electron density of the central bases (see “Materials and Methods”). Difference electron density maps following refinement of all ordered protein and DNA residues revealed a missing scattering component in the center of the Cre-HJ complex that we believe to be bound peptide (Fig. 4). The peptide electron density is continuous over regions that could accommodate segments of about three amino acids, but is not interpretable. Despite repeated attempts to improve the diffraction resolution limit, alter the crystallization conditions (temperature, peptide concentration, etc.), and co-crystallize with alternative peptides, the electron density in the center of the complex is reproducible, but the quality is consistently too low to allow us to unambiguously fit a peptide inhibitor. It is clear, however, that models of peptides fit into this density would all be consistent with the peptide contacting solvent-exposed base pairs at the branch point of the junction, in agreement with the 2AP quenching data described above.

The peptide difference density is clustered into two primary regions of the HJ structure (which are related by 2-fold symmetry to two identical regions) labeled A and B in Fig. 4. Region A is close to the crossing strand of the junction and adjacent to the “active” recombinase subunit that would normally catalyze strand exchange in the HJ intermediate. Region B is stacked on the A2’-T2’ base pair, where peptide intercalation between A2’ and T1’ has moved T1’ by a considerable distance. The struc-
tural changes that occur in the Cre-loxPHJ complex as a result of peptide binding are discussed in more detail below.

There are two likely explanations for the uninterpretable electron density corresponding to bound peptide. First, the nucleotides at the center of all published Cre-HJ complexes (Table II) have significantly weaker electron density and higher B-factors than the average DNA residue. This is likely because of the small number of interactions, direct or solvent-mediated, between Cre and the DNA in this region. The second reason is that the peptide is most likely statically disordered with respect to the C2221 crystal lattice. The recombinase tetramer forms a “cage” around the junction center that shelters the strand-exchange region of the complex (and any components bound within the cage) from crystal packing, imposing

| Table I |
| Crystallographic data and refinement of Cre-loxPHJ-peptide complex |
| Space group | C2221 |
| Unit cell (a, b, c, Å) | 106.078 121.529 177.799 |
| X-ray source | CHESS A1 |
| Resolution (Å) | 30–2.8 |
| Completeness (%) | 96.2 (91)* |
| R<sub>sym</sub> | 6.0 (19.7) |
| R<sub>work</sub> | 0.196 |
| R<sub>free</sub> | 0.285 |
| R.m.s.d. bond length (Å) | 0.014 |
| R.m.s.d. bond angles (deg) | 1.71 |

* Values in parentheses refer to the highest resolution shell.
*<sup>b</sup> R<sub>sym</sub> = Σ[hI<sub|h</sub> - (I<sub|h</sub>)<sup>2</sup>]/ΣI<sub|h</sub>, where (I<sub|h</sub>)<sup>2</sup> is the average intensity over symmetry equivalents.
*<sup>c</sup> R<sub>work</sub> includes the 95% of reflection data used in refinement.
*<sup>d</sup> R<sub>free</sub> includes the 5% of reflection data excluded from all refinements.

FIG. 3. Quenching of 2-aminopurine fluorescence upon addition of WKHYNY peptide. Cre K201A-HJ3 complex with the four branch point adenines replaced by 2-aminopurine (marked X in Fig. 1B) was titrated with peptide and fluorescence emission intensity at 390 nm (320 nm excitation) is plotted against peptide concentration. The data are fit to a Hill equation with n = 4. Error bars represent 2 S.D. from sixty total intensity measurements over two independent titrations.

FIG. 4. Difference electron density at 2.8-Å resolution in the center of the Cre-loxPHJ-peptide complex. The density is contoured at 2.3 times the r.m.s. value of the map. Corresponding density is not observed in the Cre-HJ1, Cre-HJ2, or Cre-loxPHJ structures that were crystallized in the absence of peptide inhibitor under similar conditions (Table II). Density regions marked A and B are discussed in the text. Cre recombinase subunits are not shown. This figure was created with Pymol (43).
the symmetry of the lattice on its contents. The difference density shown in Fig. 4 is necessarily 2-fold symmetric because a crystallographic dyad passes through the center of the complex. The observed density probably represents an ensemble of conformations of two or more peptides bound to the junction that have been statistically averaged over the crystal. Differ-ence electron density maps computed at the same resolution for nearly isomorphous Cre-HJ complexes crystallized in the absence of peptide are featureless in this region (11, 28), supporting the assertion that the density represents bound, but disor-dered, peptide inhibitor.

**Peptide Binding Alters the Cre-bound HJ Conformation**—Although the Cre-loxPHJ-peptide structure does not provide an atomic resolution model for understanding how the peptide recognizes junction DNA, it does reveal the consequences of peptide binding on the DNA substrate geometry. The Cre-HJ intermediate is composed of four Cre subunits, each bound to one arm of the 4-way junction produced by the first round of strand exchanges. Two of the symmetry-related Cre subunits are active to cleave the “crossing strands” in the junction (de-strand exchanges. Two of the symmetry-related Cre subunits are active to cleave the “crossing strands” in the junction (de-

| Name used in text | Cre HJ DNA | Ref. | PDB code |
|--------------------|------------|------|----------|
| Cre-HJ1            | Cre R173K  | HJ1; immobile | Gopaul et al. (11) | 3CRX |
| Cre-HJ2            | wt-Cre     | HJ2; nicked, symmetric | Gopaul et al. (11) | 2CRX |
| Cre-loxPHJ         | Cre R173K  | loxPHJ; wt sequence | Guo (28) | 1X00 |
| HisCre-loxPHJ      | His-Cre    | loxPHJ; C13→G mutant | Martin et al. (22) | 1KBU |
| Cre-loxPHJ-peptide | wt-Cre     | loxPHJ; wt sequence | This work | 1XNS |

The second large change in the loxPHJ crossover region apparent from Fig. 5A is a shift in the center of the continuous junction strand away from the center of the complex. This change is coupled to the crossing strand shift, as residues T1’ and A1’ are part of a concerted movement that includes residues A1, C2, and A3 described above. The rearrangement of T1’ is the most severe, with complete loss of A1’-T1’ base pair hydrogen bonding. This distortion is particularly striking for two reasons. First, the Cre-HJ1, Cre-HJ2, and Cre-loxPHJ structures all contain junction arms that are completely Watson-Crick base paired at the branch point. Second, the shift in T1’ appears to be caused by insertion of bound peptide into this position, corresponding to difference density region B in Fig. 4.

The second comparison we made is to a 2.2-Å resolution structure of His8-Cre bound to a loxP-HJ described by Baldwin and co-workers (HisCre-loxPHJ; Table II). This reaction intermediate structure contains an N-terminal His8-tagged, but other-wise wild-type Cre recombinase and a loxPHJ construct similar to that shown in Fig. 1F, but lacking 5’-T overhangs and containing a point mutation in one of the recombinase-binding arms. Two distinct conformations for the HJ DNA branch point bases were described in the HisCre-loxPHJ structure. The minor conformers overlap reasonably well with Cre-HJ1, Cre-HJ2, and Cre-loxPHJ, as previously noted for Cre-HJ1 (22). The Cre-HJ1, Cre-HJ2, and Cre-loxPHJ complexes do not, however, overlap well with the major conformer of HisCre-loxPHJ in the strand exchange region of the junction, although the structures are nearly superimposable throughout the remainder of the complex. The differences observed between the major conformer of HisCre-loxPHJ and the Cre-HJ1, Cre-HJ2, and Cre-loxPHJ structures are located in both the crossing and continuous junction strands, and are similar to those discussed above for the Cre-loxPHJ-peptide complex comparison to the same structures.
Interestingly, there is much closer similarity between the HisCre-loxPHJ complex and the Cre-loxPHJ-peptide complex described here for this region of the structure (Fig. 5B). The trajectories of the junction crossing strands are similar (but not identical) and the continuous DNA strands superimpose reasonably well. Most intriguing is the similar positioning of T1' and consequent disruption of stacking and base-pairing in both the peptide complex and the HisCre-loxPHJ complex. It is therefore tempting to speculate that the HisCre-loxPHJ structure may in fact be a “peptide-inhibited” form of this intermediate, where the His6 hexapeptide provided in cis plays the role of a self-inhibitor. This could in principle explain how a transient reaction intermediate readily crystallizes and remains stabilized against cleavage and resolution to duplex products in the crystal.
Peptide Binding Distorts Active Site Geometry—Comparison of the active sites where cleavage and strand exchange would normally be catalyzed in this reaction intermediate (the “cleaving” subunits) reveals a large conformational change in the scissile DNA strand (G2, T3, and A4) up to and including the scissile phosphate (Fig. 6A). This change is an extension of the shift observed in the position of the crossing junction strands discussed previously and shown in Fig. 5A. A consequence of this alternative conformation is that the Sp non-bridging oxygen atom of the scissile phosphate is rotated into the line of attack of the conserved nucleophile, Tyr224, generating a geometry that appears less favorable for catalysis than that observed in the Cre-HJ1 or Cre-loxPHJ structures.

Comparison of the active sites that are normally inactive for cleavage (the “non-cleaving subunits”) also shows a distortion in the DNA strand on the 3’-side of the scissile phosphate, but the shift is in the opposite direction relative to the same comparison in the cleaving subunit (Fig. 6, A versus B). This change is a consequence of the continuous junction strand displacement shown in Fig. 5A. Interestingly, the scissile phosphate is also rotated in this active site relative to that seen in the Cre-loxPHJ structure, but in the opposite sense to that shown in Fig. 6A. Here, the Sp non-bridging oxygen rotates down and away from Tyr224, creating an improved geometry for in-line attack.

In both the cleaving and non-cleaving active sites, there is minimal change in the positions of the active site residues as a result of peptide binding to the Cre-bound junction. The largest change is in the position of Tyr224 in the cleaving active site, but this tyrosine adopts an ensemble of positions between the scissile phosphate and the 5’-adjacent phosphate in the DNA intermediate structures determined thus far (39, 40) and the conformation observed in the Cre-HJ-peptide complex described here is well within this range.

DISCUSSION

The biochemical and structural data presented here strongly support a model in which peptide inhibitor binds at the center of the Cre-bound HJ intermediate. The planar, open form of the Cre-bound HJ intermediate and crystallographic data indicate that the peptide interacts with solvent-exposed bases at the branch point of the junction, which might explain the high frequency of Trp, Tyr, and Phe residues in the most potent inhibitors that have been identified.

In principle, binding of peptide to the center of the HJ intermediate may alone be sufficient to inhibit resolution of the junction via reciprocal strand exchange. Inhibition could occur by physically blocking the strand exchange path, effectively stabilizing the 4-way junction with respect to duplex resolution products. In this case, one might expect that the recombinases should be able to cleave the HJ intermediate in the presence of bound peptide, but religation to form the junction would be strongly favored over strand exchange ligation to form DNA duplexes. However, the peptide-inhibited Cre-HJ structure indicates that bound peptide(s) may play a more active role in inhibiting junction resolution. By inducing an alternative conformation of the DNA substrate, peptide binding modifies the active site geometry in a manner that appears to be less favorable for catalysis. In this case, both cleavage and strand exchange of the HJ intermediate would be expected to be disrupted upon binding of peptide inhibitor.

As more and more biochemical systems that involve Holliday junction intermediates are studied in detail, it is becoming increasingly clear that the planar, unstacked form of the HJ intermediate generated by the tyrosine recombinases is present in a variety of other genetic pathways and in a broad range of organisms. For example, the yeast CCE1 and bacteriophage T7 endonuclease I junction resolving enzymes have also been shown to unfold HJ substrates upon binding to generate a planar, unstacked form (35, 36). The availability of small molecules that can selectively stabilize these DNA intermediates could present important new opportunities to probe genetic mechanisms and pathways. Indeed, peptides similar to those that inhibit tyrosine recombinases are also capable of inhibiting unrelated HJ-resolving systems that involve planar HJ intermediates. Recent studies suggest that this approach may also be a fruitful source of novel antibiotics (16), because several bacterial DNA-repair pathways require faithful resolution of HJ intermediates to allow normal chromosome segregation and cell growth (5).

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Peptide Trapping of the Holliday Junction Intermediate in Cre-loxP Site-specific Recombination

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