The arginine residue at position 308 in the Flp recombinase corresponds to the only invariant arginine within the Int family of recombinases. Alterations of this residue result in Flp variants that retain substrate recognition, but form weaker protein-DNA complexes than wild type Flp. Furthermore, their DNA cleavage activity is significantly diminished. A conservative change of R308K results in a functional Flp variant; however, this protein has a lowered temperature optimum for recombination. The Arg-308 mutants can be stabilized on the DNA substrate through cooperativity with a partner Flp mutant that is tight binding. Thus, interactions between Flp monomers must be a relevant feature of the normal recombination reaction.

The chemistry of the site-specific recombination reactions catalyzed by the λ Int protein, the P1 Cre protein, and the 2-μm circle Flp protein is identical (1, 2). All three reactions involve cleavage of the DNA substrate and transient covalent attachment of the recombinase to DNA through a 3'-phosphotyrosyl linkage (3–5). Recombination proceeds by pairwise exchange of single strands, that is, by formation and subsequent resolution of a Holiday intermediate (6–10). The only invariant residues among these proteins and other members of the Int family are a triad of histidine, arginine, and tyrosine (11). These correspond to His-305, Arg-308, and Tyr-343 of Flp, respectively. This absolute conservation may reflect the common chemistry of recombination. If so, these 3 residues are likely to be part of the active site of the Int family recombinases.

We showed earlier that Tyr-343 of Flp was not required for DNA binding, but was essential for DNA cleavage (12). The His-305 mutants of Flp were capable of substrate binding (although with lower affinities than wild type Flp) and were proficient for DNA cleavage; yet, they could not execute the strand exchange and reunion step (13). One previously studied Flp mutant altered at Arg-308, Flp(R308G), showed weaker substrate binding than wild type Flp when assayed in partially pure preparations (13). However, the role of Arg 308 in catalysis, if any, remained unclear. We now describe functional analyses of three more Arg-308 variants of Flp. Our results show that Arg-308 is not essential for general substrate recognition, but contributes to the tightness of the Flp-DNA complexes. In addition, efficient strand cleavage is contingent upon the presence of Arg-308. We propose possible mechanisms by which Arg-308 could be involved in coupling tight substrate binding to the first step in catalysis, namely DNA cleavage.

MATERIALS AND METHODS

Plasmids—The plasmids used for expression of Flp in Escherichia coli and for the in vivo assay of recombination have been described previously (14, 15). In these plasmids expression of Flp was driven either by the temperature inducible λ P. promoter or by the IPTG inducible lacI′W3 promoter.

Mutagenesis—Mutations were introduced by the double primer method (16) or by the selective mutagenesis scheme that eliminates wild type parental template (17). Accuracy of all mutations was confirmed by DNA sequencing after subcloning of the appropriate Flp fragments into the expression plasmids.

Purification of Flp—Wild type and mutant extracts were purified by slight modifications of the procedure of Prasad et al. (12). These extracts, termed "partially pure," contained approximately 25% Flp. For some experiments they were further purified on an affinity column by procedures analogous to previously published ones (18). The affinity column was prepared by ligating the synthetic double-stranded oligodeoxynucleotide

\[
\text{5'-CCGAAGTTCCTATTC-3'}
\]

\[
\text{3'-ATAAGGCTTCAAGG-5'}
\]

to an average size of 100 to 150 mer and coupling it to CNBr (cyanogen bromide)-activated Sepharose. The Flp binding site on the oligomer is not cleavable by Flp. Partially pure extracts of Flp or Flp(R308K) were adsorbed on the column in low salt buffer (50 mM Tris-HCl, pH 7.5, and 25 °C, 250 mM NaCl, 1 mM EDTA, 20 mM mercaptoethanol, 20% (v/v) glycerol). The column was washed with several column volumes of the loading buffer, followed by buffer containing 400 mM NaCl. Flp was eluted with buffer containing 1.0 M NaCl. The purity of the protein, as estimated from Coomasie Brilliant-Blue-stained gels, was >90%. The following modifications were introduced for purification of Flp(R308G), Flp(R308P), and Flp(R308Q). Initial adsorption to the column was done in buffer containing 100 mM NaCl, and the washing buffer contained only 250 mM NaCl. The proteins were eluted with buffer containing 1.0 M NaCl.

Recombination Assays—In vivo recombination assays were done as described previously (14, 15). In vitro recombination conditions were similar to those described by Gates and Cox (19). The reactions contained 25 mM Tris-HCl, pH 8.0, and 25 °C, 200 mM NaCl, 1 mM EDTA, 20% (v/v) glycerol, 10% (w/v) polyethylene glycol, the DNA substrate, and Flp protein in a total volume of 30 μL. Reactions were terminated by addition of sodium dodecyl sulfate (0.2%, final concentration), phenol-chloroform-extracted, ethanol-precipitated, vacuum-dried, and electrophoresed on agarose gels. DNA bands were visualized by ethidium bromide staining or by autoradiography.

DNA Binding, DNase Footprinting, and Strand Cleavage—Binding reactions were done according to Prasad et al. (12). DNase footprinting reactions (without isolation of individual complexes) were done by the methods of Prasad et al. (12) and Andrews et al. (20). Footprinting of isolated complexes followed the procedure outlined by Andrews et al. (21). Strand cleavage assays were done as described by Seneff et al. (22).
Cooperativity in DNA Binding and DNA Cleavage—Reaction mixtures for binding or for cleavage were set up according to standard protocols, except that no Flp protein was added. At the start of the reactions, the requisite amounts of the Flp variants were added to the appropriate tubes and mixed quickly but gently. Half of the individual reaction mixtures were quickly withdrawn, mixed appropriately, and incubations were continued under standard conditions for binding or for cleavage.

General Methods—Bacterial transformations, isolation of plasmid DNA, restriction enzyme digests, and other miscellaneous methods were done by published procedures (22). DNA sequences were determined by the method of Maxam and Gilbert (24) or Sanger et al. (25).

RESULTS

Recombination Properties of Flp Variants Altered at Arg-308—The mutant Flp genes were expressed in E. coli from the λ P, promoter at 42 °C by inactivating the temperature-sensitive repressor (cl434) and assayed for recombination in vivo by scoring for the deletion of the kanamycin resistance marker bordered by two direct copies of the Flp site (14). All four variants studied, Flp(R308G), Flp(R308K), Flp(R308P), and Flp(R308Q), were inactive in recombination by this assay (data not shown). However, an apparent contradiction arose when purely prepaes of these proteins were assayed for activity in vitro at 30 °C. In contrast to Flp(R308G), Flp(R308P), and Flp(R308Q), which remained true to their in vivo behavior, Flp(R308K) showed recombination in in vitro assays (Fig. 1). This result implied that Flp(R308K) may be temperature-sensitive. This notion was verified by fusing the gene for Flp(R308K) to the lac u55 promoter and testing recombination in vivo following induction of the protein with isopropyl-1-thio-galactoside. In this assay recombination in vivo behavior, Flp(R308K) showed recombination in in vitro assays. Hereafter, the recombinationally inactive Flp variants will be referred to as the Arg-308 mutants of Flp. The rather special properties of Flp(R308K) will be discussed separately.

Binding of Arg-308 Mutants of Flp to DNA—The first step in recombination is the recognition of the substrate by the recombinase and the production of an active synaptic structure. The results of a gel retardation assay for the three protein-DNA complexes (cI, cII, and cIII) formed upon binding of Flp to its substrate (21) are shown in Fig. 2. These assays were done at 30 °C using partially pure protein preparations (approximately 20 to 30% Flp or Flp variants). None of the three Arg-308 mutants of Flp, Flp(R308G), Flp(R308P), and Flp(R308Q), had lost the ability to recognize the normal Flp substrate. They generated the three complexes of the expected gel mobilities, but did so at relatively high protein concentrations. These complexes were not as tight as those formed by type Flp. In DNase protection experiments no clear-cut footprints were seen for Flp(R308G) or Flp(R308P), and only a faint protection pattern was observed for Flp(R308Q) (Figs. 3 and 4). The band corresponding to substrate cleavage by Flp (see the section on DNA cleavage) was not detectable for Flp(R308G) or Flp(R308P); an extremely faint cleavage band could be seen in the bottom strand footprints of Flp(R308Q).

For better resolution of the protection assay, we also compared the DNase footprints of the gel-isolated complexes cII and cIII formed by Flp(R308G) and Flp(R308Q) with those of wild type complexes (Fig. 5). For Flp(R308Q), some differences in the levels of DNase protection from the wild type at certain positions were noticeable. For example, the top strand footprints (see Fig. 5; right panel), revealed poorer protection of the 1a symmetry element and the spacer region of the substrate by Flp(R308Q). On the other hand, the level of DNase protection provided by Flp(R308G) was weaker over the entire Flp site than that provided by Flp(R308Q). Nevertheless, certain interesting similarities were noticeable between the two patterns. The top strand footprint of complex cIII formed by Flp(R308G) showed almost a complete lack of

FIG. 1. In vitro recombination assays. The protein preparations used in the assay shown here contained approximately 20–30% Flp or the Flp variants. The results were the same when 90–95% pure Flp preparations were used (obtained by affinity chromatography; see "Materials and Methods"). The substrates for the recombination were an unlabeled circular substrate with a single Flp site (S1), and a linear substrate containing two inverted Flp sites (S2) end-labeled with 32P. The diversity of products from iterative rounds of recombination makes the assay very sensitive. Reactions were carried out at 30 °C for a range of protein concentrations. The number above each lane is the amount of Flp or Flp variant added in microliters (2 μl corresponds to approximately two to four Flp monomers/recombination site). The left panel shows gel-fractionated recombination mixtures stained with ethidium bromide. The right panel is an autoradiogram of the same gel.
FIG. 2. Substrate binding by Arg-308 mutants of Flp. The Flp site-containing DNA fragment used for the binding assays is shown at the top. An EcoRI site and a HindIII site mark the right and left ends of this fragment. The 13-base pair symmetry elements that interact with Flp are indicated (1a, 1’a, and 1’b). The wavy lines indicate vector-derived sequences. The top or the bottom strand was selectively labeled by filling in the EcoRI site or the HindIII site, respectively, with α-32P-labeled deoxyribonucleosidetriphosphates using Klenow polymerase. The points of cleavage by Flp within the core region of the substrate are marked by an F. The sites at which the restriction enzyme XhoI cuts are shown by an X. Binding assays were done at 30 °C as described previously and the three protein-DNA complexes, cI, cII, and cIII, were separated by electrophoresis in 5% polyacrylamide gels (12). The amounts of protein used (nanograms) in the binding reactions are indicated at the top of the respective lanes. The assays contained approximately 0.05–0.1 pmol substrate/reaction. The extent of binding with wild type Flp is shown at the bottom far right for comparison.

FIG. 3. Top strand footprinting. DNase footprinting was performed on the top strand-labeled substrate by previously described protocols (12, 20). The region of protection was mapped from Maxam-Gilbert sequencing ladders run adjacent to the footprinted samples. The amounts (nanograms) of Flp or Flp variants used in the assay are indicated above the corresponding lanes. The band resulting from Flp catalyzed cleavage is indicated by an F.
Substrate Binding and Cleavage by Arg-308 Mutants of Flp

protection within 1a (Fig. 5, middle panel). This was somewhat analogous to the low degree of protection seen in the same region of complex cIII formed by Flp(R308Q). In the bottom strand footprints of cII and cIII derived from Flp(R308G), positions of increased DNase sensitivity in the core region were also evident (Fig. 5; left panel).

DNA Cleavage by the Arg-308 Mutants—Once an active synaptic complex is formed, the next step in recombination is cleavage of the DNA backbone with concomitant covalent attachment of the recombinase to DNA. Strand cleavage was assayed with a DNA fragment containing the Flp site (shown in Fig. 2), end-labeled on the bottom or on the top strand. The assay permits the detection of approximately 1–2% of the level of wild type cleavage. The results of cleavage using the top strand labeled substrate and partially pure Flp variants are shown in Fig. 6. No cleavage was seen with Flp(R308G) or Flp(R308P); a small amount of cleavage was detectable with Flp(R308Q). Similar results were obtained with the bottom strand labeled substrate (data not shown). The extent of cleavage with Flp(R308Q) was no more than 3–4% of that of wild type Flp. This low level of cleavage does not correlate with the amount of the substrate complexed with the protein. In these experiments, the levels of protein used were sufficient to drive over 30–40% of the substrate into cII and cIII. Recent assays using highly purified Flp preparations (90–95% pure; see "Materials and Methods") have provided general confirmation of these observations (data not shown). Some experiments revealed a hint of the cleavage product with Flp(R308G) (data not shown). This activity was at least three to four times less than that of Flp(R308Q) and was barely above the lower end of the sensitivity of the assay. Thus, mutations of Arg-308 in Flp almost entirely eliminates the substrate cleavage function of the protein.

DNA Binding of Flp(R308G) and DNA Cleavage by Flp(R308Q) Are Enhanced by Cooperativity with Flp(Y343F)— Earlier work on recombination had implied that interactions between monomeric units of Flp may be an important aspect of recombination (26–28). If this were true, we might expect the Arg-308 mutants to be anchored more tightly on the DNA when provided with a strong binding protein as its partner. We, therefore, performed mixing experiments in which the binding reactions for Flp(R308G) and Flp(R308P) were laced with relatively small amounts of Flp(Y343F), a mutant that...
binds DNA well but cannot cleave it (12). The results obtained with Flp(R308G) (Fig. 7, A and B) revealed cooperativity of binding between Flp promoters. The strong binding partner, Flp(Y343F), stabilized the weaker one, Flp(R308G), on the DNA, such that significant levels of complexes cII and cIII were formed. DNA cleavage, if any, observed under these conditions could be attributed only to Flp(R308G). However, under these cooperative binding conditions, no cleavage was observed (data not shown). The results with Flp(R308P) were generally similar to those with Flp(R308G). Cooperativity in binding was evident; but no detectable cleavage resulted from this cooperativity (data not shown). We also tested whether Flp(Y343F) could enhance the very low levels of cleavage normally seen with Flp(R308Q). The protein concentration required for detectable cleavage by Flp(R308Q) was sufficient high to drive almost all the substrate into complexes cII and cIII (Fig. 7C). The changes in the relative levels of cleavage upon addition of increasing amounts of Flp(Y343F)
Substrate Binding and Cleavage by Arg-308 Mutants of Flp

FIG. 8. Effects of temperature on the activity of Flp(R308K). Substrate binding (A), cleavage (B), and recombination (C) were tested at the indicated temperatures. The protein preparations used in these assays were purified to 90–95% homogeneity using an affinity matrix (see "Materials and Methods"). Binding and cleavage reactions were done using standard conditions, except for the differences in temperature (Fig. 7D). The Flp-DNA complexes are labeled cI, cII, and cIII. The cleavage fragment produced by Flp and the product of XbaI digestion are marked F' and X, respectively. Recombinations were done using two linear substrates, each containing the one Flp site. One of the two substrates was end labeled with 32P (S1). The second, unlabeled substrate cannot be seen in the autoradiogram. The 32P-labeled recombinant products are named R1 and R2.

DISCUSSION

We have studied the properties of four variants of the Flp recombinase in which Arg-308 (1 of the 3 invariant residues of the Int family) has been changed by site-directed mutagenesis to glycine, lysine, proline, or glutamine. All changes except the conservative change to lysine abolish recombination. The lysine mutant (Flp(R308K)) shows a lower temperature optimum for recombination than wild type Flp. Earlier in vivo DNA binding assays for Flp had suggested that wild type Flp may be less active at 42 °C than at 30 °C (29). In vitro results now confirm this notion. Flp(R308K) is more exquisitely sensitive to temperature than wild type Flp.

The recombinationally inactive mutants, Flp(R308G), Flp(R308P), and Flp(R308Q), can all recognize their target DNA site and form enzyme-substrate complexes. However, DNase I challenge showed that the complexes formed by these mutants are less tight than the wild type complexes. DNase footprints of isolated protein-DNA complexes formed by Flp(R308G) and Flp(R308Q) indicated certain nucleotide positions within the substrate that were poorly protected by these proteins. Between the two mutants, the footprints of Flp(R308G) were fainter than those of Flp(R308Q). We interpret these results as follows: the general contacts between Flp and its DNA substrate are not completely eliminated by the absence of Arg-308. However, the positively charged side chain of Arg-308 is important in reinforcing the initial protein-DNA interactions into a productive recombination complex. The side chain of glutamine is only an inadequate substituent in this regard, whereas those of glycine and proline are essentially nonfunctional.

The most striking aspect of the Arg-308 mutation is their pronounced effect on substrate cleavage. Although Flp(R308Q) is competent for cleavage, it is highly inefficient. Little or no cleavage activity was detectable with Flp(R308G) or Flp(R308P). Thus the Arg-308 side chain has apparently a dual role in recombination, in stabilizing Flp-DNA interactions and in promoting DNA cleavage.

One plausible mechanism that can couple the binding and catalytic functions of Arg-308 would involve hydrogen bonding between the Arg-308 side chain and the phosphate of the DNA backbone at the position of strand cleavage. This would not only contribute to the strength of the protein-DNA complex, but would also make the phosphate a better target for nucleophilic attack by Tyr-343 (the invariant tyrosine of the Int family), which is the residue involved in covalent attachment to DNA (5). We wish to reiterate the analogy, pointed out by guest on March 24, 2020
out by Parsons et al. (13), of DNA cleavage by Flp to strand scission reactions carried out by pancreatic ribonuclease and by Staphylococcal nuclease (30). An important mechanistic feature of the nuclease reactions is hydrogen bonding between positively charged amino acid side chains and the phosphate at the cleavage point (K-41 in the case of pancreatic ribonuclease and R-35 and R-87 in the case of Staphylococcal nuclease).

Although a mechanistic explanation for the role of Arg-308 in recombination is attractive, we cannot rule out a possible structural explanation. It is known that binding of Flp to its substrate bends DNA and that mutants of Flp that fail to induce bending are inactive in recombination (31). Our results would be consistent with the possibility that the lack of Arg-308 eliminates some important protein-DNA or protein-protein contact that directly affects the architecture of the synaptic complex and indirectly affects catalysis. The stimulation of Flp(R308Q)-mediated DNA cleavage by Flp(Y343F) without substantially increasing the levels of protein-DNA complex would fit the structural explanation nicely. Solution of the crystal structure of Flp and Flp-DNA complexes may provide the correct choice between the two alternate, but not mutually exclusive, explanations offered here.

We had shown earlier that His-305 of Flp (the 3rd invariant residue of the Int family) is not required for substrate binding or DNA cleavage, but is essential for the final step of recombination, namely strand exchange and recombination (13). It is difficult to assess whether Arg-308 participates in this step as well. Two observations are consistent with Arg-308 being required for strand exchange and reunion. First, in recombination reactions, significantly more DNA cleavage can be detected in the Flp(R308K) reactions than in the wild type reactions. Second, Flp(R308Q) shows low levels of DNA cleavage, yet produces no completed recombinants even in very sensitive assays for recombination.

The cooperativity of substrate binding and cleavage seen with mixtures of Flp(Y343F) and Arg-308 variants of Flp underscores the importance of protein-protein interactions in generating productive Flp-DNA complexes. Based on the Flp-binding and recombination properties of synthetic Flp sites containing point mutations in one or both of the symmetry elements 1a and 1'a (see Fig. 2), Prasad et al. (27) surmised that intrasubstrate and intersubstrate cooperativity is intrinsic to recombination. Their suggestion that the source of this cooperativity could be interactions between promoters of Flp is substantiated by our binding and cleavage experiments using pairs of appropriate Flp variants. Interactions between Flp monomers is a basic feature of the model for recombination proposed by Senecoff et al. (28) as well.

As pointed out earlier, Arg-308 is part of the invariant triad of the Int family recombinases. It would indeed be surprising if the role of Arg-308 in the Flp reaction is not analogous to its role in recombinations catalyzed by other members of this family.

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