The Role of the Conserved Trp$^{330}$ in Flp-mediated Recombination

FUNCTIONAL AND STRUCTURAL ANALYSIS*

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The active site of Flp contains, in addition to a trans-donated nucleophilic tyrosine, five other residues that are highly conserved within the Λ-integrase family of site-specific recombinases and the type IB topoisomerases. We have used site-directed mutagenesis and x-ray crystallography to investigate the roles of two such residues, Lys$^{229}$ and Trp$^{330}$. Our findings agree with studies on related enzymes showing the importance of Lys$^{229}$ in catalysis but demonstrate that in Flp-mediated recombination the primary role of Trp$^{330}$ is architectural rather than catalytic. Eliminating the hydrogen bonding potential of Trp$^{330}$ by phenylalanine substitution results in surprisingly small changes in reaction rates, compared with dramatic decreases in the activities of W330A, W330H, and W330Q. The structure of a W330F mutant-DNA complex reveals an active site nearly identical to that of the wild type. The phenylalanine side chain preserves most of the van der Waals interactions and is responsible for maintaining the copy number of this plasmid and is responsible for maintaining the copy number of this plasmid from Saccharomyces cerevisiae and is responsible for maintaining the copy number of this plasmid in vivo (1). Although many site-specific recombinases require co-factors for activity, Flp-mediated recombination is remarkably simple and requires only the enzyme and target DNA, which makes it a valuable genetic tool. The usefulness of Flp has been enhanced by the isolation of a variant of Flp ("Flpe") that displays significantly more robust activity than WT Flp at 37 °C (2). Although Flp shares many mechanistic and structural similarities with other recombinases and with type IB topoisomerases, in other aspects it is unique, such as in the assembly and regulation of its active site. It has thus been unclear to what extent the results of biochemical studies on other members of these families could be extrapolated to the Flp system.

The reaction catalyzed by Flp and other tyrosine recombinases minimally requires four protein monomers and two DNA segments containing specific sequences recognized by the enzyme (Fig. 1a). The reaction proceeds in a stepwise fashion, with only one strand of each duplex cleaved at a time. Thus, only two of the four protomers are active in each step of the reaction. For Flp, the required DNA site, named FRT (Flp recognition target), is made up of two 13-bp Flp-binding sequences inverted around an 8-bp spacer region, with a third nonessential Flp-binding site separated by 1 bp from the rest of the sequence. However, whereas most other enzymes in this family assemble the catalytic center in cis, using residues from one protomer, Flp uses a unique in trans mechanism, with its active site consisting of residues from two protomers (3, 4). The α-helix (helix M) containing the catalytic tyrosine (Tyr$^{343}$) is donated to an adjacent protomer and cleaves DNA in conjunction with the other active site residues provided by that protomer. Corresponding to half of the sites activity, only one DNA cleavage reaction takes place within an Flp dimer, two of which come together to form a synaptic complex. Each of the two exposed 5'-hydroxyl groups then attacks the phosphotyrosyl linkage in the other Flp dimer of the complex. The resulting Holliday junction intermediate can subsequently isomerize and activate the two protomers whose catalytic tyrosines were dormant during the first half of the reaction. These two tyrosines then engage in a second round of DNA cleavage and ligation reactions, ultimately yielding recombined products. Interestingly, structural and biochemical studies have shown significant differences in the mechanisms by which different recombinases control the activity of their catalytic sites (5–9).

The DNA relaxation reaction catalyzed by type IB topoisomerases also proceeds through a 3'-phosphotyrosyl intermediate, and the catalytic similarities between these two families of enzymes correspond to a conserved core structure that harbors the active site (10). Several structures from both families have been determined, including Λ-integrase (11), Cre (12–15), HP1 integrase (16), XerD (17), and Flp (4) from the site-specific recombinase family and vaccinia topoisomerase (18) and human topoisomerase I (19–21) from the topoisomerase family. Despite low sequence homology among these proteins, the secondary and tertiary structures of their catalytic domains are strikingly similar, and they probably share a common ancestor (10, 22). There are, however, clear differences among these enzymes as well. For example, the topoisomerases act as monomers, whereas the recombinases act as tetramers, and Flp...
differs from other members of both families in the trans assembly of its active site.

The active sites of both families include, in addition to the nucleophilic tyrosine, five other highly conserved amino acid residues (reviewed in Refs. 23–25). In Flp, these are Arg191, Arg308, His305, Lys223, and Trp330. The two arginines and the histidine, but not the lysine and the tryptophan, have been extensively studied in this system (26–30). The arginines presumably position the scissile phosphate and stabilize the build-up of negative charge in the transition state. The histidine (replaced by lysine in topoisomerases) is appropriately positioned in the Flp and Cre complex structures to act as the general base that accepts a proton from the attacking tyrosine, although mutagenesis data suggest that it is not essential (27, 30, 31). The lysine is essential for catalysis in other recombinases and has been shown to act as a general acid in the cleavage step in the vaccinia topoisomerase reaction, protonating the leaving 5'-hydroxyl group with the help of one of the arginines (8, 32–35). The tryptophan is replaced by histidine in most related enzymes, although tryptophan is found at this position in Cre as well. The DNA complex structures of Cre and human topoisomerase I show that the side chain of this tryptophan/histidine forms a hydrogen bond with a nonbridging oxygen of the scissile phosphate, whereas in Flp-DNA structures, Trp330 sometimes forms a hydrogen bond with the free 5'-OH in the active site. Mutation of the equivalent histidines to alanine in vaccinia topoisomerase (His365) and human topoisomerase I (His632) decreased the reaction rates substantially, whereas the effects of glutamine and asparagine replacements were less drastic (36, 37). Experiments on vaccinia virus topoisomerase using phosphorothioate-containing substrates showed that this histidine forms a catalytically important hydrogen bond to one of the nonbridging phosphate oxygens (38). All of these results supported a hypothesis that the key feature of the tryptophan/histidine is its ability to hydrogen-bond with DNA. However, this question had not been addressed experimentally in the tyrosine recombinases.

To investigate the roles that Lys223 and Trp330 play in Flp-mediated recombination, mutagenesis and activity studies have been performed on these two residues in FlpE. Whereas our results confirmed the importance of Lys223, they also provided some surprising insights into the function of Trp330, indicating that it might be more important in maintaining a viable active site through its van der Waals interactions with neighboring amino acids rather than stabilizing a reaction intermediate through its hydrogen bonding capability. We confirmed this by determining the structure of the W330F protein complexed with DNA.

EXPERIMENTAL PROCEDURES

Protein Cloning and Mutagenesis—In our experiments, FlpE was used as the WT Flp. A Flp clone was constructed and kindly provided by Dr. Mike Cox. A C-terminal His tag plus a single glycine linker region was added using PCR, and the tagged protein was cloned into pET24d vector. Point mutagenesis was performed using the QuickChange™ site-directed kit from Stratagene, with the primers ordered from Operon. The results were confirmed by DNA sequencing.

Protein Expression and Purification—The protein was expressed and purified using a new method recently published (5). Two columns were used: a Talon column that bound the His tag and a DNA affinity column containing Flp-binding sequences. The whole purification process took only 1 day (beginning with frozen cell pellets), yielding an average of 1 mg of protein from 1 liter of cell culture. The protein was stored at −80 °C. It was later dialyzed against 50 mM HEPES (pH 7.1), 1 mM EDTA, 1 mM dithiothreitol, and 15% glycerol and aliquoted before usage. The purification and dialysis were performed at 4 °C.

Role of the Conserved Trp in Flp-mediated Recombination

DNA Recombination Assays—Plasmid pFJS39 was originally provided by Dr. Michael Cox (39). It was amplified in DH5α cells and purified with Qiagen Miniprep kits. 15 pmol of SacI-digested pFJS39 plasmid was labeled with γ-32PATP and T4 polynucleotide kinase. Qiagen nucleotide removal kits were used to remove the enzyme and nucleotide afterward. The recombination assay was performed at 30 °C in a 10-μl reaction volume with 2 μM radioactively labeled SacI-cleaved pFJS39, 120 mM Flp protein, 25 mM MgCl2, 5 mM sodium acetate, 120 mM NaCl, 0.8% glycerol, 2 mM EDTA, and 1 mg/ml bovine serum albumin. The concentration of the DNA was calculated based on the assumption that no DNA was lost during the purification procedure after labeling, and the actual concentration might be slightly lower than 2 μM. The reaction was terminated with 0.4% SDS, and the products were separated on a polyacrylamide gel. The reaction conditions were similar to those used by Yates et al. (39) with minor modifications. Although polyethylene glycol was used in previous studies to enhance activity, it did not increase the reaction rate in our assays and only induced the appearance of reaction intermediates. It was therefore omitted from the reaction buffer.

DNA Cleavage Assays—Synthetic oligonucleotides were purchased from the Kec biotechnology facility at Yale University and purified by urea polyacrylamide gel electrophoresis. S3 (TAA TTC AGT GGA AGT TCC TAT TTC T) was labeled with γ-32PATP and purified as described under “DNA Recombination Assays.” It was then annealed with S4P (5'-phospho-CTA GAA GAA TAG GAA CTT CCA CTG GAT TA) to form a DNA substrate containing one Flp-binding site. The final substrate for the DNA cleavage assays consisted of both radioactively labeled and unlabelled suicide substrates at a ratio of 1.9. Each 10-μl reaction contained 4 mM DNA substrate, 120 mM Flp protein, 25 mM MgCl2 (pH 8.0), 200 mM NaCl, 12% glycerol, 2 mM EDTA, and 1 mg/ml bovine serum albumin. The assay was carried out at 30 °C and terminated with 0.4% SDS. The products were separated on a 15% SDS-PAGE gel. Fluorescence was quantified using a Molecular Dynamics PhosphorImager. The data were processed using Origin software, and the curves were fit to a hyperbola equation to attain the apparent rate constant for a second order reaction.

Crystallography and Structure Determination—The complex of WT FlpE and T4D4 (T4: TAA GGT CTT ATT CT; B4: TTT AAA AOA ATA GTA ATC) was crystallized from the original Flp-Holliday junction complex (4). A slightly higher protein concentration, 0.48 mg/ml, was used. Crystals appeared in hanging drops containing 20 mM HEPES (pH 7.0), 11% polyethylene glycol 5000 monomethyl ether, 20% xylitol, 100 mM NaCl, 2 mM dithiothreitol, 10 mM CaCl2, approximately 1 week after the trays were set up. Data were collected from crystals with a size of 0.2 × 0.2 × 0.4 mm at the BioCARS 14BM-C beamline at the Advanced Photon Source using an ADSC Q4 CCD detector. The crystals were in space group P211, with unit cell dimensions of a = 80 Å, b = 117 Å, c = 142 Å, and β = 97.3°. The diffraction was anisotropic, with <h01> falling below ~2 at 2.7, 3.0, and 2.9 Å along the three axes. The data were processed with the HKL suite (40). Initial phases were provided by the WT FlpE structure, which crystallized isomorphously (Protein Data Bank number 1M6X) (5). The structure was refined with CNS_SOLVE (41), using a combination of 2- and 4-fold noncrystallographic symmetry restraints. Differences in the refined structures of the WT and mutant were in agreement with difference Fourier maps (ΔFWT − ΔFW330) and (ΔFWT − ΔFW330) (5).

In addition to changes in the active site discussed under “Results,” the DNA strand in the catalytic site of the inactive monomer D could be modeled better as nicked rather than intact. Structure figures were generated with Ribbons (42). Buried surfaces were calculated using CNS_SOLVE with the side chain atoms of the His330 residue of FlpE (43). The total buried surface area was calculated to be 1200 Å2. The result was the total buried surface area from molecule 1 and molecule 2, and should be divided by 2 when considering only one molecule in the interface.

RESULTS

Flp Purification—One difficulty in studying Flp recombinase is the usually cumbersome protein purification procedure, which is further complicated by the protein’s intrinsic instability (43, 44). We were able to improve the efficiency of this process with the use of two affinity columns: Talon column for a newly added C-terminal His tag and DNA affinity column containing multimers of Flp-binding sites. The addition of the His tag was based on the observation that the C terminus of the enzyme is exposed and far from the active site and DNA-binding regions in the Flp-DNA complex structure. The DNA...
column was similar to those in previous studies except that the DNA molecules consisting of multiple Flp-binding sites were prepared with template-free PCR instead of ligation. Purified protein was obtained within 1 day. Flpe is used in our studies, and the wild type in this paper refers to His6-tagged Flpe without further mutations. The four point mutations (P2S, L33S, Y108N, and S294P) found in Flpe are removed from the catalytic center and do not appear to affect the static structure of the enzyme-DNA complex.

Trp^330 and Lys^223 Are Important for Activity—Single mutations were introduced at Trp^330 and Lys^223 to study the roles of these two residues in Flp-mediated recombination. Trp^330 was substituted with alanine, histidine, glutamine, and phenylalanine, whereas Lys^223 was replaced by alanine and arginine. The effects of these mutations were first tested in full recombination assays. The linearized plasmid pJFS39 contains one FRT site (shown as a gray box) with a symmetric spacer. Depending on the orientation of the two substrates in the synaptic complex, recombination results in two products that are either the same length as the substrate (left) or different lengths (right). c, 120 nm protein and 2 nm DNA were used in a 24-h reaction. The first lane contains no protein; the second lane contains WT Flpe; the next four lanes contain mutations at Trp^330 as shown, and the last two lanes contain mutations at Lys^223. The reaction was terminated with 0.4% SDS, and the products were separated by agarose gel electrophoresis. Double-stranded cleavage products result from slow hydrolysis of the intermediate (see “Results”). d, recombination experiments with higher protein and DNA concentrations: 360 nm protein and 10 nm DNA were used in a 24-h reaction. The “intermediate” visible in the K223R lane is probably a Holliday junction.

Although recombination catalyzed by the wild type protein reached equilibrium within several hours under our conditions, overnight reactions were carried out for the mutants to allow detection of low activity levels (Fig. 1c). Protein and DNA concentrations were also raised in one experiment to further increase the sensitivity (Fig. 1d). W330A, W330Q, and K223A
failed to generate any detectable amount of recombined products. W330H was capable of mediating recombination at a much slower rate, whereas K223R showed an extremely low activity only in the assay where the protein and DNA concentrations were increased. In contrast to the poor performances of the other mutants, W330F displayed an activity level indistinguishable from the wild type in these experiments, a surprising observation in light of the previous hypotheses that the hydrogen bond between the Trp330 side chain and the DNA would be important for catalysis.

The fact that mutating Lys 223 to alanine abolished activity whereas K223R restored some enzymatic capability is consistent with previous findings that this lysine acts as a general acid in vaccinia topoisomerase. In Flp, the side chain of Lys223 is also involved in direct contact with a base in the minor groove and therefore may be important for DNA binding. K223A and K223R showed decreased affinities for the DNA column during protein purification (data not shown), suggesting that part of the activity loss might result from impaired DNA binding capabilities.

The long incubation time for the overnight reactions with WT and W330F Flp led to the appearance of some doubly cleaved side products. These double strand cleavage products, like the reaction intermediates, have been observed by others and probably result from slow hydrolysis of the covalent phosphotyrosine intermediate (39, 45).

Comparison of Wild Type and W330F in Recombination Assays—A more detailed comparison was carried out between the activities of the wild type and the W330F mutant by measuring the formation of recombined products at time points before equilibrium (Fig. 2). No detectable difference was observed between the reactions catalyzed by the two proteins, suggesting again that the role of Trp330 in Flp-mediated recombination might be different from previous hypotheses. However, the full recombination reaction requires many steps, and the rate-limiting one may actually be a conformational change in the Holliday junction (46). This could mask a small decrease in the mutant’s catalytic activity. A second group of experiments, DNA cleavage assays, was used to determine whether there is any defect in the ability of W330F to catalyze the chemical reactions.

Comparison of Wild Type and W330F in Cleavage Assays—DNA cleavage is the first chemical step in the reactions mediated by this family of enzymes. Our assay used suicide substrates shown in Fig. 3a. The attack of Tyr343 between the Flp-binding site and the spacer region results in a covalent protein-DNA linkage and the excision of two thymines at the end of the DNA top strand, which diffuse into the bulk solution.

Fig. 2. Time course of recombination assays. Top panels, reactions using WT Flp (a) and W330F Flp (b) were terminated with SDS at the time points shown, and the products were separated by agarose gel electrophoresis. Conditions were the same as in Fig. 1c. Bottom panels, The percentage of total DNA converted to product amino acid plotted versus time.
The reaction is trapped at this stage due to the absence of the 5'-hydroxyl group on the first thymine, which is necessary for the reverse reaction or the subsequent ligation reaction of the recombination process. Because of half of the sites activity, a theoretical 50% of the substrate duplex will be covalently linked to the enzyme when the protein is present in excess. A modest decrease in reaction rate was observed for the W330F mutant, compared with that of the wild type (Fig. 3). The appearance of the covalent protein-DNA product fit better to a second order reaction curve than to a first order one, consistent with the requirement for two DNA-bound Flp protomers to form a single active site. The apparent rate constant for the wild type was estimated to be $1.0 \times 10^8 \text{ M}^{-1} \text{ min}^{-1}$, whereas that for the mutant was roughly 5-fold lower, $0.18 \times 10^8 \text{ M}^{-1} \text{ min}^{-1}$. This decrease probably reflects a slight defect in catalysis rather than in DNA binding, because these assays were carried out with a large excess of protein, above the estimated $K_d$ (47), and because our preliminary mobility shift assays using a single Flp-binding site indicated no difference in affinity between the two proteins (data not shown).

Radioactive and nonradioactive cleavage assays were also performed on the other mutants. The results were consistent with those of recombination assays, except that W330Q was able to yield covalent protein-DNA adduct at a rate that is slower than the wild type but slightly faster than W330H (Fig. 3b), in contrast to the apparently higher activity level of W330H in the overall recombination assay. This suggests that W330Q is even more defective in the later stages of recombination than in the cleavage step.

When compared with the dramatic activity decrease in W330H and W330Q, the small reaction rate difference between the wild type and W330F mutant suggests that hydrogen-bonding capability is not the critical feature of Trp³³⁰. Examination of the previous Flp-DNA complex structure revealed an extensive network of amino acids contacted by Trp³³⁰ through van der Waals interactions within or close to the catalytic site. The most essential role of Trp³³⁰ in the reaction may be maintaining a stable local architecture. To investigate whether the high activity level of the mutant stemmed from the ability of phenylalanine to retain those van der Waals contacts, W330F mutant protein was crystallized with DNA, and a structure of W330F-Holliday junction complex was determined.

**Structure of W330F Mutant Protein Complexed with DNA**—W330F Flpe was crystallized with the same DNA substrate as used in the WT Flp and Flpe complex structures (4, 5). Phases were provided by a newly determined structure of a wild type Flpe-DNA complex (5). The overall structure of the mutant is the same as that of the wild type Flpe. The complex has approximate 2-fold symmetry and consists of a mutant tetramer bound to a DNA Holliday junction (Fig. 4a). Two catalytic centers contain a covalent phosphotyrosyl linkage and a free 5'-OH, whereas the other two contain a continuous or nicked DNA strand with Tyr³⁴³ displaced from the scissile phosphate. The mutant structure was refined to R and R-free values of 23.6 and 27.5%, respectively (Table I). 87.6% of the nonglycine protein residues are in the most favored conformational regions of the Ramachandran plot (as defined by Procheck (48)). One of the two active protomers (“chain C,” with Tyr³⁴³ donated by chain C) was significantly better ordered than the other. Discussions of the active site geometry thus pertain to this protomer.
Role of the Conserved Trp$^{330}$ in Flp-mediated Recombination

The slightly higher resolution of the W330F Flpe structure, compared with WT Flpe, allows us to model the C-terminal His$_6$ tag. The His$_6$ tag of protein chain B is involved in crystal packing. It forms a short $\alpha$-helix, with two histidine side chains stacked against the bases at the end of a DNA arm from the neighboring Holliday junction. It is interesting to note that in some other structures where ordered His$_6$ tags are seen, they are in extended conformations (Protein Data Bank numbers 1GKK and 1LW6) (49, 50). Thus, His$_6$ is clearly a sequence that can adopt different secondary structures in different environments.

Other than a small shift in the position of the two inactive protomers, which is probably due to minor differences in crystal packing, the only significant differences between the mutant and WT Flpe structures are at Trp$^{330}$ and in its immediate vicinity. A prominent peak at the position of the Trp$^{330}$ side chain was observed in a difference map comparing the data of the wild type and the mutant, corresponding to the alteration of tryptophan to phenylalanine and a small change in the $\chi_1$ dihedral angle. Other peaks correspond to a shift in the proximal residues of the trans helix, including Tyr$^{343}$, and the scissile phosphate to which it is covalently attached. There are only rather subtle changes that are difficult to interpret at this resolution in the other active site side chains (Fig. 5). The 5'-hydroxyl group formed a hydrogen bond with the side chain of Trp$^{330}$ in the Flp structure and was only 3.6 Å away from the tryptophan N$_e$ atom in the WT Flpe model. This hydrogen bond is necessarily broken in the W330F structure. The 5'-hydroxyl is not well defined in the electron density, but it is possible that it could form an alternative hydrogen bonding interaction with Arg$^{191}$.

The phenylalanine side chain maintained most of the van der Waals interactions between Trp$^{330}$ and neighboring residues. Trp$^{330}$ interacts with several amino acids residing in its own protomer and on the trans-helix donated from the adjacent one, burying a total of 383 Å$^2$ in the active site containing the phosphotyrosyl linkage. Phe$^{330}$ still contacts the majority of these residues, with a buried area of 340 Å$^2$, a slight decrease due to the smaller size of the phenylalanine side chain. Of the amino acids contacted by residue 330, Arg$^{306}$ and Tyr$^{343}$ are directly involved in catalysis, whereas the others are near the active site. Three (including Tyr$^{343}$) lie on the trans helix, suggesting that Trp$^{330}$ helps position this helix and Tyr$^{343}$ for DNA cleavage. The loss of activity in the W330A, W330H, and W330Q mutants may reflect the loss of some or most of these stabilizing hydrophobic interactions.

Docking Helix M—The van der Waals interactions of Trp$^{330}$ may be particularly important in Flp because of its trans cleavage mechanism. The correct docking of the Tyr$^{343}$-bearing helix appears to be crucial to Flp-mediated recombination and is clearly regulated by the protein to achieve half of the sites activity. To further investigate the docking of helix M, Asn$^{325}$, whose side chain forms two hydrogen bonds to the trans polypeptide segment (Fig. 6), was mutated to alanine. The N325A mutation caused a significant decrease in both the overall recombination rate and the initial DNA cleavage rate (data not shown).

We also mutated two residues (Thr$^{224}$ and Ser$^{331}$) that lie...
shown. Asn$^{325}$ from the purple protomer forms two hydrogen bonds with the scissile phosphate. The side chains of residues discussed here are shown. Ser$^{336}$ and Ala$^{339}$ on helix M interact with Trp$^{330}$, which is replaced by a phenylalanine in the mutant structure shown here. His$^{309}$ and His$^{345}$ are also involved in correctly placing Tyr$^{343}$ for catalysis. Thr$^{224}$ and Ser$^{351}$ contact the DNA near the active site.

near the conserved pentad and contact the incoming DNA strand near the 5'-hydroxyl to see if they were also important in positioning catalytic sites (Fig. 6). However, the activity of the T224A and S331A mutants in recombination assays was not significantly different from that of WT Flp (data not shown).

DISCUSSION

The biochemical and structural evidence presented here demonstrates that the most important feature of the conserved Trp$^{330}$ in Flp-mediated recombination is its ability to form extensive van der Waals interactions rather than its ability to form a hydrogen bond with the DNA. It may be that the hydrogen bond donated by this residue in WT Flp contributes relatively little to the rate-limiting step of catalysis. This is in contrast to the vaccinia virus topoisomerase case, where the equivalent histidine was clearly implicated in forming a hydrogen bond to the nonbridging oxygens of the scissile phosphate during the transition state of the cleavage reaction (38). Differences in Trp$^{330}$ in Cre and the corresponding histidine (His$^{632}$) in human topoisomerase I are both hydrogen-bonded to a nonbridging oxygen atom on the scissile phosphate, whereas in Flp complexes the Ne atom of Trp$^{330}$ is a bit farther away from the scissile phosphate (4, 5, 12–14, 19–21). In the original Flp-DNA complex, Trp$^{330}$ forms a hydrogen bond with the free 5'-hydroxyl group instead, whereas in the Flp-DNA structure it is roughly equidistant between the 5'-hydroxyl and a nonbridging phosphate oxygen (3.6 versus 3.8 Å). We cannot exclude the possibility that the loss of a hydrogen bond to the 5'-hydroxyl in the W330F complex is compensated by formation of an alternate interaction. In the new three-dimensional model of the mutant protein-DNA complex, a small swivel movement of the poorly ordered 5'-hydroxyl group could bring it within hydrogen bonding distance of Arg$^{191}$. The slight activity drop in the W330F mutant could therefore be due to the slight deficiency of a smaller phenylalanine side chain in maintaining all of the original van der Waals interactions or some minor defect resulting from the loss or switch of the hydrogen bond acceptor or both. It is not known whether the histidine or glutamine side chain of W330H and W330Q maintained a hydrogen bond with the 5'-hydroxyl group, although model building indicated that they could.

The van der Waals interactions involving Trp$^{330}$ may be particularly important in the Flp case because of this enzyme’s unique trans cleavage mechanism. The importance of the correct docking of the tyrosine-bearing helix M is further illustrated by the experiments on N325A, which is also involved in intermolecular contacts with the trans polypeptide segment. Previous mutational studies also highlighted residues involved in the packing of this helix into the active site cavity of the neighboring protomer. Trp$^{330}$ packs against one face of this helix, contacting Ser$^{336}$ and Ala$^{339}$ in addition to the catalytic Tyr$^{343}$. Mutations at Ser$^{336}$ and Ala$^{339}$, which are strictly conserved in Flp-like tyrosine recombinases from other yeast species, caused defects in DNA cleavage and the formation of dimeric complexes (27, 51) (shown in Fig. 6). The roles of His$^{309}$ and His$^{345}$, also involved in contacts between helix M and its “host” protomer, have been extensively investigated as well (52). Together with our experiments on Trp$^{330}$ and Asn$^{325}$, these results show that the correct positioning of Tyr$^{342}$, which is essential to the activity of Flp, involves multiple residues inside or close to the active site.

These intermolecular contacts must be strong enough to properly position helix M in the two active catalytic centers yet weak enough to be reversible, since they are not seen in the two inactive catalytic centers. In fact, the half of the sites activity of Flp is thought to be regulated by the disruption of these contacts and displacement of Tyr$^{342}$ due to the increased distance between the two contributing protomers in the inactive catalytic centers (5). In Cre, where the active site is assembled in cis, Trp$^{315}$ and its surrounding residues bury nearly the same amount of interface area in both active site states (~385 Å$^2$), despite changes in the protein backbone and some side chain conformations. It would thus be interesting to know whether or
not a W315F mutation in Cre behaves similarly to the W330F mutation in Flp.

Some variability was seen within the topoisomerase family as well, where the histidine that is equivalent to Trp330 of Flp has been studied extensively. In vaccinia virus topoisomerase, H265A, H265Q, and H265N mutations reduced the activity by 100–, 4–, and 5-fold, respectively. In human topoisomerase I, the mutations H632A, H632Q, H632N, and H632W resulted in decreases of 6000–, 100–, 180–, 12,000-fold in the cleavage reaction rate. The different extents to which the two alanine mutations affected the enzymatic activity suggest that the importance of this histidine's role may vary. For Flp, it is difficult to accurately quantify extremely low activity, because it requires relatively long reaction times. This is complicated by the protein's instability and side reactions such as hydrolysis of the phosphotyrosyl bond. Judging from the sensitivity of our recombination assay, the W330A mutation slowed down the appearance of product by at least 2500-fold, a severe reduction in activity rate. The different extents to which the two alanine mutations reduced the activity by H265A, H265Q, and H265N mutations.

We thank Michael Cox and Elizabeth Wood for help with data collection. Some variability was seen within the topoisomerase family as well, where the histidine that is equivalent to Trp 330 of Flp could act as a general acid in the reaction. Instead, during the appearance of product by at least 2500-fold, a severe reduction in activity rate. The different extents to which the two alanine mutations reduced the activity by H265A, H265Q, and H265N mutations.

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