Characterization of a Mutation of Bacteriophage λ Integrase
PUTATIVE ROLE IN CORE BINDING AND STRAND EXCHANGE FOR A CONSERVED RESIDUE*

Troy Bankhead and Anca M. Segall‡

From the Department of Biology and Molecular Biology Institute, San Diego State University, San Diego, California 92182-4614

Site-specific recombination is involved in processes ranging from resolution of bacterial chromosome dimers to adenovirus-associated viral integration and is a versatile tool for mammalian genetics. The bacteriophage λ-encoded site-specific recombinase integrase (Int) is one of the best studied site-specific recombinases and mediates recombination via four distinct pathways. We have characterized a mutant version of λ Int, IntT236I; this mutant can perform the bent-L pathway only, whereas the corresponding IntT236A mutant can perform bent-L, excision and integration pathways. Experiments with both IntT236I and IntT236A show that the hydroxyl group of threonine is necessary for wild-type recombination. Substitution of the threonine by serine leads to nearly complete rescue of the mutant phenotypes. In addition, our data show that the IntT236I mutant is defective partially due to obstructive steric interactions. Comparisons of crystal structures reveal that the threonine at residue 236 may play an important role in stabilizing recombination intermediates through solvent-mediated protein-DNA interactions at the core-binding sites and that the hydroxyl group is important for effective cleavage and Holliday junction formation. Our data also indicate that Int contacts the core sites differently in intermediates assembled in excisive versus bent-L recombination.

The Int protein of bacteriophage λ is the archetype member of the Int family of site-specific recombinases now known as the tyrosine recombinases. All of these enzymes use a common mechanism to carry out various biological functions (1). Int mediates site-specific recombination by assembling a tetramer and mediates recombination via four distinct pathways. We have characterized a mutant version of λ Int, IntT236I; this mutant can perform the bent-L pathway only, whereas the corresponding IntT236A mutant can perform bent-L, excision and integration pathways. Experiments with both IntT236I and IntT236A show that the hydroxyl group of threonine is necessary for wild-type recombination. Substitution of the threonine by serine leads to nearly complete rescue of the mutant phenotypes. In addition, our data show that the IntT236I mutant is defective partially due to obstructive steric interactions. Comparisons of crystal structures reveal that the threonine at residue 236 may play an important role in stabilizing recombination intermediates through solvent-mediated protein-DNA interactions at the core-binding sites and that the hydroxyl group is important for effective cleavage and Holliday junction formation. Our data also indicate that Int contacts the core sites differently in intermediates assembled in excisive versus bent-L recombination.

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The DNA substrates of Int-mediated SSR, attP, attB, attL, and attR (Fig. 1), contain two 9-bp core sites separated by a 7-bp overlap region where cleavage, strand exchange, and recombination occur. To what extent is normal, attB and attL sites also contain arm-binding sites and accessory protein recognition sequences (see Fig. 1). A number of catalytic residues important for recombination by tyrosine recombinases have been described. These include the active site tyrosine (Tyr-342) and the arginine-histidine-arginine (Arg-His-Arg) triad that functions to stabilize the transition state of the cleavage reaction by forming hydrogen bonds to the non-bridging oxygen atom of the scissile phosphate (7). A highly conserved lysine (Lys-201 in P1 Cre and Lys-235 in λ Int) has been described as an acid in the transesterification reaction where it may function to protonate the leaving 5′-hydroxyl group (8, 9). In addition to these main catalytic residues, it is also clear from sequence conservation and information gained from crystal structures that other residues play an important role in the recombination reaction.

The use of mutants of λ integrase has provided insight into the structure-function relationship of Int. For example, the delimitation of the functional domains of Int was aided through the use of Int mutants defective in catalyzing excision in vivo (10). The recent flurry of crystal structures of several Int family proteins has been very informative. These include the catalytic domain of Int (11), the catalytic domain of the Hemophilus influenzae phage HP1 Int (12), the XerD protein of Escherichia coli (13), and a series of co-crystals of the bacteriophage P1 Cre protein bound to DNA (14–16). These structures show that the fold of the catalytic domain is highly conserved among these recombinases. Despite many differences in the primary sequence, highly conserved patches of residues can be found throughout the catalytic region (7).

In order to study further the structure-function relationships of λ integrase, we have characterized a mutant Int, IntT236I, along with corresponding alanine and serine mutants, IntT236A and IntT236S, respectively. Among the members of the tyrosine recombinase family, approximately 60% contain residues at this position that can form hydrogen bonds with DNA, of which 75% contain threonine or serine residues (only five members contain serine). Therefore, this threonine is highly conserved in the Int family of recombinases. Of the tyrosine recombinases that lack a threonine or serine at position 236, another 23% have a threonine or serine at positions 234 or 237.

The IntT236I mutation was isolated in an in vivo screen for recombination-defective Int mutants using excision between...
non-wild-type attL and attR sites (10). We have characterized this protein in vitro and found that it is capable of carrying out only the bent-L pathway and only at or below 30 °C, whereas IntT236A can perform bent-L, integrative and excisive reactions at all temperatures tested, albeit with reduced efficiency. In contrast, IntT236S can carry out bent-L, straight-L, and excisive recombination at near wild-type levels. Our experiments show that IntT236I displays reduced DNA cleavage and strand exchange and forms unstable intermediates, whereas the catalytic activity of IntT236A is intermediate between the IntT236I mutant and the wild type. However, both IntT236I and IntT236A exhibit a reduction in core-binding and protein-protein interactions. We propose that the temperature-sensitive mutant is defective partially due to obstructive steric interactions between the recombinase and the core sites, and the isoleucine may interfere directly with the active site tyrosine.

Our results show that the hydroxyl group of the threonine is important for performing wild-type DNA cleavage and for forming Holliday junctions. Our data together with comparisons of crystal structures lead us to propose that the threonine at residue 236 may play an indirect role in stabilizing recombination intermediates through solvent-mediated protein-DNA interactions.

### EXPERIMENTAL PROCEDURES

**Strains and Site-directed Mutagenesis of Int**—All mutant Int genes in this study were expressed in *E. coli* HN1463 (thiA− hupA− hupB−) cells. The clone of the IntT236I mutant gene was the generous gift of J. Gardner and Yiping Han. The IntT236A and IntT236S mutant genes were generated using the QuickChange site-directed mutagenesis kit (Stratagene). Plasmid vectors containing mutant Int genes were the pLex58 vector (17) for the IntT236A and IntT236S mutants and the pYWH7 vector (10) for the IntT236I mutant. Wild-type Int was expressed in *E. coli* BL21 (DE3 pLysS) cells. Purified Int was the generous gift of S.-W. Yang and H. Nash (National Institutes of Health), and Xis was purified as described.²

**Optimisation of Int—*E. coli* HN1463 (thiA− hupA− hupB−) cells (500 ml) were grown in LB supplemented with ampicillin (100 μg/ml Sigma) at 37 °C to mid-log phase. Int expression was induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (β-gal) (in 10 mM Tris-HCl, pH 7.4). The cellular debris was removed by centrifugation at 45,000 rpm for 45 min. The supernatant was frozen at −80 °C, and the pellet was re-extracted in 200 μl of extraction buffer. Int was isolated from these extracts by mixing 300 μl of phosphocellulose equilibrated in buffer X-400 (50 mM Tris-Cl, pH 8, 400 mM KCl, 1 mM EDTA, 10% glycerol, 10 mM β-mercaptoethanol) with 800 μl of cell extract. After rotating for 1 h at 4 °C, the resin was pelleted gently; the supernatant containing unbound proteins was removed, and the resin was washed with 600 μl of buffer X-400 for 30 min. Two sequential elution steps were performed with 150 μl of buffer X containing 1 mM KCl. Int concentration was determined by Western blot. Band shift assays in which the ability to form UMCs on a wild-type attL and single arm site-binding assays were also performed, and wild-type and mutant proteins exhibited comparable binding activities. Protein concentration values refer to Int monomers.

**Gel Mobility Shift and in Vitro Recombination Assays**—Linear substrates containing the attL or attLenP1 sequence were 5'-end-labeled using T4 polynucleotide kinase (New England Biolabs) and γ-[^32]P-ATP (PerkinElmer Life Sciences). All reactions and binding reactions were performed in 20 μl. Recombination reactions contained 1 nM labeled att site (187 bp for attL and 136 bp for attR) and 4 nM unlabeled att site (486 bp). Binding reactions contained 1 nM labeled att site, unless specified otherwise. Recombination reactions were stopped by the addition of 5 μl of 2% SDS containing bromphenol blue. These samples were loaded onto 5% polyacrylamide Tris/SDS gels and electrophoresed in Tris/Tricine/SDS containing bromphenol blue. The following gel systems were used: 10–20% polyacrylamide Tris/SDS gels and electrophoresed in Tris/Tricine/SDS buffer at 100 mA constant current for 5 h. For the bent-L pathway, reactions were incubated at 25 °C for 90 min, unless otherwise specified. Gel mobility shift reactions were assembled as above and were layered without loading dye directly onto 5% native polyacrylamide, 0.5% TBE gels (29:1 acrylamide/bisacrylamide). Gels were run at 165 V at room temperature for 3 h. Band shift assays using the attL substrate with arm-type-binding site contained only 500 ng of nonspecific DNA. All reactions also contained 44 mM Tris-Cl, pH 8, 60 mM KCl, 0.05 mg/ml bovine serum albumin, 11 mM Tris borate, pH 8.9, 1 mM EDTA, 13.6% (v/v) glycerol, and 5 mM spermidine. Int and IHF were present at 60 and 37 nM, respectively. Recombination reactions were stopped by the addition of 5 μl of 2% SDS containing bromphenol blue. The following gel systems were used: 10–20% polyacrylamide Tris/SDS gels and electrophoresed in Tris/Tricine/SDS buffer at 100 mA constant current for 5 h. For the bent-L pathway, reactions were incubated at 25 °C for 90 min, unless otherwise specified. Gel mobility shift reactions were assembled as above and were layered without loading dye directly onto 5% native polyacrylamide, 0.5% TBE gels (29:1 acrylamide/bisacrylamide). Gels were run at 165 V at room temperature for 3 h. Band shift assays using the attL substrate with arm-type-binding site contained only 500 ng of nonspecific DNA.
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for several reasons. First, we do not know the fraction of active Int in the preparation. Second, 4 Int monomers are expected to be required for catalysis (14, 15, 19), and synaptic complexes in at least 1 recombination pathway contain Int tetramers. Third, we use nonspecific DNA in our reactions to minimize nonspecific aggregation of Int, and some Int molecules almost certainly bind to this DNA rather than to the att sites. Fourth, Int is very inefficient at performing some assays, such as the half-site cleavage assay, which depends on very weak interactions between Int and one of its core sites.

_Holliday Junction Formation and Cleavage Assays—_Full-site cleavage reactions were assembled as for recombination assays except that labeled phosphorothiolate substrates were used. For those reactions that were treated with proteinase K, 2 \(\mu\)g of proteinase K was added followed by 10 \(\mu\)g of proteinase K. Reactions were incubated at 37 °C for an additional 30 min and then loaded onto 5% polyacrylamide Tris/SDS gels and electrophoresed in Tris/Tricine/SDS buffer at 100 mA constant current for 5 h. The substrates used in the half-site cleavage reactions were the following annealed oligonucleotides: 5′-AATGAATCCTGTTGGAACGGCTTTT-3′ and 5′-GTATAAAAAAGCAGGCTTCAACG-3′. The substrates were single 5′-end-labeled at the top strand. Half-site cleavage reactions were assembled as for recombination assays. The reactions were stopped with SDS and then digested with proteinase K at 37 °C for 30 min before they were loaded onto a 20% polyacrylamide, 7 M urea gel. Reactions were electrophoresed at 25 watts constant power for 2 h. Holliday junction intermediates were trapped using a peptide inhibitor, WKHYVY (20). Reactions were assembled as for recombination assays, and peptide inhibitor was added at a final concentration of 10 \(\mu\)M for bent-L recombination and 100 \(\mu\)M for excision.

_Protein-Protein Cross-linking Assays—_Reactions were assembled as for gel mobility shift assays (see above) except that the concentration of att site was 4 nM. After 30 min of incubation at room temperature, 1,6-bismaleimidohexane (BMH) (Pierce) diluted in Me2SO was added to a final concentration of 20 \(\mu\)M. Laemmli’s sample buffer (Bio-Rad) was added to the samples, which were then boiled for 10 min. Cross-linking products were separated on a 4–12% Tris glycine gel (NOVEX) at 125 V for approximately 2 h. Proteins were electroblotted to a nitrocellulose membrane (Bio-Rad) using the NOVEX blot module, and Int was detected with polyclonal rabbit anti-Int antibody and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Jackson Laboratories). The nitrocellulose membrane was treated with 10 ml of SuperSignal peroxide substrate (Pierce) for 5 min before it was exposed to Kodak XAR film.

RESULTS

T236I Confers a Temperature-sensitive Defect—In order to understand the features of Int necessary for catalysis and to separate them from those necessary for regulating directional recombination, we set out to characterize a number of Int mutant proteins that mediate only bent-L pathway of Int-mediated SSR (5, 10). These mutants were originally isolated in vivo (10). Int mutant-assembled complexes may be less stable, with IntT236I forming intasomes near wild-type levels (data not shown). In addition, the bands corresponding to IntT236A and IntT236I intermediate complexes of bent-L recombination at different temperatures (Fig. 3A). Like wild-type Int, IntT236I is capable of forming two UMCs at 0 °C but forms a single UMC at 25 °C and cannot form UMCs at 37 °C (Fig. 3A). IntT236A is capable of forming two UMCs at both 0 and 25 °C but is also capable of forming intasomes at 37 °C, although not as well as wild-type Int (data not shown). In addition, the bands corresponding to UMCs formed by both the IntT236I (3A) and IntT236A (data not shown) mutant proteins migrate slightly slower than those formed by the wild type, suggesting that they confer an altered conformation to the UMCs. IntT236S was able to form attL and attR intasomes near wild-type levels (data not shown). However, the mobility of UMCs formed by IntT236S was also altered compared with those formed by wild-type Int. When band shift reactions were electrophoresed on a long gel, IntT236S complexes were closest in mobility but not identical to those assembled by wild-type Int (Fig. 3C).

The fact that IntT236I and IntT236A mutant proteins formed more UMCs at 0 °C than at 25 °C suggested that these mutant-assembled complexes may be less stable, with IntT236I
being less stable than IntT236A. We tested the stability of complexes by performing competition assays. Mutant and wild-type Int protein were incubated at room temperature with labeled attLtenP1 sites for varying lengths of time after which an excess of cold attLtenP1 site was added. We found that both the IntT236I and IntT236A mutants exhibit a slight decrease in stability (data not shown). These results imply that the threonine itself may be important in protein-DNA interactions since the decrease is also seen in the IntT236A mutant.

We also tested the ability of these mutants to assemble UMCs with an attR DNA substrate. The attR UMCs represent early intermediates formed in the excision pathway of Int-mediated SSR. IntT236A is able to carry out excisive recombination, whereas IntT236I is unable to catalyze this pathway (see Table II). The results show that both mutants are somewhat defective in forming these UMCs, although IntT236I is more defective than IntT236A (Fig. 3B). Both mutants assemble complexes with a slightly slower mobility, as seen in the previous experiments (Fig. 3A), implying that the complexes have an altered structure.

**Loss of Threonine Confers a Core-binding Defect and Reduced Protein-Protein Interactions**—The assembly of synaptic complexes in the straight-L pathway requires stable, high affinity interactions between Int and its core-binding sites but is independent of strand exchange (23). We tested the possibility that the reduced stability displayed by IntT236I and IntT236A mutants is due to a reduction in core binding by determining their ability to form bimolecular complexes (complexes containing two DNA substrates noncovalently juxtaposed by Int). Bimolecular complexes in the straight-L pathway are assembled when two attL substrates are bound by Int in the absence of IHF protein. The results show that IntT236I is most deficient in forming these complexes, whereas IntT236A and IntT236S are less deficient (Table II). This decrease in core binding was also confirmed through band shift assays in which the binding protein HU was used in place of IHF (data not shown; see Ref. 21). These results indicate that Thr-236 plays a role in core binding and possibly in synapsis. This finding is supported by earlier work from Han et al. (10), which showed that T236I exhibited a 10-fold drop in the level of challenge phage repression when core-containing attL inserts were used. Experiments in which the core sequences were scrambled showed that whereas T236I is defective for core binding, it still retains the ability to recognize and bind to cores sites (10). It was theorized that this mutant was defective in synapsis since it could assemble stable attL complexes but was defective for excision in vivo (10).

**Protein-protein cross-linking studies were performed in or-**
FIG. 4. BMH cross-linking of wild-type and mutant Int proteins. All reactions contained 75 nM Int and 1 μg of salmon sperm DNA. For reactions containing substrate, attL was added to 2 nM final concentration, and reactions were incubated for 30 min prior to the addition of cross-linker. BMH cross-linker was added at 1 μM final concentration. Reactions were incubated 5 min and then quenched with β-mercaptoethanol. The series of bands labeled with an * denote possible cross-reacting species that do not appear reproducibly.

FIG. 5. Comparison of cooperativity by wild-type or mutant Int. The DNA substrates contained only the three arm-type-binding sites from attL (coordinates +12 to +110 with respect to the 0 position in the core; top strand cleavage takes place at −2). Reaction mixtures contained 1 nM labeled DNA, 500 ng of salmon sperm DNA, and 70 nM Int. Bands representing arm sites occupied by one, two, or three Int monomers are depicted in the cartoons shown to the left (cartoons refer strictly to the stoichiometry rather than to the actual position occupied by Int monomers). Lane 1 is a substrate control.
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FIG. 6. Half-site and full site cleavage assays of wild-type Int or mutant protein. A, sequence of the half-site DNA substrate. Arrow points to the site of cleavage. B, half-site cleavage assay. Reactions contained 2 nM labeled half-site DNA and 0.3 μg of salmon sperm DNA. Lane 1 contains DNA only. Reaction mixtures were incubated for 60 min and then stopped with 0.2% SDS followed by proteinase K treatment for 30 min. Reactions were electrophoresed on a 7% urea gel. C, cleavage efficiency in the context of a full attLtenP’1 site in recombination conditions. Full site cleavage assays contained 4 nM unlabeled attLtenP’1 site (496 bp) and 1 nM labeled attLtenP’1 phosphorothiolate DNA (187 bp), as well as 0.3 μg of salmon sperm DNA. Lane 1 contains labeled attLtenP’1 phosphorothiolate DNA only. The identified recombination product is due to the presence of unlabeled attLtenP’1 non-phosphorothiolate DNA, which can undergo strand exchange with one strand of the labeled DNA (the strand containing the phosphotyrosyl linkage with Int).

TABLE III

Reduction in the rates of cleavage and Holliday junction formation for mutant proteins

|       | Bent-L Cleavage | Bent-L HJ formation | Excision Cleavage | Excision HJ formation |
|-------|-----------------|---------------------|------------------|----------------------|
| IntT236S | 1.4             | 2.2                 | 2                | 3                    |
| IntT236A | 3               | 12                  | 4                | 16                   |
| IntT236I | 7               | 25                  | 9                | 200                  |

All values represent the fold decrease compared to wild-type in the rates of the denoted step. Initial reaction rates were determined from time courses up to 20 min in conditions of maximal DNA cleavage and Holliday junction formation.

junctons. Because very few Holliday junction intermediates accumulate in Int-mediated recombination normally, we treated recombination reactions with a peptide inhibitor, WKHYN, which stabilizes these intermediates and allows their investigation (20). The completed Holliday junctions are insensitive to proteinase K, whereas if ligation of even one strand in the junction is not completed because Int remains attached, the resulting species migrates more slowly due to the added protein mass and is sensitive to proteinase K (Fig. 7, compare lane 2 with lane 3 or lane 6 with lane 7). As expected because of their decreased DNA cleavage capabilities, both mutant proteins formed fewer Holliday junctions than Int+,

In previous recombination assays, we noticed the absence of Holliday junction intermediates in reactions with the two mutant proteins. For this reason, we specifically set out to investigate whether the mutants were defective in forming Holliday...

in order to allow optimum cleavage of the scissile phosphate by the active site tyrosine.

DISCUSSION

The experiments described here address the role in Int-mediated recombination of the conserved threonine at position 236. Substitutions of the threonine confer a core-binding defect that probably accounts for the decrease in the ability of mutant proteins to form at least some reaction intermediates. Directly or indirectly, the defects in core binding lead to decreased rates of DNA cleavage and Holliday junction formation.

Substitution of the threonine by isoleucine is more deleterious than substitution with alanine. IntT236I can perform only bent-L recombination, and only in a temperature-sensitive manner, whereas IntT236A has considerably more activity. This suggests that substitution by isoleucine results in steric obstruction that translates into a larger defect in formation of intermediates or in formation of unstable recombination intermediates by the protein at higher temperatures. Analysis of the Cre co-crystal structures indicates that this interference is probably located at a protein-DNA interface in the core region (Fig. 8). Although IntT236I is temperature-sensitive in intermediate formation, both IntT236I and IntT236A form fewer and somewhat less stable intermediates when compared with the wild-type, most likely due to the reduced core binding by the mutant proteins. This also appears to change the overall structure of the UMC complex. The altered mobility of complexes assembled by IntT236I and IntT236A is partially corrected in the case of the IntT236S mutant, further supporting that the lack of a hydroxyl group at this position affects UMC formation and geometry. Cross-linking of the mutant proteins revealed a decrease in protein-protein interactions (Fig. 4). All...
three mutants exhibit at least some reduction in core binding and protein-protein interactions, suggesting that both the presence of the hydroxyl group and its precise positioning play a role in these attributes.

To explain the role of this threonine, we took into consideration two features of the Cre co-crystal structures. 1) Thr-236 of λ Int corresponds to Thr-202 in Cre and is positioned in the middle of a loop between the β2 and β3 strands; the β3 strand may be involved in protein-protein contacts with the carboxyl-terminal N helix of the adjacent monomer (16). This contact between the Cre monomers has been previously described by Sherratt and colleagues (28) in Xer recombination as a “ball and socket” connection. 2) Van Duyne and colleagues (16) have described a kink formed by the binding of Cre to the DNA substrate, at a position where the two DNA strands are separated by water molecules (Fig. 8C). The water molecules may function to stabilize this opened region of the DNA helix by hydrogen bonding with the exposed nucleotide bases. The kink is located in the binding site for the Cre monomer that would cleave the second set of DNA strands, to resolve the Holliday junction intermediate rather than to form it. This possibility will be tested directly in the future.

It should be noted that a highly conserved lysine found throughout the tyrosine recombinase family (Lys-235 in λ Int scissile phosphate. The greater defect in DNA cleavage seen with the IntT236I mutant may be due to the steric interference between the mutant isoleucine residue and the DNA backbone, thereby misplacing the active site tyrosine to a greater extent than would be observed for the IntT236A mutant (Fig. 8B).

The IntT236I and IntT236A mutants are more defective in Holliday junction formation than expected simply based on their defect in DNA cleavage. We interpret this observation to mean that the core interactions mediated by Thr-236 continue to be important after strand cleavage, during the strand exchange, and/or ligation steps of recombination. This possibility will be tested directly in the future.

![Fig. 8. Co-crystal images of Cre bound to the loxP site.](image) The threonine on the activated monomer is on the left and that of the "non-cleaving" monomer is on the right. A, image showing the position of Thr-202 (yellow; analogous to IntT236) and Tyr-324 (red; analogous to IntY342) in both monomers of Cre. B, detailed view showing the proximity of the T202 residue (yellow) to the phosphotyrosine bond between Cre and DNA (tyrosine is red). C, detailed view showing the position of Thr-202 with respect to the DNA substrate. Water molecules (green) that most closely border each threonine residue are shown. All images were generated using RasMol.
and Lys-201 in Cre) and the type Ib topoisomerase family is located adjacent to the Thr-236 residue. This lysine has been described as an acid during cleavage, and in vaccinia topoisomerase its role is to promote the leaving group in DNA (8, 9, 29, 30). Although it is possible that substitution of the neighboring threonine could alter the function of the lysine, this effect would not explain many of the findings in this study. It is unlikely that substitution with an alanine residue would disturb the function of an acid. Moreover, the cleavage assays utilizing phosphorothiolate DNA substrates disprove the idea that disruption of this lysine would lead to a reduction in cleavage, since the leaving group in the phosphorothiolate-containing DNA substrates does not require protonation. More than 50% of tyrosine recombinases have a threonine or serine immediately downstream (41%) or immediately upstream (14%) of the conserved lysine. Based on our data, the neighboring hydroxyl group of Thr/Ser may contribute to the positioning of the conserved lysine. However, a significant subset (23%) of family members, including the vast majority of the bacterial chromosome dimer-resolving enzymes, contain the motif GKGXK in this region, suggesting an alternate positioning mechanism.

Our original interest in this threonine residue was sparked by the fact that IntT236I cannot perform excisive, integrative, or straight-L recombination, while remaining able to perform at least detectable levels of bent-L recombination. The IntT236A mutant protein, in contrast, is better at performing excisive than bent-L recombination, performs integrative recombination at least as well or better than the wild type, and is incapable of mediating straight-L recombination. When testing intermediate steps of recombination, we found that the IntT236A mediates DNA cleavage more or less equally well in bent-L and excisive recombination and is marginally worse at forming Holliday junction intermediates in excisive recombination than in bent-L recombination. Our interpretation of these data is that the architecture of protein-DNA intermediates differs in bent-L and excisive recombination and that there is more steric hindrance between the isoUCeu at position 236 and DNA in excision intermediates than in bent-L intermediates. The alanine substitution does not interfere with protein-DNA interactions but rather reflects the effect of the missing hydroxyl group. The proposal that the architecture of intermediates differs in different recombination pathways is also supported by the finding that bent-L intermediates are more forgiving of Int modification, particularly at Cys-217, than excisive intermediates\(^2\) and by the finding that the four recombination pathways are inhibited to different extents by peptide inhibitors (20).

In summary, several of the conserved catalytic residues of the tyrosine recombinases, specifically the Arg, His, Arg, and Tyr residues, have been studied and characterized in great detail. However, the conserved threonine, while highly conserved only in a subclass of the family of tyrosine recombinases, has not been studied extensively. Our detailed look at the function of this residue gives further insight into the mechanism of site-specific recombination.

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