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Intra- and intersubunit interactions controlling assembly of active synaptic complexes during Hin-catalyzed DNA recombination

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Abstract

Serine recombinases, which generate double strand breaks in DNA, must be carefully regulated to ensure that chemically-active DNA complexes are assembled correctly. In the Hin-catalyzed site-specific DNA inversion reaction, two inversely-oriented recombination sites on the same DNA molecule assemble into a synaptic complex that uniquely generates inversion products. The Fis-bound recombinational enhancer, together with topological constraints directed by DNA supercoiling, function to regulate Hin synaptic complex formation and activity. We have isolated a collection of gain-of-function mutants in 22 positions within the catalytic and oligomerization domains of Hin using two genetic screens and by site-directed mutagenesis. One genetic screen measured recombination in the absence of Fis and the other assessed SOS induction as a readout of increased DNA cleavage. These mutations, together with molecular modeling, identify important sites of dynamic intrasubunit and intersubunit interactions that regulate assembly of the active tetrameric recombination complex. Of particular interest are interactions between the oligomerization helix-E and the catalytic domain of the same subunit that function to hold the dimer in an inactive state in the absence of the Fis-enhancer system. Among these is a relay involving a triad of phenylalanines that are proposed to switch positions during the transition from dimers to the catalytically-active tetramer. Novel Hin mutants also are described that generate synaptic complexes that are blocked at step(s) prior to DNA cleavage.

Keywords

site-specific DNA recombination; serine recombinase; Fis; hyperactive mutants; DNA invertase

Introduction

Reactions involving DNA breakage and rejoining must be carefully controlled to avoid undesirable chromosome breaks or rearrangements. Careful regulation of serine

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recombinases (Fig. 1a) is particularly important because this family of site-specific recombinases generates double strand breaks at the two recombining DNA segments during the process of DNA exchange.¹ For example, the Hin serine recombinase, which is the subject of this report, creates double strand breaks in the chromosome of *Salmonella enterica* when it inverts a 1 kb segment that regulates flagellin synthesis (see Fig. 1b).^{2; 3} In contrast, members of the tyrosine recombinases family sequentially cleave and ligate single strands of DNA generating a Holliday intermediate during the DNA exchange reaction.¹

Serine recombinases are catalytically silent when bound to DNA as a dimer because the active site serine is not positioned appropriately to attack DNA (Fig. 1e).⁴ In order to become chemically active, two recombinase dimers must first assemble into a tetrameric synaptic complex containing the two recombining sites (Fig. 1f).¹ For DNA invertases like Hin or Gin, the formation of the active synaptic complex, called an invertasome, requires the Fis regulatory protein bound to a cis-acting recombinational enhancer sequence (Fig. 1d).^{5; 6; 7} The enhancer is normally located about 100 bp from the *hixL* recombination site within the invertible segment but can function many kilobase-pairs away and on either side or orientation on a plasmid substrate. When the Hin dimers associate at the Fis-bound enhancer they undergo a remarkable quaternary change to generate the synaptic tetramer in a process that involves multiple conformational steps.^{8; 9; 10; 11} The tetramer cleaves all four DNA strands at the center of the two *hix* sites resulting in each subunit linked to the cleaved 5' end by a serine-phosphoester bond.^{10; 12; 13} DNA exchange occurs by rotation of one pair of newly synapsed subunits relative to the other pair within the tetramer.^{10; 14; 15; 16; 17} The translocation of subunits brings the covalently-linked DNA segments into the recombinant configuration for ligation.

The requirement for the proper assembly of the supercoiling-dependent invertasome structure prior to the chemical and mechanical steps of the reaction ensures that the two recombining DNA segments are located on the same chromosome and are aligned appropriately to generate inversion upon DNA exchange.² We previously have characterized a strong hyperactive Hin mutant containing a single histidine to tyrosine substitution at residue 107 in the long helix-E, which connects the catalytic domain (residues 1–99) to the DNA binding domain (residues 135–190) (Fig. 1a,e,f).¹⁸ This mutant catalyzes unregulated recombination, including deletions and intermolecular reactions, without the requirement for the Fis/enhancer system or DNA supercoiling. Mutants isolated in other serine recombinases, such as the related DNA invertase Gin and resolvases like those encoded by the Tn3 and $\gamma\delta$ transposons and a *S. aureus* plasmid (Sin), also promote promiscuous recombination reactions in the absence of otherwise required co-factors or accessory recombinase binding sites (Fig. 1a).^{19; 20; 21; 22; 23; 24; 25} However, multiple mutations are usually required to generate strong hyperactivity in the transposon resolvases.

An understanding of the regulation of the Hin-catalyzed DNA inversion reaction requires knowledge of the molecular forces that are involved both in maintaining the Hin dimer in an inactive state and the conformational conversion to the synaptic tetramer that is competent for DNA cleavage and exchange. One approach to identify such forces is to obtain mutants that exhibit relaxed or altered regulatory properties. A second approach is to evaluate specific residues, using available atomic structures or from work in related serine recombinases, which could potentially control conformations or enzymatic properties of Hin. These approaches have identified 22 residues within the Hin catalytic domain that directly or indirectly control its activity. Whereas wild-type Hin is extremely inefficient at assembling synaptic complexes containing associated *hix* sites without Fis, many of the regulatory mutants support robust assembly of stable synaptic complexes. Most of the synaptic complexes are active for DNA cleavage and exchange, but some accumulate synaptic complexes at stages prior to DNA chemistry. Such mutants have not been

characterized previously in Hin and provide a means to investigate the structure(s) of synaptic complex intermediates that have not advanced to a cleavage-competent state. We can explain the phenotypic properties of many of the mutants by structural modeling of the conformational transitions involved in assembly of the active synaptic complex. Most significantly, we have identified a set of unappreciated intrasubunit interactions that appear to play a large role in regulating the conformational transitions from inactive dimer to activated tetramer in Hin.

Results

Isolation of hyperactive mutants

Two genetic screening strategies and site-directed mutagenesis were employed to obtain gain-of-function Hin mutants. The most extensive screens were for Hin mutants that were able to promote inversion in the absence of Fis. In most of these screens the *hin* gene under the control of the *tac* promoter was mutagenized by error-prone PCR and transformed into a *fis* mutant reporter strain containing *lacI^{qs}* and *λfla-lac406OFF* (Fig. 1b). Basal expression of wild-type *hin* was sufficient to promote extremely low rates of inversion as recognized by weakly Lac⁺ colonies after >40 h on Lac MacConkey agar. Fis-independent Hin mutants promoted faster rates of inversion. The most active mutants, such as Hin-H107Y, M115V, and L77I generated intense red (Lac⁺) colonies after <16 h, an *in vivo* rate that was comparable to Hin-wt in the presence of Fis (Table 1). In all cases, Fis-independent Hin mutants exhibited faster *in vivo* inversion rates when Fis was present.

A second screen measured SOS activation as recognized by induced expression of λ P_L-*lacZ* controlled by the λ^{434} repressor (Fig. 1c). Basal expression of Hin-wt under control of the *lacI^{sq}* gene weakly induced P_L-*lacZ*, forming light red colonies after 35 h that are believed to reflect low levels of Hin-catalyzed DNA breaks. Unlike transposition systems, where transposase-induced SOS induction requires the presence of transposon ends^{26; 27; 28}, Hin induces SOS without introduction of an inversion substrate, presumably because the recombinase can react with pseudo-*hix* sites within the chromosome. Hyperactive mutants were identified as those that formed red colonies faster than Hin-wt, with the strongest mutants forming intense red colonies after 25 h (Table 1). All mutations obtained by the SOS assay, except for Hin-F105V, also were isolated by the Fis-independent screen.

The 27 gain-of-function mutants are classified into 4 groups (Table 1) based on their location within the modeled Hin structure and proposed mechanism of action. Representative Hin mutants from each of the groups were purified and their ability to promote DNA inversion or partial reactions were evaluated *in vitro*. Rates of DNA inversion were assayed using our standard inversion substrate pMS551 by following a change in the restriction pattern as a function of DNA inversion (Fig. 2a). Because moderate amounts of ethylene glycol stimulate inversion reactions in the absence of Fis for many of the mutants, Fis(−) reactions were performed with 15% ethylene glycol. Fig. 2a illustrates *in vitro* inversion reactions with Hin-wt and 5 representative hyperactive mutants. The presence of ethylene glycol enables a very low rate of a basal (no Fis) reaction with Hin-wt, but the inversion rate is only about 1% of the Fis(+) rate assayed without ethylene glycol. In general, Fis(−) inversion reactions performed with ethylene glycol better reflected their relative *in vivo* rates. *In vitro* inversion rates of all mutants (except for the catalytically-inactive Hin-R66D) were further stimulated by Fis, although in a few cases, Fis-activation was most evident in reactions lacking ethylene glycol.

DNA cleavage rates on supercoiled DNA substrates produced in Mg²⁺-deficient reactions containing 30% ethylene glycol were measured in the presence or absence of Fis (Fig. 2b). All mutants generated primarily double-strand cuts at the *hix* sites as evidenced by the lack

of an increase in nicked plasmids. Although most mutants generated predominantly double-strand cuts at both *hix* sites, some produced substantial amounts of single-*hix* site cleavage (linear products). Thus, the normally moderate coordination of cleavage between *hix* sites is sometimes disturbed by the mutations, but there are no examples where the normally high coordination of double-strand cleavage at each *hix* site is significantly altered.

The ability of the *Hin* mutants to form synaptic complexes in the absence of *Fis* and DNA supercoiling was evaluated after a 30 min incubation of *Hin* with 5'-radiolabeled 36 or 50 bp *hixL* oligonucleotides by native PAGE performed in the presence of 10% glycerol. *Hin*-wt generates a trace or no detectable synaptic complexes, and the mutants exhibit variable efficiencies (e.g., Fig. 3f). Cleavage of the *hix* sites within the synaptic complexes were determined by extracting the complexes from the gel in the presence of SDS and electrophoresing the radiolabeled DNA on a denaturing gel (e.g., Fig. 3g). Variable cleavage efficiencies by the mutants were observed that did not necessarily correlate with their efficiency of synaptic complex formation.

Mutations in the helix-E oligomerization interfaces

Mutations at 5 residues within the N-terminal half of helix-E, which spans the dimer and tetramer interfaces, cause *Hin* to become hyperactive (Fig. 3a and b). *Hin*-H107Y, A113T, and M115V are among the strongest *Fis*-independent mutants both in vivo (Table 1) and in vitro Fig. (3c–g), with *Fis*(–) inversion rates increased over 200-fold compared to WT for H107Y and M115V. *Hin*-M115V and M115I are as efficient as H107Y in forming stable synaptic complexes Fig. 3f) but, whereas most of the H107Y, A113T, and M115V recombinases are engaged in a covalent complex with the cleaved DNA, only about 10% of M115I complexes contain cleaved *hix* sites (Fig. 3g). The inefficiency of DNA cleavage by M115I synaptic complexes is also observed in *Fis*(–) cleavage reactions on supercoiled DNA in vitro and is consistent with its intermediate *Fis*-independent inversion activity in vivo (Table 1) and in vitro (Fig. 3c). Where tested (*Hin*-H107Y, A113T, and M115V), the cleaved synaptic complexes support efficient ligation upon addition of Mg^{2+} , and the products contain both recombinant and parental forms, indicating they are competent for subunit rotation (ref.¹⁸ and not shown).

Hin-M101I near the N-terminal end of helix-E is a weak hyperactive mutant with respect to *Fis*-independent activity (Table 1, Fig. 3c–e). M101I is inefficient at forming synaptic complexes, but about half of the complexes contained cleaved *hix* sites. *Hin*-F105V was isolated solely because of its SOS-induction phenotype. It does not display significant *Fis*-independent activity in vivo, and purified preparations of *Hin*-F105V exhibited low activity (Fig. 3c). DNA cleavage reactions with purified *Hin*-F105V in the presence or absence of *Fis* produced larger amounts of single-*hix* relative to double-*hix* cleaved products as compared to *Hin*-wt and other mutants (not shown), which may reflect its in vivo SOS-inducing phenotype. As elaborated in the Discussion, structural modeling indicates that Phe105 engages in alternative interactions during the dimer-tetramer transition, suggesting it may be a particularly important residue controlling *Hin* activity.

Mutations at Glu122, located in the C-terminal half of helix-E (Fig. 3a), were introduced based on the $\gamma\delta$ resolvase dimer-site I crystal structure showing H-bonding between the equivalent glutamic acid and Ser10 in one of the subunits⁴ and the reported hyperactivity of E124Q in both Tn3 and $\gamma\delta$ resolvases.^{20; 22} Different substitutions at *Hin* Glu122 give variable activities with E122Q generating the strongest hyperactive phenotype (Table 1 and Fig. 3c). Tyrosine and cysteine substitutions produce mild hyperactive phenotypes, whereas the properties of arginine, methionine, and valine substitutions are similar to wild-type, and tryptophan strongly inhibits inversion (data not shown). Both *Hin*-E122Q and E122Y generate substantial amounts of synaptic complexes, but only a very small fraction (~5%) of

the *hix* DNA within E122Y complexes is cleaved (Fig. 3f and g). Moreover, Hin-E122Y synaptic complexes formed with 36 bp *hix* fragments reproducibly migrate faster than those by Hin-E122Q or most other hyperactive mutants (most apparent after long electrophoresis times). The fully cleaved Hin-E122Q complexes ligate into both recombinant and parental DNA configurations upon addition of Mg^{2+} and dilution of ethylene glycol, confirming they are competent for subunit rotation and DNA exchange (not shown).

Mutations in the F88 hydrophobic core

Mutations within 6 residues, Leu64, Leu67, Leu77, Ile78, Leu81, and Phe88, whose side chains are oriented into a hydrophobic core (Fig. 4a and b; Fig. 8c, below), generally lead to strong Fis-independence and SOS induction in vivo (Table 1). We refer to this region as the F88 core because of the predicted role of its large side chain in organizing a hydrophobic pocket and the prominent role Phe88 has on Hin activity (elaborated in Discussion). This hydrophobic core includes residues from helices B, C, and D along with $\beta 3$ and $\beta 4$ (Fig. 1a). Hin-G68S is considered part of this group because Gly68 appears to be a key residue within the loop between helices C and D that seals the bottom of the pocket. All mutants except G68S have conservative hydrophobic changes, usually to smaller side chains. For example, substitution of Phe88 with the smaller hydrophobic residues Leu, Ile, and Val all lead to strong Fis-independent activity. However, introduction of a tryptophan at residue 88 inactivates Hin (data not shown), presumably because the larger side chain cannot be accommodated.

In vitro Fis(+) and Fis(−) inversion and cleavage rates of F88 core mutants are increased to variable extents over Hin-wt (Fig. 4c–e). However, none reach the robust Fis-independent activities of the most hyperactive helix-E mutants. The most active F88 core mutant in vitro, L64I, exhibits nearly 50-fold increased Fis-independent inversion rates over wild-type Hin. Leu64 is likely to play a particularly important role as it is predicted to connect the F88 hydrophobic core to the helix-E of the partner subunit near the center of the dimer interface (see Discussion). The efficiencies of synaptic complex formation and DNA cleavage within synaptic complexes also vary among the F88 core mutants (Fig. 4c and h).

Mutations in β -strand 4

Mutations at two histidines on $\beta 4$, which are predicted to be solvent exposed, give rise to hyperactive Hin proteins (Table 1 and Fig. 4c, f and g). His87 and His89 are on either side of Phe88 on the peptide chain and thus could indirectly affect the structure of the F88 pocket. They also are predicted to contact residues from $\beta 5$ and the loop connecting to Ser99, which is the major peptide backbone pivot point during the quaternary transition from dimers to synapsed tetramer (see Discussion).¹⁰ Hin-H87R, which was one of the most commonly isolated mutants, and H87K exhibit relatively strong Fis-independent activity and efficiently form cleaved synaptic complexes (Fig. 4c, g and h).

Other Hin gain-of-function mutants

Five Hin mutants, R48Q, N54S, V71A, K72E, and E80R, which are located in disparate regions of the protein, were isolated based on their mild Fis-independent activity in vivo (Table 1). Of the purified proteins, all but Hin-R48Q exhibit mild hyperactivity in the presence or absence of Fis (data not shown). Hin-R48Q, which is distinguished within the set due to its high in vivo SOS activity, was less active in vitro than Hin-wt. Hin-N54S and K72E form up to 40% synaptic complexes, and 40–75% of these have cleaved *hix* sites. Lys72 is within the interface formed between synapsed subunits in the activated tetramer.⁸ Lys72 also is modeled to be in position to form an inhibitory salt bridge with Glu116 from the same subunit during the transition from dimer to active tetramer (not shown but see Discussion). Asn54 is in a region that is involved in regulatory interactions in resolvases and

Sin,^{21; 29; 30; 31} but in our model of the Hin invertasome (in preparation), Asn54 is not near a protein interface. Asn54 is near the predicted path of the enhancer DNA in the invertasome model, but the most distinguishing property of Hin-N54S over wild-type is its enhanced synaptic complex formation on oligonucleotide substrates where the enhancer is not present. Val71 contacts a number of residues during the conformational transitions, some of which include sites where other hyperactive mutations have been isolated (see Discussion).

Hin-R66D, obtained by site-directed mutagenesis, was very efficient at forming synaptic complexes. An arginine at the equivalent position of Hin residue 66 is conserved among serine recombinases and functions in DNA catalysis.^{10; 25} As expected, Hin-R66D is catalytically inactive and the *hix* DNA remains uncleaved in the synaptic complexes. As elaborated in the Discussion, Arg66 is modeled to engage in multiple protein and DNA interactions during the dimer-to-tetramer transition.

Sensitivity of hyperactive mutant dimers to CHAPS

Previous studies have shown that the zwitterionic detergent CHAPS destabilizes the Hin dimer such that at high detergent concentration only the monomeric form is present in solution.³² Under low CHAPS conditions Hin-wt binds to a half-*hix* site (Fig. 5a) as a dimer due to cooperative interactions between subunits.³³ However, a small amount of monomeric complexes form as the concentration of CHAPS in the binding reaction is increased (Fig. 5b, Hin-wt panel). We asked whether differences in subunit interactions within dimers of hyperactive mutants could be revealed by evaluating the efficiency of monomer binding to half-*hix* sites as a function of increasing CHAPS concentration. Because dimeric interactions are reconfigured during remodeling into the synaptic tetramer, increased sensitivity to CHAPS may be related to enhanced formation of synaptic complexes.

Many of the hyperactive mutants exhibited increased sensitivity to CHAPS relative to Hin-wt (Fig. 5b, c, and d). Prominent monomer binding is observed by mutants containing changes within residues of the helix-E that are within the dimer interface, such as at Phe105, Ala113, and Met115. Hin-H107Y exhibits partially elevated monomer binding at the higher CHAPS concentrations, consistent with the more peripheral location of His107 within the dimer interface (see Discussion). Mutations within the F88 pocket also lead to robust monomer binding (Fig. 5d, and L64I and F88L in panels b and c). While the Leu64 side chain is modeled to be against the dimer interface in the Hin dimer, as is the leucine in the analogous position in resolvase, Phe88 and Leu77 are located well away from the interface. Thus, disruptions within the F88 pocket can destabilize dimer interactions even though the respective residues do not directly contact the partner subunit. Moreover, hyperactivating mutations at His87 also lead to increased monomer binding, suggesting these residues also indirectly affect dimer stability. Gain-of-function mutations within residues located in other regions of the Hin dimer structure that could directly or indirectly engage in dimeric interactions, such as Arg66 and Glu122, also stimulate monomer binding in high CHAPS (Fig. 5d) (see Discussion). On the other hand, mutations at surface-exposed residues located well outside of the dimer interface, like N54S and K72E, have little effect on monomer binding to a half-*hix* site in high CHAPS (Fig. 5d).

Importance of serine 99

Structural data from $\gamma\delta$ resolvase reveals that the peptide backbone undergoes dramatic changes at Gly101, which is immediately prior to helix-E (ref.¹⁰ and see Discussion) during formation of the tetramer. We therefore evaluated a set of Hin mutations at the equivalent residue Ser99 for their effects on DNA inversion and synaptic complex formation. None of the residue 99 substitutions exhibited detectable Fis-independent inversion activity over Hin-wt. Fis-activated inversion activities of mutants containing leucine, lysine, and arginine

substitutions at residue 99 were slightly diminished, and a cysteine substitution was slightly elevated in comparison with Hin-wt. On the other hand, a glycine, glutamic acid, or proline at position 99 inactivated Hin for recombination, but did not disrupt dimer binding to *hix* (Fig. 6a and data not shown). The inhibitory effect of the glycine was unexpected because a glycine is found in this position in the $\gamma\delta$ and Tn3 resolvases (Fig. 1a). Hin-S99K formed low amounts of synaptic complexes that migrated slightly faster than those of Hin-H107Y, F88L, and H89R, and the *hix* DNAs were uncleaved (Fig. 6b and c). Hin-S99R formed a low amount of synaptic complexes that migrated as a doublet with the DNAs in the faster migrating species being uncleaved and those in the slower migrating complexes being partially cleaved. We suspect that the difference in electrophoretic migrations reflects alternative conformations that are correlated with the cleavage-competent state of the synaptic complex. These data suggest that residue Ser99 plays an important function during the Hin dimer-to-tetramer transition.

Serine 98 is also modeled to be exposed in the Hin dimer and tetramer structures, but a glycine or lysine at this position had little detectable effects on Hin activity. Residue 97 is a threonine whose side chain is predicted to be oriented into the F88 pocket. Hin-T97A is inactive for recombination, probably because it disrupts the protein conformation. Ala100 is predicted to define the start of helix-E, and threonine, cysteine, or lysine mutations at this position all strongly disrupt Hin-catalyzed inversion activity (S. Merickel, E.R.S., J.K.H., and R.C.J., unpublished).

Combinatorial mutants involving residue 99

S99K, which creates a Hin mutant that weakly forms synaptic complexes and appears to inhibit tetramers from adopting a DNA cleavage-competent configuration, was combined with hyperactivating mutations at other locations to investigate epistatic relationships. A Hin-F88L/S99K double mutant rapidly generates synaptic complexes that migrate slightly faster than those formed by Hin-F88L alone (Fig. 6a and b). Unlike many of the hyperactive mutants, including Hin-H107Y, synaptic complex formation by Hin-F88L/S99K occurs efficiently without glycerol in the gel matrix, and the *hix* DNA remains uncleaved (Fig. 6c and d). The ability of Hin-F88L/S99K to efficiently assemble stable uncleaved synaptic complexes was confirmed by the addition of the active site mutation S10G. Hin-F88L/S99K/S10G remains capable of efficiently assembling tetrameric synaptic complexes (Fig. 6a). This ability to assemble catalytically inactive synaptic complexes contrasts with Hin-H107Y, where addition of S10G abolishes formation of detectable synaptic complexes (Fig. 6a).¹⁵

Addition of S99K to H89R also increases efficiency of synaptic complex formation, and the resulting Hin-H89R/S99K complexes are uncleaved and migrated faster (Fig. 6b and c). On the other hand, addition of S99K to Hin-H107Y is not sufficient to block formation of *hix*-cleaved synaptic complexes, but rates of DNA cleavage are reduced (Fig. 6a and d). Addition of S99E to Hin-F88L and to Hin-H107Y effectively blocks synaptic complex formation, but dimer binding to *hix* is not affected (not shown).

Discussion

The Hin recombinase must transition through many conformational states beginning with the catalytically-inactive dimer bound to individual *hix* recombination sites through to the activated synaptic tetramer. Synapsis of the two Hin-bound *hix* sites and remodeling of wild-type Hin occur at and require the function of the Fis-bound enhancer DNA segment. Once all four DNA strands are cleaved by the activated Hin tetramer, DNA strand exchange proceeds by rotation of the newly associated subunits over the flat interface created within the tetramer. Our genetic and biochemical studies of Hin, combined with similar studies in

other serine recombinase systems, indicate that a large number of interactions intrinsic within the recombinase structure function to restrict formation of the active tetramer. Most of the gain-of-function mutations isolated in Hin can be explained by directly or indirectly destabilizing the dimer or other transition intermediates and/or by stabilizing the active tetramer. The biochemical properties resulting from specific amino acid residue substitutions are discussed below in the light of structural models of intermediates along the pathway to formation of the recombinationally-competent synaptic tetramer. Taken together, our studies on Fis-independent mutants identify both intrasubunit and intersubunit interactions that form energetic barriers to these structural transitions, which are normally overcome by the action of the Fis/enhancer system.

Our interpretation of the functional consequences of the mutations relies heavily on structural modeling of conformational intermediate steps along the pathway to the activated synaptic tetramer and therefore should be considered with appropriate caution. The model of the starting Hin dimer was based on the $\gamma\delta$ resolvase dimer (1GDT) bound to DNA.^{4; 8; 32} Crystal structure snapshots have identified inactive tetrameric structures for the catalytic and oligomerization (helix-E) domains of TP901 integrase (3BVP)¹¹ and one pair of subunits in a $\gamma\delta$ resolvase tetramer (2GM5),⁹ which were both crystallized without DNA and likely represent a similar intermediate step in the assembly of the active tetramer. Similar Hin tetramers have been modeled based on the TP901 (this paper) and a symmetrized form of 2GM5.⁸ This tetrameric state is referred to as the 'pre-cleaved' or 'open tetramer,' reflecting the less compact structure as compared to the final DNA-cleaved or 'closed' tetramer, where the 5' ends of the cleaved DNA are covalently-associated with Ser10. The latter tetramers, which are in a conformation competent for subunit rotation about the flat interface, were modeled from the $\gamma\delta$ resolvase-Hin chimera crystal structure (2GM4)¹⁰ as described.^{8; 14} The subunit designations and interfaces for the dimer and tetramer conformations are diagrammed in Fig. 7a. A body of site-directed crosslinking data supports the Hin models of the dimer and active tetramer conformers.^{8; 14; 15; 32} Conformational intermediates between the dimer, open tetramer, and closed tetramer were modeled using the Yale Morphing Server.³⁴ As elaborated below, many of our Hin hyperactive mutants are able to form DNA-cleaved tetramers without the assistance of the Fis/enhancer system. However, some mutants (F88L/S99K, H89R/S99K, M115I, and E122Y) form stable tetramers that are blocked at a step preceding DNA cleavage, and thus may be trapped in an open tetramer-like conformation.

Amino acid residue changes within helix-E

A large class of Hin hyperactive mutants contained changes within the oligomerization helix-E. This is understandable since residues within the N-terminal 2/3 of helix-E participate in almost all the intersubunit contacts in both the dimer and tetramer and are dramatically remodeled during formation of the synaptic tetramer. In the DNA-cleaved tetramer helix-E residues comprise most of the newly created synaptic interfaces (between subunits A and B and subunits D and C) as well as the rotating interface (between subunit pairs AB and DC) (Fig. 7b). As expected, most changes at residues along helix-E that make intersubunit interactions or contacts to the catalytic domain of the same subunit are detrimental to Hin activity, particularly those changes that introduce polar groups,^{32; 35} but a subset of structurally conservative changes lead to hyperactivity in Hin and other serine recombinases.^{18; 19; 20; 21; 22; 25; 36}

Histidine 107—Hin-H107Y is one of the strongest Fis-independent mutants we have isolated in Hin, and its biochemical properties have been studied in detail.¹⁸ Hin-H107Y readily assembles synaptic complexes with all four DNA strands cleaved. Crosslinking studies have shown that the Hin-H107Y tetramer structure closely fits with the DNA-

cleaved tetramer structures of resolvase and that the complex supports rotation of the AB subunit pair relative to the DC subunit pair.^{8; 14} The Hin models predict that a tyrosine substitution at residue 107 will both disrupt the dimer and stabilize the tetramer. In the Hin dimer model, His107 (subunit A) is oriented such that the imidazole ring could hydrogen bond with its partner subunit (D) at Asp93 or Ser94 and may be in van der Waals contact with Ile95 (Fig. 7c). A tyrosine is predicted to impose a modest steric clash with the partner subunit, or be reoriented into the solvent, and thus destabilize the dimer, consistent with the mildly increased sensitivity of the Hin-H107Y dimer structure to CHAPS (Fig. 5b–d). Around the open tetramer intermediate, the His107 rings from newly synapsed subunits (A–B and C–D) closely pass by each other and then become well stacked and likely stabilize the DNA-cleaved tetramer (Fig. 7d; see Supplemental Movie 1). The tyrosine aromatic ring in the hyperactive mutant may afford greater stability to the active tetramer due to increased pi-stacking forces.^{37; 38} The importance of a histidine or aromatic amino acid at Hin residue 107 is emphasized by the fact that six other substitutions generate catalytically-inactive mutants.^{32; 35}

Interestingly, the residue at the Hin 107 position is not well conserved among serine recombinases, yet is a frequent target of hyperactivating mutations. Similar histidine-to-tyrosine gain-of-function changes have been isolated in the DNA invertases Gin and Cin, along with a weaker phenylalanine mutation in Cin.^{19; 36} Tn3 resolvase and TP901 integrase have a threonine at the analogous position with an isoleucine in Tn3 generating hyperactivity²⁰. A recent X-ray structure of a Sin hyperactive mutant with a Gln to Arg change at the analogous position²¹ revealed the arginine side chains in a pi-stacking interaction across the synaptic interface of the tetramer in a similar manner as His107 in our Hin model (P. Rice, personal communication).

Methionine 115—Our hyperactivating mutations at Met115 also are predicted to destabilize, but not completely disrupt, the dimer conformation and affect stability of the tetramer. The dimer model shows that Met115 buries about 80 Å² of surface area as it inserts into a cleft formed by Val71, Leu64, Asp65, and Leu74 of the partner subunit (Fig. 7e). A valine or isoleucine would likely not optimally fit, resulting in a less stable dimer, consistent with the results of the CHAPS assay (Fig. 5b–d). During the dimer-tetramer transition the Met115 side chain must move out of the dimer subunit cleft and eventually slide into a new pocket bordered by Ala100, Met101, and Phe104 within the synapsed subunit (Fig. 7f and Supplemental Movie 2). Hyperactivating mutations to smaller hydrophobic residues on the wall of the cleft also have been obtained at Val71 (alanine) and Leu64 (isoleucine), consistent with a role of the Met115-inserting cleft in stabilizing the dimer structure. In the model of the final DNA-cleaved tetramer complex, the methionine side chain buries about 100 Å² within the synaptic interface (subunits A–B or D–C). The residue at position 115 also comprises part of the rotating interface so maintaining the hydrophobic character is expected to be critical for recombination activity.

Hin-M115V is extremely hyperactive, readily forming DNA-cleaved synaptic complexes without Fis. This mutation also creates a strong Fis-independent phenotype in Gin.³⁹ On the other hand, Hin-M115I is considerably less hyperactive for recombination. Hin-M115I readily forms tetrameric synaptic complexes that exhibit an altered electrophoretic migration relative to those of Hin-M115V or H107Y and are impaired in progressing to the DNA-cleaved state. We suspect that the larger and less flexible isoleucine side chain inhibits the transition from the pre-cleaved open tetramer to the cleavage-competent closed tetramer conformation.

Methionine 101—The residue corresponding to Met101 in Hin is well conserved among serine recombinases, being a methionine in all the examples listed in Fig. 1A. M101I is a

relatively weak hyperactivating mutant in Hin, and the same substitution has been reported to lead to hyperactivity in $\gamma\delta$ and Tn3 resolvases, and Sin.^{21; 22; 24} The Met101 side chains at the beginning of helix-E are close to one another in the dimer (Fig. 7g), as demonstrated experimentally by the formation of M101C disulfide-linked dimers³², and would be one of the first residues of opposed dimers to contact each other in the early steps of synaptic complex formation. The Met101 side chain also must undergo considerable changes during the transition into the open and then closed tetramer conformations. In the open tetramer, Met101 is modeled to make a number of “trans-diagonal” contacts with the N-terminal end of helix-E of the trans-diagonal subunit (subunits A–C and D–B) including the opposing Met101 (Fig. 7h). The trans-diagonal contacts rearrange during the steps from open to closed tetramer configurations to position Met101 at the edge of the rotating interface (subunits A–D and B–C) (Fig. 7f). Nevertheless, a cysteine at position 101 remains within an Å8 crosslinking distance with the trans -diagonal subunit in certain rotational conformers.¹⁴

Alanine 113—The Hin-A113T substitution leads to surprisingly strong hyperactivity, but unlike the residues discussed above, Ala113 is not predicted to make intersubunit contacts. Rather, the side chain is oriented into the core of the same subunit (Fig. 7c). A threonine at this position would be expected to clash with Val75 on helix-D and in turn may induce a modest disruption to the dimer, as implied from the mild CHAPS sensitivity (Fig. 5d). During the transition between the open and closed tetramer complexes, the residue 113 side chain becomes sandwiched between Val71 and Lys72 from the same subunit as the D helices pivot in relation to the E helices (see below and ref.¹⁰). Thus, the properties of Hin-A113T may be related to the modest hyperactivity of Hin mutants V71A and K72E.

The helix-E phenylalanine triad and the Phe88 pocket

Identification of Hin-F105V together with a set of mutants with hyperactivating changes at and around Phe88 identifies a previously unappreciated intrasubunit connection that regulates the activity of Hin. Phe105 is a member of a triad of phenylalanines (residues 104–106) emanating from helix-E. The Phe105 ring in the dimer is inserted within a hydrophobic cleft in the same subunit referred to as the Phe88 pocket. As elaborated below, during formation of the active synaptic tetramer Phe105 rotates out of the Phe88 pocket and Phe106 subsequently inserts into the pocket (Fig. 8a and b, Supplemental Movie 3). This Phe105–106 intramolecular relay, together with a dynamic series of intermolecular interactions by Phe104 and Phe105, appears to be an integral part of the conformational transitioning from inactive to active complexes. The remarkably strong regulatory phenotypes of the Phe88 core mutants that are modeled to directly or indirectly affect this intramolecular relay indicate that this system is a critical feature controlling Hin activity, and we propose that a similar relay of hydrophobic residues likely performs a similar function in the other serine recombinases.

Figure 8a shows a surface representation of the catalytic domain of the dimer subunit model that depicts the pocket into which the Phe105 ring inserts. Phe88 defines the back wall of the pocket and is predicted to play a prominent role in organizing the 12 branched chain amino acid residues surrounding it (Fig. 8c). Including Phe88, changes in 7 of these residues lead to Fis-independence (Table 1 and Fig. 4). Ile78, where a valine causes a strong hyperactivating mutation, is on one surface bordering the inserted Phe105 ring (Fig. 8c). Leu64 is on the other side and connects the Phe88 pocket with helix-E of the dimer partner at Met115 and Ala111. The very strong Fis-independent phenotype of the isoleucine substitution at residue 64 is consistent with the destabilization of the dimer (Fig. 5b–d), and the smaller side chain may facilitate sliding of the Met115 side chain of the dimer subunit past residue 64 during formation of the open tetramer intermediate (Fig. 7e and f). Other

positions of hyperactivating mutations are Leu77 and Leu81, located behind and in contact with Phe88, and Leu67 plus Gly68, which help define the bottom of the pocket. In most cases substitution of a smaller hydrophobic residue was isolated, which may “loosen” the pocket to facilitate the switching of phenylalanines from helix-E. Even an isoleucine in place of Leu77, which is located behind Phe88, has a destabilizing effect on the dimer (Fig. 5d), presumably by indirectly affecting the structure of helix-E through the insertion of Phe105. Gain-of-function mutants with changes within the region analogous to the Hin F88 core have been reported in Tn3 resolvase and Sin.^{20; 21} These include mutations at equivalent hydrophobic residues and even mutations that correspond to the Hin hyperactivating mutation G68S (G68S in Sin and G70A/C in Tn3 resolvase).

During the remodeling from dimers to active synaptic tetramer, Phe106 is predicted to rotate into the Phe88 pocket (Fig. 8b) and the excluded Phe105 ring becomes stacked on the Phe105 ring from the diagonally opposed subunit (subunits A–C and D–B) (Fig. 7d and Supplementary Movie 3). The trans-diagonal stacking of Phe105 rings across the rotating interface could function as a ratchet to help restrict subunit rotations to steps of 180°. We postulate that the Hin-F105V mutation may enhance initiation of the dimer-tetramer transition because the valine may more easily slip out of the F88 pocket, consistent with the mutant dimer being very sensitive to CHAPS (Fig. 5d). However, because Hin-F105V exhibits relatively low activity in cleaving and recombining DNA or forming stable tetramers, it may have difficulty transitioning into a tetrameric synaptic complex. Its SOS induction phenotype (Table 1) could be correlated with its uncoupled DNA cleavage phenotype, leading to an accumulation of products cut at only one *hix* site, or perhaps be due to enhanced processive subunit rotation when DNA cleavage at both *hix* sites does occur. Most substitutions at Phe105 and 106 lead to recombination-defective DNA invertases; for example, a leucine or isoleucine in Hin or a tyrosine in Gin at the Phe106 position results in catalytically-inactive recombinases.^{19; 32; 35}

The Phe104 rings are positioned across from each other in the dimer structure model, consistent with the formation of disulfide-linked dimers by Hin-F104C.³² During formation of the active tetramer, the Phe104 side chains are modeled to move out of a pocket formed by Ile95, Met101 and Phe104 in the partner subunit of the dimer, engage in a series of intermediate contacts with the diagonally-opposed synapsing subunit (A–C and D–B), and end up in the closed tetramer making extensive contacts with the newly synapsed partner subunit (subunits A–B and D–C; (Fig. 7f)) plus comprise part of the rotating interface. Not surprisingly, a variety of substitutions in place of the phenylalanine at residue 104 result in inactive mutants.^{32; 35; 40}

The triple Phe motif in helix-E is also found in Gin, but different hydrophobic residues are present in the other serine recombinases (Fig. 1). Hyperactive mutants of Gin (phenylalanine to valine),¹⁹ Tn3 (valine to methionine),²⁰ and Sin²¹ (isoleucine to valine or threonine) have been isolated at the analogous Hin Phe105 position. Clefts analogous to the modeled Phe88 pocket in Hin are also present in X-ray structures of different conformers of serine recombinases. A somewhat larger cleft than modeled for Hin is present in the $\gamma\delta$ resolvase dimer crystal structure (1GDT), and both a valine (equivalent position to Hin Phe105) as well as a leucine (equivalent position to Hin Met109) from helix-E are inserted. The TP901 precleaved tetramer structure (3BVP) has a very prominent cleft together with a phenylalanine and leucine in similar positions as Phe105 and 106 in the Hin open tetramer, respectively. Additionally, the DNA-cleaved resolvase tetramer structure (1ZR4) has Val108 (analogous to Hin Phe106) inserted into its pocket. These similarities suggest that the triad of hydrophobic residues on helix-E, together with the surface pocket within the catalytic domain, is likely to have a major role in controlling the structural transitions in other serine recombinases.

A hinge at residue 99

We found that substitutions at Ser99 gave a variety of phenotypes ranging from those which completely inactivate Hin-catalyzed recombination (glycine, glutamic acid, and proline), to those which exhibit near wild-type activity (leucine, cysteine), to those which weakly enhance formation of a tetrameric synaptic complex that is unable to cleave DNA (lysine and arginine). The latter phenotype was particularly evident when S99K was coupled with the hyperactivating mutations F88L or H89R, which are themselves weak to moderately active in forming synaptic complexes. The Hin-F88L/S99K and H89R/S99K double mutants very efficiently form synaptic complexes that are largely blocked for DNA cleavage (Fig. 6). The uncleaved synaptic complexes blocked by S99K have altered electrophoretic migrations relative to cleaved synaptic complexes formed by most other mutants, consistent with a different nucleoprotein complex conformation. Both Hin-F88L/S99K and H89R/S99K could efficiently cleave and recombine supercoiled DNA under Fis-activating conditions, suggesting that the Fis/enhancer system functions to advance remodeling of tetramers to the chemically active stage.

In their comparisons of the dimer and various tetramer conformers of $\gamma\delta$ resolvase crystal structures, Steitz and co-workers noted that a rotational hinge exists at Gly101, corresponding to Ser99 in Hin (Fig. 8a–c), where the peptide backbone angles change during the scissor-like opening of the E helices plus rotation of the catalytic domain relative to the E helices.¹⁰ Moreover, they proposed that a glycine to serine would force the peptide backbone angles into a configuration that would favor the tetramer. Supporting this idea, G101S is one of the several activating mutations used to generate the resolvase tetramer crystal structures, and Ser101 is present in the $\gamma\delta$ resolvase-Hin chimera in which a partially remodeled tetrameric structure was crystallized.^{9; 10}

The polypeptide segment at Ser99 was difficult to accommodate in the Hin dimer model, and the backbone angles at Ser99 remained outside of the allowed Ramachandran constraints in the final refined dimer model but not in the tetramer models. Thus, S99G in Hin may effectively stabilize the dimeric configuration, resulting in too great an activation barrier to transition into the tetramer even with Fis activation. A proline would presumably inhibit the peptide backbone rotation required for tetramer assembly. Along with affecting peptide backbone angles, arginine, lysine, or glutamic acid residues at position 99 could form inhibitory H-bonds with surrounding residues at various steps in the assembly pathway leading to the observed blocks in the progression to the DNA-cleaved tetramer. As noted above, mutations in resolvases and Sin at or close to the hinge residue also affect their ability to assemble synaptic complexes without accessory factors.^{20; 21; 22; 23; 24} In addition, ϕ C31 integrase mutations at Val129, whose position is equivalent to Gly101 in Hin, have been reported to generate uncleaved synaptic complexes.⁴¹

Hin hyperactivating mutations were isolated in two surface residues, His87 and His89, which are located in the short β 4 strand that runs antiparallel to the loop adjacent to Ser99 and the β 5 strand, respectively. Changes at these residues, particularly to highly polar side chains, may indirectly affect the integrity of the Ser99 hinge region (Fig. 8a and b). A leucine at residue 89 is modeled to impose adjustments to residues in β 5 that are connected to His107 across the dimer interface. Hin-H87R was one of our most frequently isolated and most active mutants in both the Fis-independent and SOS screens. Other than an alanine to a threonine change in Tn3 resolvase²⁰ at the equivalent of Hin His89, hyperactivating mutants have not been reported at these positions in the other serine recombinases. However, the residue at position 89 in wild-type Gin is already an arginine, which causes a mild hyperactive phenotype in Hin.

Additional intersubunit contacts controlling Hin activity

On one side of the $\gamma\delta$ resolvase dimer structure the highly conserved residue Glu124 is H-bonded to the active site Ser10 and conserved Arg68 of the partner subunit (see Fig. 8d depicting the Hin dimer model).⁴ Substitutions of the equivalent residues Glu122 and Arg66 in Hin lead to strongly enhanced tetramer formation. Modeling of the conformational steps leading to the DNA-cleaved tetramer complex indicates that connections between Glu122 and Ser10 in the dimer would need to be released almost immediately. The Glu122 side chain then passes close to a number of residues, including Lys63, on the partner subunit at conformers around the open tetramer, but is rotated away from the catalytic domains in the final closed tetramer. Thus, it is understandable that various amino acid substitutions at Glu122 have very different phenotypes. Whereas a glutamine gives a strong Fis-independent phenotype and efficiently forms cleaved synaptic complexes, most substitutions give a weak Fis-independent phenotype; some also exhibit reduced Fis-activated inversion rates and a tryptophan abolishes recombination. In vitro analysis of Hin-E122Y revealed that it could efficiently form tetrameric synaptic complexes, but these exhibited an altered migration and were unable to advance to the cleavage competent structure in the absence of Fis. These properties suggest that tetramers formed by Hin-E122Y may have difficulty transitioning past the open conformation. Arg66 is almost certainly a critical residue for DNA catalysis as resolvase Arg68 is bonded to the non-esterified oxygen at the cleavage site in the cleaved tetramer structures.^{10; 25} We postulate that the loss by Hin-R66D of a salt bridge between Arg66 and Glu122, whose presence would stabilize the dimer, contributes to its enhanced ability to form tetrameric synaptic complexes. Even though the *hix* DNA is not cleaved by Hin-R66D because of the critical role of this residue in catalysis, the electrophoretic migration of the synaptic complexes suggests they are in a closed tetramer conformation. The identical E124Q mutation in resolvase also leads to hyperactivity,^{20; 25} and R68H (equivalent position to Hin Arg66) was one of the mutations present in the resolvase tetrameric crystal structure without DNA (2GM5),⁹ so these residues are also likely to be playing a general role in controlling conformational changes in serine recombinases.

Materials and Methods

Mutagenesis of Hin and screen for gain-of-function mutations

The entire coding sequence of Hin from pMS571 (pBR322, *tacPO-hin*)⁵ was randomly mutagenized by PCR containing 100 μ M MnCl₂ and Taq DNA polymerase to generate mutant libraries. Approximately 100 libraries were created from unique PCRs that were then cloned into empty pMS571 vector backbones. A few Hin mutants were isolated after treatment of pMS571 with hydroxylamine or growth in a *mutD* strain.⁴² To screen for mutants with increased Fis-independent inversion activities, libraries were transformed into the Fis⁻ tester strain RJ1652, or RJ1652 containing a pACYC184 based plasmid encoding a defective Fis mutant (D20K, V22H, V22Q)⁴³ in hopes of isolating potential allele-specific Fis suppressor mutants. RJ1652 is *E. coli* F' *proAB*⁺ *lacI*^{qs} *lacPL8*, λ *fla-lac406 off*, *fis::kan-767* Δ (*pro-lac*) *recA56 srl rpsL ara*; Hin-catalyzed inversion switches the invertible segment in λ *fla-lac406* such that *lacZ* is expressed (Fig. 1b).⁴⁴ Transformants were plated on selective MacConkey-lactose agar media (Difco BD), and colonies were screened between 24–48 h for increased red color formation or sectoring as compared to Hin-wt. Approximately 3000 colonies were screened per transformation of each library, for a total of 300,000 colonies. Plasmid DNA from colonies with faster color development was isolated and re-transformed to confirm the phenotype. To screen for mutants with increased SOS activity, libraries were transformed into RJ1657 (F' *proAB*⁺ *lacI*^q *ZU118* NK8027: Δ (*lac-pro*) *rpsL thi* Δ (*gal-λG*) *pL-lacZ cI*⁺₄₃₄ *pRS7*; NK8027 containing the SOS-controlled λ *pL-lac* fusion^{45; 46} (Fig. 1c) was provided by N. Kleckner, Harvard University). About 75% of mutants obtained from PCR reactions contained one or two additional silent or substitution

mutations. Multiple mutations were separated such that each studied mutant, except for intentionally constructed combinations, contains a single amino acid substitution.

In vivo assays of Hin activity

Hin mutant plasmids were transformed into the *fis*⁺ inversion tester strains RJ1471 (RJ1652 *fis*⁺) or RJ3635 (F' *proAB*⁺ *lacI*^{qs} *lacPL8* Δ *lac proA::IS10 recF::kan* *lfla-lac406* off), the *Fis*-independent inversion tester strain RJ1652, or the SOS tester strain RJ1657 and plated on MacConkey-lactose agar media containing ampicillin to obtain well isolated colonies. Color development was scored between 15 and 48 h of growth at 37°C as described in the Table 1 legend.

Purification of Hin protein

Hin mutant proteins were obtained using a native purification procedure for all in vitro activities reported in this paper. Mutant derivatives of pRJ1518 (pET11a-*hin*),⁴⁷ were transformed into RJ3387 (BL21 (DE3) *fis::kan-767 endA::Tn10*) and grown in LB to OD₆₀₀ = 0.7–1.0. Cultures were cooled to 15°C, IPTG added to 0.2 mM, and incubated overnight at 10°C. Cells were harvested, resuspended in HB+0.15 M NaCl (50 mM HEPES, pH 7.5, 4 mM CHAPS, 20% glycerol, 1 mM EDTA, 5 mM DTT, and 150 mM NaCl), lysed using a French press, and centrifuged 30 min at 15K in a SS34 rotor. An additional 350 mM NaCl was added to the supernatant which was mixed with 2 ml of heparin-Sepharose slurry (GE-Amersham) equilibrated in HB + 0.5 M NaCl and allowed to batch bind at 4°C for 1–2 hours. The resin was added to a column and washed extensively with HB+0.5 M followed by 0.6 M NaCl. Protein was eluted with HB+1.2 M NaCl, peak fractions pooled, and dialyzed into HB+1 M NaCl and 50% glycerol.

In vitro recombination, binding, and synaptic complex assays

Hin-catalyzed DNA cleavage and inversion reactions were performed as described using the supercoiled plasmid substrate pMS551, which contains 2 *hixL* sites and the enhancer at the native spacing of 99 bp from *hixL1*.^{5; 32} Cleavage and inversion rates were calculated using data points within the linear range of activity, and results were averaged from at least 3 experiments and presented as % cleaved plasmids/min (cleaved at one or both *hix* sites) and as inversions/molecule/min.⁴⁴ In cases where a lag in activity was observed, the rates were calculated from time points where the product was increasing linearly.

Hin dimer binding and synaptic complex formation on 5'-³²P-labeled 36 bp or 50 bp *hixL* substrates were performed as described.¹⁸ Native gel electrophoresis to evaluate synaptic complex formation was typically performed (including all figures in this paper except Fig. 6a) with 10% glycerol in the polyacrylamide gel. The differences in electrophoretic migration of synaptic complexes observed for some mutants depended on DNA fragment length, with uncleaved synaptic complexes generated on 36 or 50 bp *hix* fragments migrating faster or slower than cleavage-competent synaptic complexes, respectively. Mutant and substrate-dependent migration differences of dimer and tetramer DNA complexes have been noted in other studies.^{18; 21; 25} DNA cleavage within Hin synaptic complexes was evaluated by extracting the complex in the presence of 1% SDS followed by electrophoresis in denaturing polyacrylamide gels. For CHAPS sensitivity assays, 2–16 mM CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) was included in the binding reaction that otherwise contained 20 mM HEPES (pH 7.5), 80 mM NaCl, 5% glycerol, 1 mM DTT, 0.1 mM EDTA, 40 µg/ml dI•dC, and 5'-³²P-labeled 183 bp *hixL* half-site substrate (amplified from pMS570⁵) plus 10–30 nM Hin dimer, depending on the mutant preparation. Electrophoresis was through 6% native polyacrylamide gels in 0.5× TBE.

Hin models

Hin models of the DNA-bound dimer based on pdb codes 1GDT⁴ (catalytic domain + helix-E) and DNA-cleaved tetramers based on 1ZR4¹⁰ and 2GM4⁹ and the Hin DNA binding domain 1IJW⁴⁸ have been described previously.^{8; 14} A new model of the open (precleaved) Hin tetramer was constructed based on the 2Å. 1TP901 integrase tetramer 3BVP.¹¹ TP901 integrase is 38% identical to Hin over the N-terminal 134 amino acid residues with a 9 amino acid insertion between analogous Hin residues 11 and 12 and an additional 2 amino acids between residues 54 and 55 (Fig. 1a). Our previous Hin-2GM5 subunit model⁸ was aligned to each of the four TP901 catalytic domains using LSQMAN and then subjected to energy minimization using CNS.⁴⁹ Because the TP901 integrase tetramer structure is symmetric, a Hin model of the open tetramer derived from this structure is probably better than our previous model, which was based on only one the synapsed subunit pair of the asymmetric crystal structure 2GM5 of $\gamma\delta$ resolvase.⁹ The loop segment prior to helix-E did not required manual adjustment and exhibits better geometry⁵⁰, and the helix-E interface is shifted slightly in the TP901-based model relative to the 2GM5-based model. The Yale Morph Server³⁴ was used to generate 26 intermediate structures between a presynapsed dimer complex, the open tetramer and the closed (DNA-cleaved) tetramer. Areaimol in CCP4⁵¹ was used to calculate buried interfaces and PDBsum⁵² used to identify protein-protein contacts and calculate solvent excluded surface areas of individual residues. Structure figures were prepared using PyMol.⁵³

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Serine recombinases and the Hin reaction. (a) Sequence of the conserved catalytic domain and helix E region of Hin and other serine recombinases. Gin is a related DNA invertase, $\gamma\delta$, Tn3, and Sin resolvases catalyze deletion, and the TP901 integrase catalyzes phage integration/excision. Common and similar residues are highlighted in blue and light blue shading, respectively, and the active site serines are demarked with black shading. Residues with green shading highlight locations where single substitutions have been reported to confer hyperactivity in the listed recombinases. Yellow shading on resolvases and Sin signify residues where hyperactivity is observed only in the presence of a second mutation. For resolvases and Sin, hyperactivity typically reflects recombination of substrates lacking

accessory sites on one of the two recombination sites. Crystal structures of dimeric and tetrameric $\gamma\delta$ resolvase, and Sin (regulatory tetramer) and TP901 (tetramer) are available; the designated secondary structure is from the $\gamma\delta$ resolvase tetramer (1ZR4.pdb), which fits well with secondary structure predictions and modeling of Hin. (b) Flagellin controlling locus in *Salmonella enterica* serovar *Typhimurium*. A 996 bp invertible DNA segment contains the *hin* gene and an outward facing $E\sigma^{28}$ promoter, which transcribes the *fljB* (H2) flagellin gene and a repressor of an unlinked flagellin gene in one orientation. The drawing schematically depicts the λ *fla-lac406* reporter construct used to monitor Hin-catalyzed inversion in vivo where the *hin* gene is inactivated, and *lacZ* is substituted in place of the repressor. *hix* recombination sites are denoted in green and blue. (c) Schematic representation of the reporter used for monitoring SOS induction by Hin. (d) Schematic representation of the Hin recombination pathway on a plasmid substrate. Hin binds to the *hix* sites and Fis dimers bind to the recombinational enhancer. The HU bending protein facilitates assembly of the invertasome in which DNA cleavage, subunit rotation, and ligation occur to invert the intervening segment of DNA (adapted from ref.¹⁴). (e and f) Structural models of the Hin dimer based on $\gamma\delta$ resolvase (1GDT) and the rotationally competent, DNA cleaved, Hin synaptic tetramer based on $\gamma\delta$ resolvase (2GM4). The locations of regulatory mutants discussed in this report are designated with red spheres and the catalytic serine 10 with a black sphere.

Fig. 2.

In vitro DNA inversion and cleavage reactions on supercoiled plasmids by wild type and representative hyperactive *Hin* mutants. (a) In vitro DNA inversion reactions. Fis and 15% ethylene glycol (EG) were added as designated. After 2 min *Hin* reactions, plasmids were digested with *Pst* I and *Hind*III to distinguish the orientation of the invertible segment, as illustrated in the drawing, and electrophoresed in an agarose gel. (par) designate products representing the parental orientation; (inv) designate products representing the inverted orientation. (b) *Hin*-catalyzed DNA cleavage reactions. The products of *Hin* cleavage reactions (2 min) were treated with proteinase K and SDS and electrophoresed in an agarose gel. The locations of the supercoiled (SC) substrate, nicked plasmid, linear plasmid containing a single double-strand break, and the vector backbone plus invertible (inv) segment resulting from two double-strand breaks are designated.

Fig. 3.

Hyperactivating Hin mutations in helix-E. (a) Location of the helix-E mutations (red spheres) on the Hin dimer and (b) DNA-cleaved tetramer models. (c) Summary of in vitro activities by helix-E hyperactive mutants. Inversion rates with and without Fis are given as inversions/plasmid molecule/min, and cleavage rates with and without Fis were calculated from the number of double and single cleaved plasmids generated per minute relative to the total DNA, excluding the open circular form. Synaptic complex (SC) formation was determined from the amount of synaptic complexes relative to the total Hin-bound DNA (panel (f)). (+++) indicates >75% of the bound DNA is assembled into synaptic complexes, (++) indicates 30–75%, (+) indicates 10–30%, and (-/+) indicates ≤5 %. SC cleavage refers

to *hix* site cleavage as determined by extracting synaptic complexes in the presence of SDS and quantifying the amount of cleaved DNA by denaturing PAGE (panel (g)). (+++) indicates >75% of the *hix* sites were cleaved, (++) indicates 25–75% cleaved, (+) indicates 5–25% cleaved, -/+ indicates <5% cleaved. All numbers are mean values from at least 3 experiments. (d and e) Representative in vitro DNA inversion reactions in the presence or absence of Fis. (f) Representative synaptic complex assays formed with 36 bp *hixL* sites. Variable migrations of the dimer-bound *hix* complexes are commonly observed and appear to reflect electrophoretic differences caused by the amino acid substitutions and perhaps from conformational differences. The slightly faster migrations of the tetrameric synaptic complexes (SC) formed by Hin-M115I and Hin-E122Q relative to the other mutants are resolved better upon longer electrophoresis (e.g., Fig. 6b). (g) DNA cleavage by Hin mutants within gel-isolated synaptic complexes. Synaptic complexes from native polyacrylamide gels as in panel (f) were extracted in the presence of SDS and subjected to denaturing PAGE. Uncleaved (50 nt) and cleaved (26 nt) 5'-³²P-labeled products are denoted.

Fig. 4.

Hyperactivating Hin mutations in the Phe88 hydrophobic core and β -4 strand region. (a) Location of the Phe88 core and β -4 mutations (red spheres) on the Hin dimer and (b) DNA-cleaved tetramer models. (c) Summary of in vitro activities by the mutants. Activities are designated as in Fig. 3c. (d–g) Representative in vitro DNA inversion reactions in the presence or absence of Fis. (h) Native polyacrylamide gel of synaptic complex reactions.

Fig. 5. Stability of Hin mutant dimers to CHAPS as evaluated by *hixL* half-site binding. (a) Sequence of the *hixL* half-site substrate used in the DNA binding assays. (b) Gel mobility shift assays on the 183 bp ³²P-labeled *hix* half site fragments with a constant amount of Hin-wt and representative mutants and different concentration of CHAPS in the binding buffer. The locations of the dimer and monomer complexes along with the unbound substrate (free) are denoted. (c) The % monomer complex relative to the bound (monomer + dimer) complexes is plotted for the different CHAPS concentrations for the wild type and mutants shown in panel b. (d) Summary of the % monomer bound at 16 mM CHAPS for different Hin mutants. Values represent the mean of at least 3 experiments.

Fig. 6. Properties of synaptic complexes by Hin mutants containing Ser99 mutations. (a) Native PAGE of synaptic complex reactions by Hin mutants. S10G inactivates the active-site serine. (b) Electrophoretic migration differences of DNA cleaved and uncleaved synaptic complexes (SC) formed on 36 bp *hixL* fragments. (c) Synaptic complexes were extracted in SDS buffer and subjected to denaturing PAGE. S99R (slow) and (fast) refer to the electrophoretic migration of the isolated synaptic complexes as in panel (b). (d) Rates of DNA cleavage on 36 bp *hixL* fragments by Hin-H107Y and F88L mutants with and without S99K. Reactions were quenched with SDS at various times, subjected to denaturing PAGE, and the amounts of cleaved and uncleaved DNAs quantified. Note that rates of formation of synaptic complexes by H107Y and F88L/S99K, as assayed by native PAGE, are similar and reach a maximum yield within several minutes.

Fig. 7. Structural interpretations of helix-E hyperactivating mutations. Schematic representations of the (a) Hin dimer and (b) DNA-cleaved tetramer. Note the chain designations (A–D), coloring of subunits, and the different subunit interfaces in the tetramer. (c) Hin dimer model highlighting the side chains of His107 along with Ala113 and Val71. (d) DNA-cleaved Hin tetramer model highlighting the stacking of residue 107 side chains (shown as red tyrosines as in H107Y) from subunits across the new synaptic interface and the dark blue Phe105 side chains across the rotating interface between diagonally-oriented subunits. See Supplementary Movie 1. (e) Hin dimer model highlighting the connection between Met115 (red spheres) on subunit D and a set of residues (yellow) across the dimer interface on the

surface of the subunit A. (f) DNA-cleaved tetramer model highlighting the connections between Met115 (red) on subunit A and residues (gold) on the synapsed subunit B. The rotating subunit C and D pair have been removed for clarity, revealing the rotating interface on the plane of the page. See Supplementary Movie 2. (g) Hin dimer model showing locations of Met101 at the top of the dimer interface. (i) Open Hin tetramer intermediate model showing Met101 (red) from subunit A engaged in a set of connections with residues from the diagonally-opposed subunit C. In the final DNA-cleaved tetramer complex, Met101 is primarily engaged in contacts with the newly synapsed subunit near the edge of the rotating interface to help stabilize the rotating subunit pair but also comprises part of the rotating interface (panel f).

Fig. 8.

The helix-E phenylalanine triad and Phe88 core hyperactivating mutations. (a) Subunit A of the Hin dimer model depicting a surface representation of the catalytic domain (residues 1–98), Ser99, and helix E (residues 100–120) as a green ribbon. The side chains of phenylalanines 104, 105 (with transparent surface inserted within the Phe88 pocket of the catalytic domain), and 106 are shown along with selected residues in the catalytic domain. The red surfaces of Phe88 and Ile78 are visible behind Phe105. (b) Subunit A of the DNA-cleaved tetramer model in a similar view as in panel a. Note that Phe105 is rotated out of the Phe88 pocket and Phe106 is now inserted. See Supplementary Movie 3. (c) Stereo pair of the Hin dimer model looking into the Phe88 core showing locations of mostly hydrophobic

side chains. Residues where hyperactivating mutations were isolated are denoted in red and labeled. The positions of side chains should be considered only as approximate. (d) Potential interactions between the side chains of Gln122 on subunit C and Arg66 and the active site residue Ser10 on subunit B on the Hin dimer model.

Table 1

Gain-of-function mutations in Hin

Mutant	Location ^a	Method of isolation ^b	<i>in vivo</i> inversion		
			Fis (+) ^c	Fis (-) ^d	SOS ^e
Hin-wt			++	-	-/+
Helix-E					
M101I	helix-E	Fis ^{ind}	++(+)	++	+
F105V	helix-E	SOS	+++	-	++
H107Y	helix-E	Fis ^{ind} , SOS	+++++	+++++	+++
A113T	helix-E	Fis ^{ind}	+++++	+++++	+++
M115I	helix-E	Fis ^{ind}	++(+)	++	-/+
M115V	helix-E	Fis ^{ind}	+++++	+++++	-/+
E122Q	helix-E	sd	++++	++	-
E122Y	helix-E	sd	+++	+	-
F88 core					
L64I	helix-C	Fis ^{ind} , SOS	+++++	++++	+++
L67A	helix-C	sd	+++	++++	+
G68S	helix-C	Fis ^{ind}	++++	+++	-
L77I	helix-D	Fis ^{ind} , SOS	+++++	+++++	+++
I78V	helix-D	Fis ^{ind}	++++	+++	++
L81I	helix-D	Fis ^{ind}	+++	++	++
F88L	β-4	Fis ^{ind} , SOS	++++	+++	+++
F88I	β-4	Fis ^{ind}	+++	++++	+
F88V	β-4	Fis ^{ind}	++++	++++	+
β-4 strand					
H87K	β-4	sd	++++	++++	-/+
H87R	β-4	Fis ^{ind} , SOS	+++++	+++	++
H89L	β-4	Fis ^{ind}	++++	+++	++

Mutant	Location ^a	Method of isolation ^b	<i>in vivo</i> inversion		SOS ^e
			Fis (+) ^c	Fis (-) ^d	
H89R	β-4	Fis ^{ind} , SOS	++	+	+
Other					
R48Q	helix-B	Fis ^{ind}	++	+	++
N54S	loop	Fis ^{ind}	++	-/+	-
V71A	helix-D	Fis ^{ind}	+++	+	-/+
K72E	helix-D	Fis ^{ind}	+++	+	-
E80R	helix-D	Fis ^{ind}	+++	+	-
R66D	helix-C	sd	-	-	-

^aLocations of the gain-of-function mutations within secondary structure elements of the resolvase-based Hin tetramer models. The helix C region adopts helix and loop structures in different conformers.

^bMutants were isolated from Fis-independent DNA inversion screens (Fis^{ind}), SOS induction screens (SOS), or by site-directed mutagenesis (sd).

^c*In vivo* inversion rates in the presence of Fis as measured by color development on lactose MacConkey media: +++++ indicates red colonies developed before 15 hours incubation, ++++ red color developed between 15–20 hours, +++ between 20–25 hours, ++ between 25–30 hours similar to Hin-wt, + between 30–36 hours, - colonies remained white after 40 hours as observed for the pKK223-3 vector (no Hin) control.

^d*In vivo* inversion rates in the absence of Fis as measured by color development on lactose MacConkey media: +++++ indicates red colonies developed before 20 hours incubation, ++++ between 20–24 hours, +++ between 24–29 hours, ++ between 29–35 hours, + between 35–40 hours, -/+ indicates red sectoring within colonies after 40 hours, - colonies remained white after 48 hours as observed for Hin-wt and the no Hin control.

^e*In vivo* SOS rates (fis⁺) as measured by color development on lactose MacConkey media: +++ indicates red colonies developed before 26 hours incubation, ++ red color developed between 26–32 hours, + between 32–40 hours, -/+ between 40–48 hours, as observed for Hin-wt, -colonies remained white after 48 hours as observed for the no Hin control.