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Brief Communication

Identification of a Potential General Acid/Base in the Reversible Phosphoryl Transfer Reactions Catalyzed by Tyrosine Recombinases: Flp H305

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SUMMARY

Flp provides a unique opportunity to apply the tools of chemical biology to phosphoryl transfer reactions. Flp and other tyrosine recombinases catalyze site-specific DNA rearrangements via a phosphotyrosine intermediate. Unlike most related enzymes, Flp's nucleophilic tyrosine derives from a different protomer than the remainder of its active site. Because the tyrosine can be supplied exogenously, nonnatural synthetic analogs can be used. Here we examine the catalytic role of Flp's conserved H305. DNA cleavage was studied using a peptide containing either tyrosine (pKa ≅ 10) or 3-fluoro-tyrosine (pKa ≅ 8.4). Religation was studied using DNA substrates with 3′-phospho-cresol (pKa ≅ 10) or 3′-para-nitro-phenol (pKa ≅ 7.1). In both cases, the tyrosine analog with the lower pKa specifically restored the activity of an H305 mutant. These results provide experimental evidence that this conserved histidine functions as a general acid/base catalyst in tyrosine recombinases.

INTRODUCTION

Site-specific DNA recombinases catalyze inversions, deletions, and insertions of DNA segments. Far more common in bacteria than eukaryotes, these reactions have a broad variety of biological consequences, including the resolution of replicon dimers, the insertion of phage DNA, and the transfer of antibiotic resistance genes [1]. One of the few eukaryotic examples, Flp from *S. cerevisiae* is responsible for maintaining the copy number of the 2 micron plasmid that encodes it. It does so by inverting a segment of DNA surrounding the plasmid's origin of replication, resulting in rolling circle replication and, ultimately, the production of multiple copies of the plasmid with only one firing of the origin [2]. Flp is a member of the tyrosine recombinase family, which includes, among others, Cre and phage λ integrase [1].

Flp-catalyzed DNA recombination proceeds through a series of phosphotransfer reactions (Figures 1A and 1B). In the cleavage steps, the DNA's 5′ hydroxyl is displaced by the enzyme's active site tyrosine, whereas in the religation steps, the tyrosine is displaced from the resulting covalent intermediate by attack of a different 5′ hydroxyl. Although recombination is normally orchestrated by a tetramer of Flp, the individual chemical steps do not require the complete oligomer [3]. Structural and biochemical studies have shown that tyrosine recombinases and the related type Ib topoisomerases surround the scissile phosphate with a constellation of conserved residues (in addition to the tyrosine) but, unlike many other phosphotransferases, do not require divalent metal ions (Figure 1) [1].

Enzymes can enhance phosphotransfer reaction rates in a number of ways. First, they can greatly enhance the reaction simply by binding the reactants such that they are appropriately oriented for the ensuing reaction. For phosphodiesters such as DNA, these reactions are thought to involve a pentacovalent transition state, in which bond formation to the nucleophile is at least to some degree simultaneous with breakage of the bond to the leaving group. The reaction could therefore be stimulated by both a general base to aid deprotonation of the nucleophile, and a general acid to protonate the leaving group. An enzyme could also preferentially stabilize the transition state, which has a slightly different geometry than the ground state and may also bear additional negative charge [4, 5].

How are these catalytic roles addressed by the conserved residues found in the active sites of Flp and related enzymes (Figure 1E)? Since all the reactants are macromolecules, the job of simply localizing the players is accomplished by formation of the protein-DNA complex as

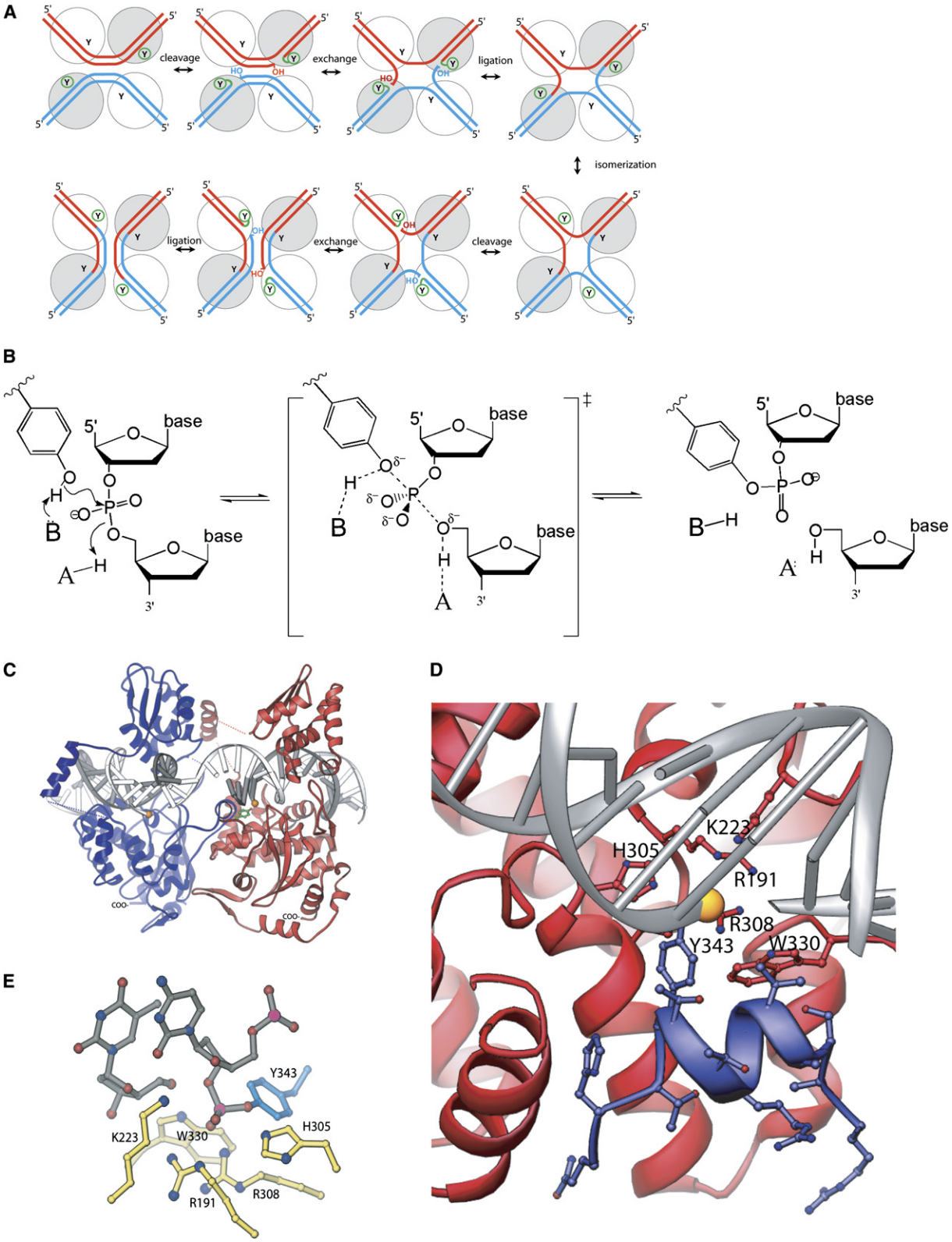

Figure 1. Site-Specific Recombination Mediated by Flp

(A) Recombination pathway. Two DNA segments and four enzyme molecules (spheres, with Y representing the catalytic tyrosine) are required. Only two protomers are active at each step, as indicated by the small circle around the Ys. The catalytic states of the individual protomers are switched in the isomerization step. The two DNA strands are sequentially cleaved, exchanged, and religated.

a whole. This may at least in part explain why these enzymes do not need Mg^{2+}, which often coordinates the attacking water molecule in hydrolases. The two arginines (Flp R191 and R308) probably help position the scissile phosphate and may also stabilize the geometry and charge of the transition state. A combination of structural data and 5′ bridging sulfur substitution studies with the type Ib topoisomerase from vaccinia virus have implicated the lysine (K223 in Flp) and possibly the 1st of the two arginines in protonating the leaving 5′ oxygen during DNA cleavage [6, 7]. The most important role of W330 in Flp appears to be structural [8]: in a full recombination assay, W330F was indistinguishable from WT, although in a cleavage assay it was ~5-fold slower. This also showed that the conformational changes required of both the DNA and the protein in a full recombination assay can mask subtle catalytic defects that are revealed in simpler (single-step) assays. In related proteins W330 is usually replaced by a histidine, which may also stabilize the transition state through a hydrogen bond to one of the nonbridging oxygens of the scissile phosphate [9–12].

Is the tyrosine assisted by a general acid/base? The active sites of the type Ib topoisomerases do not contain side chains that are good candidates for this role. Instead, a bound water molecule is thought to perform this duty [13]. No such appropriately positioned water has been noted in the tyrosine recombinases, but they do have a conserved histidine (H305 in Flp) that is well positioned to act as a general base in the cleavage reaction and, conversely, general acid in the religation reaction [14–17]. Previous qualitative studies have observed that the H305A, Q, and L mutants were defective in catalyzing the recombination reaction and tended to accumulate the DNA cleavage products [18, 19]. In Cre, the equivalent H289 has shown decreased activity as well. Both H289A and H289N mutants are ~4× less active than WT in a full recombination assay (in which the chemistry of cleavage is probably not normally rate limiting). Recently the Baldwin lab has shown that Cre H289N is 1200× slower than WT in a cleavage assay (K.A. Gelato and E.P. Baldwin, personal communication). (We have found no published measurements of cleavage rates for mutations of the conserved active site histidine in any tyrosine recombinase.) In the Flp-DNA complex model, the Nε2 atom of H305 is ~2.8 Å from the oxygen linking the tyrosine and DNA. Interestingly, this histidine lies across the DNA's minor groove, with its Nδ1 atom relatively close to the phosphates of the opposite DNA backbone (Figure 1D), making a Y-H-PO_4 arrangement reminiscent of the S-H-D triad of the serine proteases. This hypothesis could not be tested by previous biochemical experiments, and the general acid/base that assists the tyrosine remained elusive.

We have taken advantage of Flp's unique active site architecture to investigate this. In Flp, the conserved pentad of one protomer activates the scissile phosphate, while Y343 is donated by a second protomer that inserts one helix into its neighbor's active site (Figure 1) [20, 21]. The active site is thus prepared to accept an exogenous tyrosine. Cleavage and ligation were studied separately using synthetic substrates bearing tyrosine analogs with lowered pKa's that should mitigate the effects of mutating the general acid/base.

RESULTS AND DISCUSSION

DNA cleavage and ligation reactions were investigated with three variants of Flp: Y343F, Y343F/H305Q, and Y343F/K223R. All Flp proteins used also carry the four point mutations in the "Flpe" variant that enhance its thermostability [22]. We chose Q to replace H305 in order to maintain approximately the same volume and hydrogen bonding capacity while removing the labile proton. As a control, we also included a mutant of K223, which is proposed to be the general acid/base that assists the DNA 5′ oxygen in catalysis but is not expected to interact with the tyrosine [6, 7]. The K223A mutation essentially abolishes the enzymatic activity, which is partially restored in the K223R mutant that we use here in order to ensure measurable amounts of product [8]. Except for the H305-Y343 hydrogen bond, neither H305 nor K223 participates in any direct interactions with the catalytic tyrosine-containing "*trans*" helix (Figure 1D). Mutations at these residues are therefore unlikely to affect the packing of this helix into the active site pocket.

Initial DNA Cleavage

To study DNA cleavage, we supplied the tyrosine as part of a 13 aa peptide spanning residues 334–346 of the Flp sequence (Figure 1D). Previous studies have demonstrated that Flp Y343F, lacking its own nucleophile, could still facilitate the attack of alternative nucleophiles such as tyramine, glycerol, and hydrogen peroxide on the scissile phosphate [3, 23], albeit quite inefficiently. We found that this cleavage reaction is much more robust when the nucleophile is supplied on the peptide, probably due to extensive interactions between other residues on the *trans* helix and the Flp protomer it docks into. Two versions of a peptide were synthesized, one with a native tyrosine

(B) General acid/base catalysis during the DNA cleavage and ligation reactions. In the DNA cleavage reaction, a general base presumably accepts a proton from the attacking tyrosyl hydroxyl group while a general acid donates a proton to the DNA's 5′ bridging oxygen. This process is reversed in the ligation reaction.
(C) Flp cleaves DNA in *trans*. The interface between two protomers is viewed from the center of the Holliday junction. Y343 of the blue protomer, shown in ball-and-stick mode, is donated to the red protomer and cleaves the DNA bound by that one. The scissile phosphate is shown as a gold sphere.
(D) Flp Helix M (blue) packing into the active site of a neighboring Flp protomer (red). The segment shown corresponds to the peptide used in this study, and all side chains are shown.
(E) Catalytic residues. The active state of the catalytic site, containing the covalent phosphotyrosine linkage, is shown. Six conserved residues, including Y343, surround the scissile phosphate. PDB code 1M6X.

(pKa ≅ 10) and another with a 3-fluorotyrosine (pKa ≅ 8.4). Fluorinated tyrosines have been used successfully in protein kinase studies [5, 24]. The nucleophile with the lower pKa should show less dependence on a general base during the cleavage reaction.

The DNA cleavage assay corresponds to the first chemical step in the recombination pathway (Figure 2). We used a suicide substrate containing a single Flp-binding site with only two nucleotides extending past the scissile phosphate on the cleaved strand [25]. After cleavage, this dinucleotide is diluted into solution, preventing the reverse reaction and leaving the peptide covalently linked to the DNA. Studying this first step of the reaction alone avoids the conformational changes that are known to be rate limiting in a full recombination reaction [8, 26].

The results shown in Figure 2 clearly support the hypothesis that H305 is involved in general base catalysis in the DNA cleavage reaction. The rate for Y343F Flpe-catalyzed cleavage decreased with the change from tyrosine to the fluorinated analog. This is consistent with the WT enzyme utilizing a general base to aid deprotonation of the nucleophile, since the anionic form of the fluorinated analog is less nucleophilic than that of the native tyrosine. The rate for Y343F/H305Q-catalyzed cleavage increased significantly (at least 1–2 orders of magnitude; the exact ratio is hard to estimate due to the near-zero rate with the unmofidied tyrosine). Within error, the slow cleavage rate of the Y343F/K223R enzyme was unchanged. These results suggest that the lower pKa of the fluorotyrosyl hydroxyl group specifically compensated for H305Q's lack of a general base. We also tested the effects of two other changes at H305: to L and A (Figure 2D). Mutation of H305 to A, which probably introduces a packing defect in the active site, destroyed all measurable cleavage activity with either peptide. Y343F/H305L also failed to catalyze measurable DNA cleavage with the tyrosine-containing peptide. However, measurable activity was rescued by the fluorotyrosine peptide. That the cleavage defect of H305L as well as H305Q can be at least partially overcome by lowering the pKa of the nucleophile argues against the possibility that the role of general base is played by a water molecule that is hydrogen bonded to the side chain at position 305. Similar enhancement of H305Q's cleavage activity by the fluorotyrosine peptide was also seen between pH 7.5 and 9.5 (Figure S3; see the Supplemental Data available with this article online). In this assay, all combinations of Flpe and peptide variants were essentially inactive above pH 9.5

Our analysis assumes that most of the DNA substrate is bound at any given time and that the reaction is first order with respect to the concentration of protein-DNA complexes. We measured a K_d of 31.7 ± 3.96 nM for Flpe Y343F and the suicide DNA substrate (Figure S1). This is similar to previously reported values of K_d for WT Flp binding to the full-length DNA substrate [27]. This implies that under our reaction conditions ([DNA] = 10 nM and [Flp] = 360 nM), 91.5% of the DNA was bound. We also found that for DNA concentrations varying from 1 to 20 nM, the rate of product formation varied linearly with the DNA concentration, which can be taken as a surrogate for the protein-DNA complex concentration since the DNA is essentially fully occupied (Figure S2). This implies that a single Flp-DNA complex is sufficient to carry out catalysis with the *trans* helix peptide: even though dimerization is normally required for cleavage by Flp (i.e., without added peptide), it is not necessary here.

DNA Religation

If H305 accepts a proton from the tyrosine nucleophile during DNA cleavage, it presumably also donates a proton to the leaving tyrosine group during ligation, which is chemically the reverse reaction. To isolate the ligation reaction, we used activated DNA substrates in which an analog of the tyrosine leaving group was esterified to the 3′ phosphate: one, cresol (or 4-methyl-phenol), has a pKa of 10, similar to that of tyrosine, and the other, pNP (or para-nitro-phenol), has a pKa of 7.1. The latter was used as a substrate for Cre- and vaccinia topoiosomerase-mediated DNA ligation [28] and should be oblivious to the presence of a general acid under our reaction conditions (pH 8). As shown in Figure 3, ligation was initiated by adding an excess of an oligonucleotide complementary to the overhanging noncleaved strand.

Ligation reactions were carried out with the same Flp mutants as the cleavage reactions, and the results of the two sets of experiments were in good agreement (Figure 3). When comparing product formation with the cresol and pNP substrates, reactions catalyzed by Y343F and Y343F/K223R mutants displayed minor rate changes with pNP, which is a better leaving group; however, the effect of the lower pKa of pNP was far more dramatic for the Y343F/H305Q reaction. The Y343F/H305Q ligation reaction has an ~27× rate enhancement with pNP compared to cresol, while the rate enhancements for the Y343F and Y343F/K223R ligation reactions were both less than 4×, as shown in Figure 3. This implies that the phosphotyrosyl oxygen leaves more readily when H305 can participate by releasing a well-positioned proton.

The results of both sets of experiments are consistent with a general acid/base model for Flp catalysis where H305 is involved in the abstraction of a proton from and the donation of a proton to the catalytic tyrosyl oxygen in the DNA cleavage and ligation reactions, respectively. The role of H305 has been more difficult to place than that of other catalytic residues, and this is not surprising. Though H305 is clearly important for catalysis, it is less conserved and its mutants show smaller decreases in activity—especially cleavage activity—compared to the conserved lysine and arginines [18]. This suggests that, in the cleavage step, the catalytic power derived from providing a proton acceptor may be less than that derived from providing a proton donor to the leaving hydroxyl group. This may be due in part to the lower pKa of tyrosine (10) relative to that of a 5′-hydroxyl group (~15). In addition, model reactions of nucleophilic substitution at phosphodiesters show less dependence on the basicity of the nucleophile (β_{nuc} ~0.2–0.3) than the leaving group (β_{lg} ~−0.8) [4, 6]. Finally, it is worth noting that even in

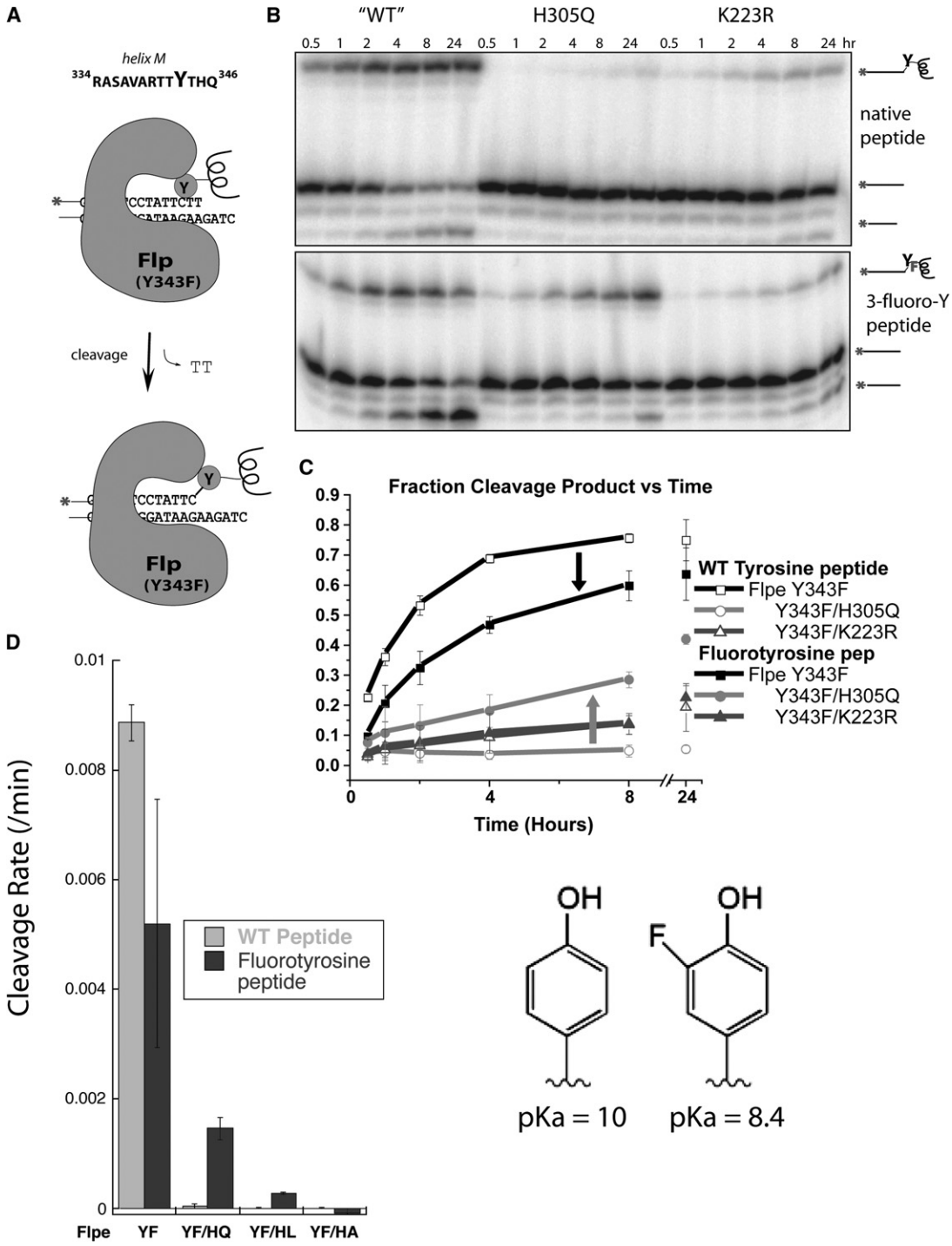

Figure 2. Peptide Cleavage of DNA Catalyzed by Flp Variants

(A) Schematic describing the cleavage reaction with a 13 amino acid peptide corresponding to residues 334–346 of Flp, including helix M and the flanking loops. Peptides bearing tyrosine or 3-fluorotyrosine were reacted with Flp variants and a DNA suicide substrate containing a single Flp binding site, which releases a TT dinucleotide upon cleavage.

(B) The products of the cleavage reactions separated on sequencing gels. Hydrolysis of the phosphotyrosine linkage happens over time, especially with the fluorinated tyrosine analog.

(C) Plot of product versus time. Product, shown as a percentage of total DNA, is the sum of the DNA-peptide complex and the subsequent hydrolysis product. Error bars represent the standard deviation of three data sets. The activity of both the Y343F and K223R decreased when the fluorotyrosine-containing peptide was used. The activity of H305Q, however, showed a significant increase.

(D) Cleavage rates with several different Flpe H305 mutants. Error bars represent the standard deviation.

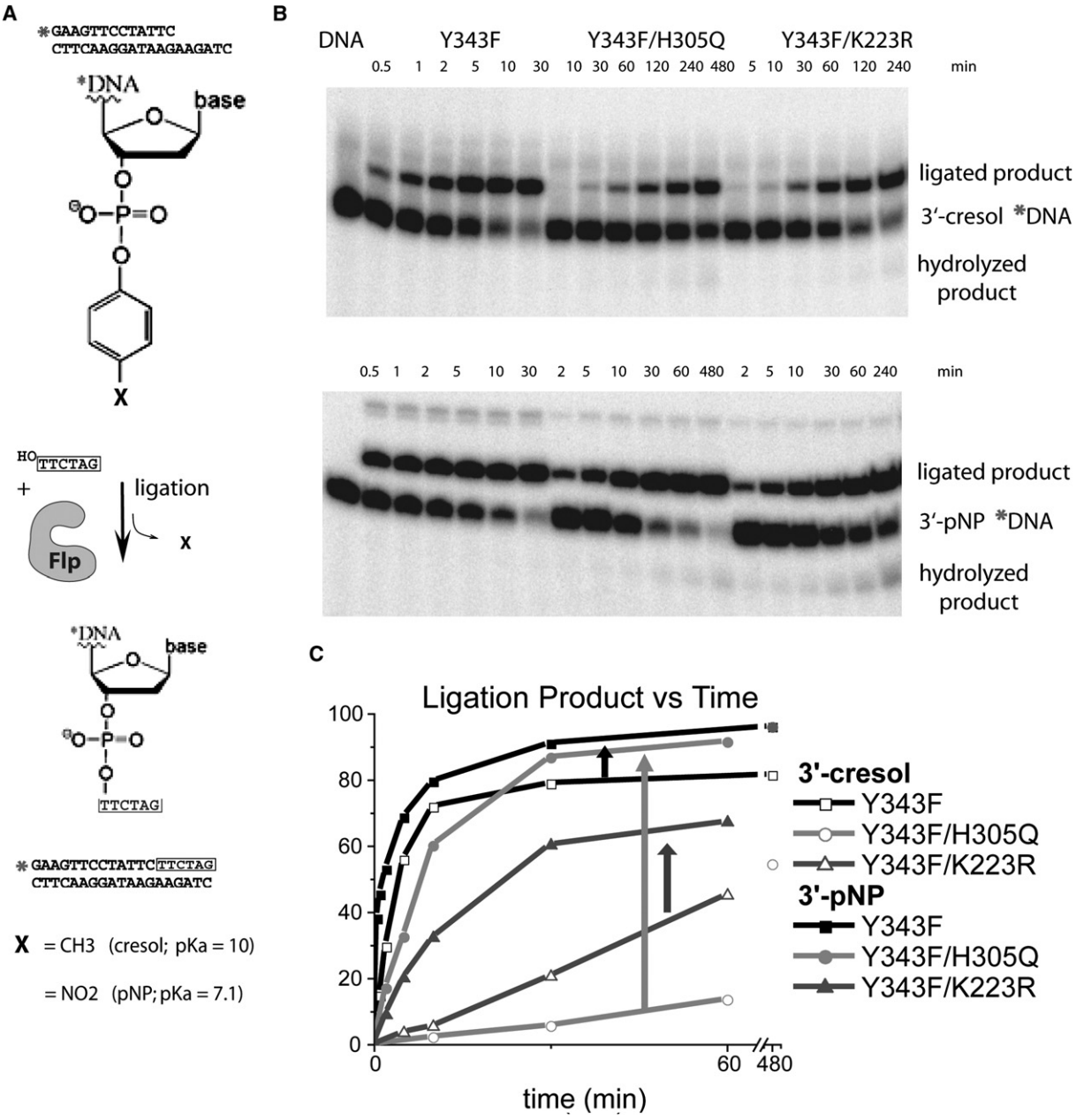

Figure 3. Ligation of Modified DNA Substrates

(A) Schematic representation of the DNA ligation assay using a substrate carrying a single Flp binding site with a tyrosine mimic (cresol or pNP) esterified to the 3′ phosphate. A short oligo complementary to the overhang of the bottom strand, which mimics the incoming strand, is added in excess, displaces the tyrosine mimic from the 3′ end of the DNA, and produces a longer ligated product.

(B) PAGE separation of ligation products. Note that different time points were taken for each Flpe variant.

(C) Quantitation of gels showing ligated product as a percentage of total DNA. Reactions catalyzed by all three proteins displayed rate increases with the 3′-pNP compared to the 3′-cresol, when the pKa of the leaving group changed from 10 to 7.1. However, H305Q showed the most dramatic increase.

the paradigmatic serine proteases, mutation or modification of the active site histidine (which assists a serine; closer in pKa to a 5′ hydroxyl) does not utterly destroy catalytic activity: k_{cat} for the His/Asp double alanine mutant of subtilisin was $\sim 10^4$-fold lower than that for the WT, but still $\sim 10^4$-fold above that for the uncatalyzed reaction [29, 30].

From comparison of Flp to the type IB topoisomerases, it is clear that there is more than one way for these related enzymes to assist the tyrosine in catalysis: H305 is important in Flp, whereas it is apparently replaced by a well-ordered water molecule in the topoisomerases [11, 13]. Recent studies on Flp's W330 have found that the relative

catalytic contribution of a specific residue may vary among related enzymes even while the general catalytic strategy is preserved. However, despite Flp's unique *trans* active site composition, the orientation of its active site components is quite similar to those of Cre and λ integrase, which cleave DNA in *cis* [16, 31]. The equivalent histidines, H289 and H308, respectively, lie in similar positions relative to the phosphotyrosine linkage and DNA backbone. Although more complicated assays may give different secondary effects of their mutation, such as altering the preferred direction of Holliday junction resolution in Cre, their close structural similarity implies that they are likely to perform a similar underlying chemical function in general acid/base catalysis [32].

SIGNIFICANCE

This work uses the tools of chemical biology to probe how Flp, a tyrosine recombinase, catalyzes phosphoryl transfer. The catalytic mechanism of these enzymes is somewhat unusual for DNA phosphotransferases in that it does not require divalent cations. We have experimentally addressed the question of which residue, if any, acts as a general acid/base to assist the tyrosine in catalysis. We were able to examine both the forward and reverse steps of the phosphoryl transfer because Flp's unique *trans* active site geometry allows it to accept an exogenous tyrosine. The critical tyrosine could thus be replaced with synthetic analogs that have altered pKas, either incorporated into a peptide, as the nucleophile in the forward reaction, or esterified to the DNA, as the leaving group in the reverse reaction. In both reactions, the tyrosine analog with the lowered pKa, which should be less dependent on a general acid/base catalyst, largely restored the activity of a Flp variant lacking the conserved histidine. Our experiments, together with previous structural and sequence conservation data, provide strong support for the hypothesis that this residue functions as the general acid/base that assists the tyrosine during catalysis.

EXPERIMENTAL PROCEDURES

Protein Mutagenesis and Purification

Y343F Flpe with a C-terminal His_6 tag was used as "wild-type" in our studies. Point mutagenesis was performed with the QuikChange Site-Directed Mutagenesis Kit from Stratagene, and the results were confirmed by DNA sequencing. The proteins were purified with a previously published protocol [8], using a Talon column for the His tag and a DNA affinity column containing Flp-binding sequences. The purification procedure for each protein was completed within 1 day starting from a cell pellet. The protein was flash-frozen in liquid nitrogen and stored at −80°C. It was later dialyzed against 50 mM HEPES (pH 7.1), 1 mM EDTA, 1 mM DTT, 15% glycerol, and 1 M NaCl overnight and aliquoted before usage.

Peptide Synthesis

The 13 residue peptides (RASAVARTTYTHQ and RASAVARTTY$_F$THQ, Y_F = 3-fluorotyrosine) were made manually by "in situ neutralization" Boc chemistry with stepwise solid-phase peptide synthesis on -OCH2-Pam-resins, as described previously [33]. Peptide compositions were confirmed by reversed-phase HPLC and LCMS on an Agilent 1100 Series chromatography instrument equipped with an MSD ion trap, using Vydac C4 columns (5 μm, 0.46 × 25 cm). Chromatographic separations were performed using a linear gradient (10%–60%) of buffer B in buffer A over 30 min at a flow rate of 1 ml/min. Buffer A = 0.1% TFA in water; buffer B = 0.08% TFA in acetonitrile. Preparative HPLC was performed on a Waters Prep LC 4000 system using a Vydac C4 column (12 μm, 2.2 × 25 cm) at a flow rate of 10 ml/min, with a gradient of 20%–50% buffer B in buffer A over 60 min. Fractions were pooled based on LC-MS analysis.

Synthesis of 3′-Cresol/pNP Substrate DNA

Oligonucleotides (6 × 1 μmol) were synthesized using 3′-PO_4 CPG (Glen Research, Sterling, VA) and standard DNA phosphoramidites on an ABI 392 automated DNA synthesizer. Oligonucleotide synthesis was completed with automated removal of the 5′-dimethoxytrityl protecting group. The resin was incubated in concentrated ammonium hydroxide at 65°C for 12 hr. DNA was precipitated with the addition of 0.1 volume of 3 M sodium acetate and 3 volumes of absolute ethanol, and the DNA pellet was resuspended in 1 ml of 2 mM $MgCl_2$, 100 mM MES (pH 5.5). Aliquots of 1 ml 99% *para*-cresol (Aldrich, Milwaukee, WI) and 0.048 g of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) (Aldrich) were added sequentially. The resulting two immiscible layers were mixed with a magnetic stir bar at 65°C to form an emulsion. After 20–24 hr, the aqueous layer was extracted three times with 2 ml each of ethyl acetate, and the DNA was ethanol precipitated. This EDC-derivatization protocol was repeated twice more. Typically, ~5% of the 3′-PO_4 (starting material) was converted to 3′-cresol DNA.

The 3′-cresol derivatized DNA was purified by anion exchange chromatography on a Hitachi analytical HPLC using a DNAPac PA-100 4 mm × 250 mm column (Dionex, Sunnyvale, CA) at 1 ml/min, with a 5%–60% 2 M sodium chloride gradient (buffered with 20 mM sodium phosphate [pH 7.0]) over 15 min. Peak fractions were pooled, precipitated, resuspended, and stored at 4°C. The synthesis of 3′-pNP substrate was similar to that of 3′-cresol.

Peptide-Mediated Cleavage Assays

Synthentic oligonucleotides were purchased from the Keck biotechnology facility at Yale University and purified by urea polyacrylamide gel electrophoresis. S3 (TAA TCC AGT GGA AGT TCC TAT TCT T) was 5′ end-labeled with ^{32}P and purified on a BioRad Spin 6 chromatography column. It was then annealed with S4P (5′-phos-CTA GAA GAA TAG GAA CTT CCA CTG GAT TA) to form a suicide substrate containing one Flp-binding site. The final substrate for the DNA cleavage assays consisted of both radioactively-labeled and unlabeled suicide substrates at a ratio of 1:9. The 50 μl reaction contains 10 nM DNA substrate, 360 nM Flpe protein, 2 mM peptide, 25 mM Taps (pH 8.0), 200 mM NaCl, 5% glycerol, 2 mM EDTA, 1 μg/ml salmon sperm DNA, and 100 μg/ml BSA. Cleavage assays with varying concentrations of DNA shown in Figure S2 contained 1–20 nM DNA substrate. The assay was carried out at 30°C and terminated with formamide. Products were separated on sequencing gels and quantified using a Molecular Dynamics phosphorimager. Experiments were conducted in triplicate, and the percent of total complex was calculated by adding together the covalent peptide-DNA complex and the hydrolysis products and dividing by the total DNA. The pH dependence assays were carried out with the following buffers—pH 6–7: 25 mM MES, 25 mM acetic acid + 50 mM Tris; pH 8–9: 50 mM TAPS; pH 10: 50 mM CAPS. Rates were calculated by fitting a line to the slope of the cleavage product versus time plot, and only early time points taken every 5–10 min from the first 30–60 min were included. Rates for H305Q, H305A, and H305L were arrived at in the same way; these and the pH dependence assays were repeated at least twice.

DNA Ligation Assay

The top strand of the activated DNA substrate was radioactively labeled and purified as described above. It was then annealed with

S43P (5′-phos-CTA GAA GAA TAG GAA CTT C). The final substrate for the ligation assays consisted of both radioactively labeled and unlabeled suicide substrates at a ratio of 1:9. The 5′ overhang was phosphorylated so it could not attack the phospho-phenyl bond. A 6 nt oligo complementing the single-strand spacer region was used as the incoming strand and supplied the 5′-hydroxyl group that cleaved the 3′-phospho-phenyl linkage, resulting in a longer ligation DNA product. The 10 μl reaction contains 10 nM DNA substrate, 360 nM Flpe protein, 6 μM incoming strand, 25 mM Taps (pH 8.0), 200 mM NaCl, 12% glycerol, 2 mM EDTA, and 1 mg/ml BSA. The protein was preincubated with the activated DNA substrate before the addition of the incoming strand, which was in large excess to the activated substrate. The assay was carried out at 30°C and terminated with 0.4% SDS. Products were separated on 15% urea gels and quantified using a Molecular Dynamics phosphorimager. Results were averaged from two sets of experiments.

Electrophoretic Mobility Shift Analysis

Flpe YF was diluted into dialysis buffer (15% glycerol, 1 M NaCl, 50 mM HEPES, 1 mM EDTA, 1 mM DTT [pH 7.1]) in two independent serial dilutions leading to final concentrations of protein ranging from 0 to 1 μM. A 20 μl sample was prepared under the same conditions as the DNA cleavage assays described above, including 1 nM radiolabelled DNA suicide substrate and 2 μl of the protein dilution. After a 2 hr incubation at 30°C, a 10 μl sample was loaded onto a native 16 × 18 cm gel containing 10% 29:1 acrylamide:bis-acrylamide (w/w), 5% glycerol, and 0.5× TBE (45 mM Tris-borate, 1 mM EDTA) at 4°C and run for 1.5 hr at 140 V. Gels were dried and visualized following exposure to phosphorimager screens (Kodak) using a Molecular Dynamics phosphorimager. Free DNA and DNA-protein complex intensities in each lane were quantified with the volume analysis function in rectangle mode of ImageQuant V5.0 (Molecular Dynamics). Free protein concentration was assumed to be equal to total protein concentration, because it was in excess over the DNA concentration. The equilibrium dissociation constant was calculated with Kaleidagraph 3.6.4 by fitting data to the equation: $\theta = (Bmax^*[P])/([P] + K_d)$, where θ = fraction DNA bound, Bmax = maximum DNA bound, and p = Flpe YF. Experiments were repeated a minimum of three times, and error bars represent the standard deviation.

Supplemental Data

Supplemental Data include three figures and are available at http://www.chembiol.com/cgi/content/full/14/2/121/DC1/.

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