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Mutants of Tn*3* resolvase which do not require accessory binding sites for recombination activity

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Tn*3* resolvase promotes site-specific recombination between two *res* sites, each of which has three resolvase dimer-binding sites. Catalysis of DNA-strand cleavage and rejoining occurs at binding site I, but binding sites II and III are required for recombination. We used an *in vivo* screen to detect resolvase mutants that were active on *res* sites with binding sites II and III deleted (that is, only site I remaining). Mutations of amino acids Asp102 (D102) or Met103 (M103) were sufficient to permit catalysis of recombination between site I and a full *res*, but not between two copies of site I. A double mutant resolvase, with a D102Y mutation and an additional activating mutation at Glu124 (E124Q), recombined substrates containing only two copies of site I, *in vivo* and *in vitro*. In these novel site I×site I reactions, product topology is no longer restricted to the normal simple catenane, indicating synapsis by random collision. Furthermore, the mutants have lost the normal specificity for directly repeated sites and supercoiled substrates; that is, they promote recombination between pairs of *res* sites in linear molecules, or in inverted repeat in a supercoiled molecule, or in separate molecules.

Keywords: γδ resolvase mutants/site-specific recombination/synapsis/Tn*3*

Introduction

Tn*3* resolvase is a member of a large family of related site-specific recombinases which includes the γδ and Tn*21* resolvases, and the DNA invertases Gin and Hin. The structure of γδ resolvase has been solved both alone and in complex with *res* binding site I (Sanderson *et al.*, 1990; Yang and Steitz, 1995). Tn*3* resolvase differs from γδ resolvase in only 35 residues, and its structure is thought to be very similar (reviewed in Grindley, 1994).

The typical reaction promoted by resolvase *in vitro* is depicted in Figure 1. A supercoiled plasmid containing directly repeated 114 bp *res* sites (analogous to the cointegrate intermediate of Tn*3* replicative transposition) is resolved into two smaller circular molecules, each with one *res* site. The two circles are linked as a simple catenane (Figure 1).

Studies on several resolvase/*res* systems *in vitro* have provided some insight into the intermediates of the reaction, and have led to models for how selectivity for recombination between directly repeated *res* sites, and unique product topology are achieved (reviewed in Grindley, 1994; Stark and Boocock, 1995). Resolution is proposed to require formation of a synaptic intermediate in which the 'accessory' binding sites II and III of the two *res* sites are intertwined, allowing juxtaposition and subsequent catalysis of strand exchange at the two site Is (Figure 1). The functions of the accessory binding sites (and resolvase bound there) are therefore proposed to be 'architectural' and regulatory. They may be crucial in holding the two copies of *res* together prior to recombination, the specific intertwining of the accessory sites required to form the synaptic complex is implicated in topological selectivity, and the structure which they create is somehow necessary for activation of the catalytic function of resolvase bound at site I (Benjamin and Cozzarelli, 1988; Grindley, 1993; Stark *et al.*, 1994; Watson *et al.*, 1996). It is not clear whether this activation is purely architectural (that is, by holding the two site Is together in a configuration suitable for catalysis), or if there are direct 'activating' contacts of resolvase subunits bound at sites II and III with those bound at site I.

The DNA invertases Gin and Hin are clearly related to resolvase in sequence and mechanism, and also are topologically selective, but recombination at the *gix* and *hix* sites does not require accessory invertase-binding sites. However, recombination does require an enhancer element which binds the host protein FIS, within the same supercoiled substrate molecule. Models for invertase recombination, in which the FIS-bound enhancer forms a synaptic complex with the *gix* (or *hix*) sites, are strikingly similar to the model for resolvase recombination described above, except that the FIS–enhancer complex takes the place of the intertwined sites II and III of *res* (Kanaar *et al.*, 1989; Heichman and Johnson, 1990). Recent mutational analyses suggest that activation involves direct contacts of specific residues of FIS with Gin/Hin subunits (Spaeny-Dekking *et al.*, 1995; Deufel *et al.*, 1997; Safo *et al.*, 1997).

It proved possible to isolate mutants of the invertases which do not require the FIS–enhancer complex to promote inversion (Haffter and Bickle, 1988; Klippel *et al.*, 1988). FIS-independent Gin mutants have been studied in detail. These mutants have lost selectivity for inverted sites; that is, they recombine sites in direct repeat or on different molecules. Also, whilst the natural reaction gives exclusively unknotted circular inversion products, the mutants

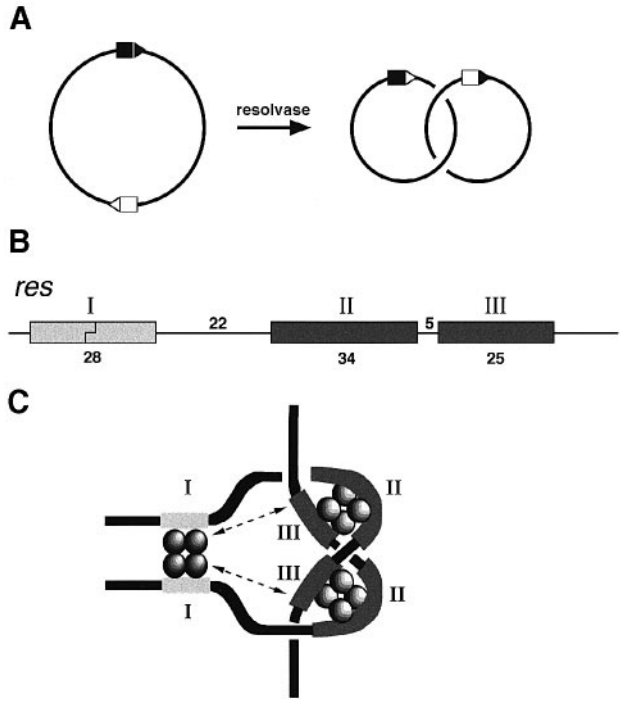

Fig. 1. (**A**) The reaction catalysed by Tn*3* resolvase. A supercoiled plasmid (represented as a circle) containing directly repeated *res* sites (represented as arrowheads) is recombined to yield two product circles, which are linked as a simple (2-noded) catenane. (**B**) The recombination site, *res*. The three resolvase-binding sites are shown as boxes (site I, pale grey; sites II and III, dark grey). The arabic numbers indicate the lengths of the various segments in base pairs. The staggered line in site I shows where resolvase breaks and rejoins the DNA during recombination. (**C**) A model for the productive synaptic complex (greatly simplified). Double-stranded DNA is represented by thick black lines; *res* accessory sites by dark grey boxes; binding site Is by pale grey boxes; and resolvase subunits by shaded balls. The dashed arrows indicate possible contacts between resolvase/DNA at the accessory sites and at the site Is.

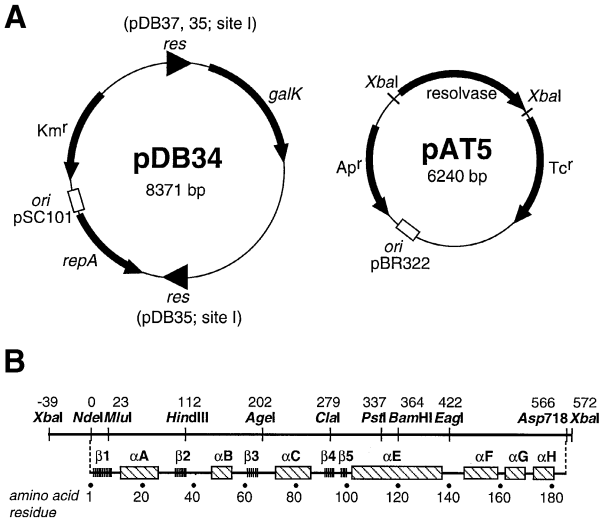

Fig. 2. (**A**) Left, the plasmids used for *in vivo* screening of resolvase mutants, pDB34 (*res*×*res*), pDB35 (site I×site I), and pDB37 (*res*×site I). See Materials and methods for further details. The *res* elements are indicated by arrowheads. The gene marked *repA* encodes an initiator protein required at the pSC101 origin of replication (Manen and Caro, 1991). Right, pAT5, the resolvase-expressing plasmid used for the *in vivo* screening experiments. (**B**) Schematic representation of the modified resolvase coding sequence in pAT5, as used for the mutagenesis. The restriction sites marked are not in the natural resolvase coding sequence, except for the *Bam*HI site, and are unique in pAT5, except for *Pst*I. The secondary structure of resolvase indicated is as in Yang and Steitz (1995). The α-helices are represented as boxes, and the β-sheet components with vertical dashes.

give products with many different topologies, consistent with recombination after synapsis by random collision (Klippel *et al.*, 1993; Crisona *et al.*, 1994).

It has been shown that the requirement of resolvase for two full *res* sites could be reduced by careful choice of *in vitro* reaction conditions, so that some recombination could be observed between a full *res* and a site I in a supercoiled plasmid (Bednarz *et al.*, 1990). However, the efficiency of reaction was very low; also, wild-type resolvase does not promote this reaction to a detectable level *in vivo* (Wells and Grindley, 1984; see below). Here, we report the isolation of resolvase mutants which have reduced or zero requirement for accessory sites to promote recombination. We describe the unusual properties of the mutants *in vitro*, and discuss implications for the mechanisms of activation and catalysis.

Results

Isolation of mutants

Our first attempts at isolation of activating resolvase mutants by chemical mutagenesis of the whole reading frame were unsuccessful (D.G.Blake, unpublished). We decided, therefore, to focus mutagenesis on a specific part of the reading frame (94–120, corresponding to amino acid residues of the E-helix and the preceding β-strand and loop; Figure 2B) which we thought most likely to yield activating mutants (see Discussion). Our strategy was to create random mutations in this region, by ligating a double-stranded oligonucleotide containing random changes from the canonical sequence into a gap made by cutting the resolvase-encoding DNA at two appropriate restriction enzyme sites. In order to allow mutagenesis of this and other selected regions of resolvase, a large part of the reading frame DNA was reassembled from double-stranded synthetic oligonucleotides, thereby introducing unique restriction sites at appropriate intervals by basepair substitutions which did not affect the amino acid translation (Figure 2B). Mutant DNA libraries were made from pAT5, a plasmid which gave a low level of resolvase expression found to be suitable for *in vivo* screening. The libraries were used to transform the *Escherichia coli* strains DS941/pDB34, DS941/pDB35 and DS941/pDB37. DS941 is a *galK*$^-$ strain; pDB34, pDB35, and pDB37 are low copy number plasmids with a *galK* gene bounded by potential recombination sites (pDB34, *res*×*res*; pDB35, site I× site I; pDB37, *res*×site I; Figure 2A). Resolution of the test plasmid separates the *galK* gene from the origin of replication and antibiotic resistance gene, and progeny cells become *galK*$^-$. *galK*$^-$ colonies are pale yellow ('white') on MacConkey agar plates containing galactose, and *galK*$^+$ colonies are red.

No mutants were found which could give sufficient resolution of a test plasmid with two copies of site I to give white colonies (~5000 mutants were screened). However, a few white colonies were observed when the test plasmid had one *res* and one site I (pDB37). Visualization of the cell DNA after agarose gel electrophoresis showed that the test plasmid was resolved in

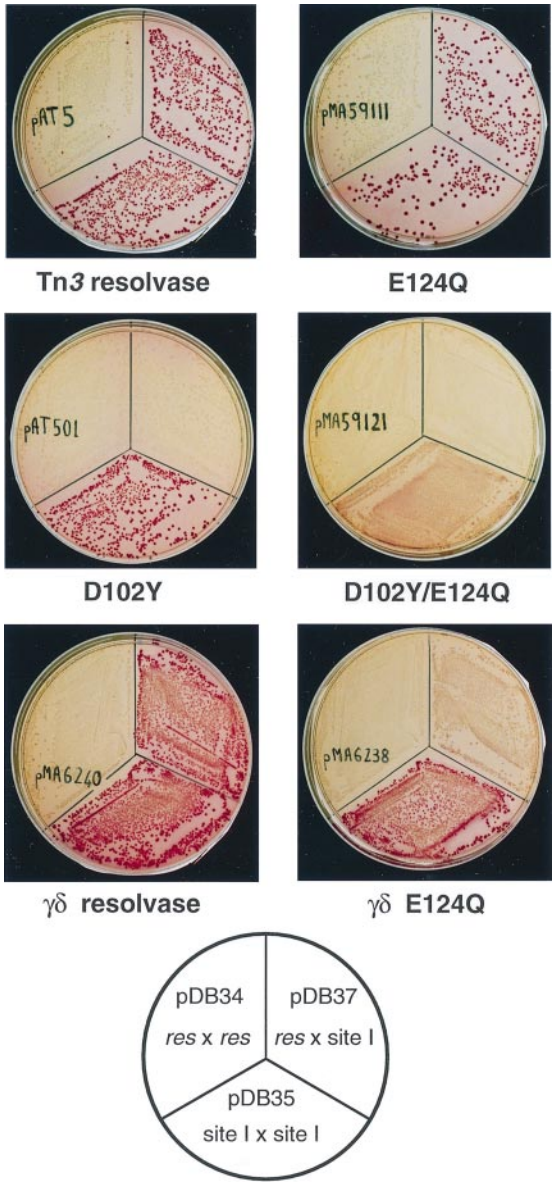

Fig. 3. *In vivo* screening of the activity of resolvases and mutants. The MacConkey agar plates are arranged so that in each panel, the upper left sector contains colonies of DS941/pDB34 (*res*×*res*) transformed with the pAT5-derived resolvase-expressing plasmid, the upper right sector is from DS941/pDB37 (*res*×site I), and the bottom sector is from DS941/pDB35 (site I×site I). The pDB test plasmid has been resolved in 'white' (pale yellow) colonies, but not in red colonies. See Materials and methods for further details.

these colonies (data not shown). The resolvase-expressing plasmids were isolated and re-tested for their ability to give white colonies on retransformation into the test strain (Figure 3). The resolvase reading frames of candidate mutant pAT5 plasmids were then sequenced.

All of the pAT5 variants isolated by virtue of their ability to resolve the *res*×site I test plasmid were found to carry mutations leading to substitutions at residue D102 and/or residue M103. The single substitutions D102Y and D102V were sufficient for a 'white' phenotype, as was M103I. D102Y had the strongest phenotype. Several mutants were isolated which had multiple changes, but all were mutant at D102 and/or M103. These were: D102A/M103I; D102Y/A113T; D102Y/Q116H; D102I/M103W; D102T/M103T; and I97V/G101S/D102Y/A113T. Further screens using PCR-based mutagenesis of the entire resolvase catalytic domain produced several 'white' mutants, which were also altered at D102 (S.Wenwieser and M.R.Boocock, data not shown).

A γδ resolvase mutant E124Q has recently been reported to be hyperactive *in vitro*; that is, it promotes recombination between *res* sites on linear molecules, unlike wild-type γδ resolvase, and also gives enhanced amounts of products in which the DNA has been cleaved in both strands at the centre of site I (M.R.Boocock, X.Zhu and N.D.F.Grindley, manuscript in preparation). We created the equivalent Tn*3* resolvase mutant (also E124Q) by cloning synthetic oligonucleotides between the *Bam*HI and *Eag*I sites of the 'cassetted' reading frame (Figure 2B). Expression of Tn*3* E124Q resolvase *in vivo* did not promote resolution of the *res*×site I or site I×site I test plasmids. Surprisingly, however, an equivalent plasmid expressing the γδ resolvase E124Q mutant did give white colonies, indicative of resolution, with the *res*×site I substrate plasmid (Figure 3).

A Tn*3* resolvase D102Y/E124Q double mutant derivative of pAT5 was made by splicing the D102Y and E124Q coding sequences. Expression of the D102Y/E124Q resolvase promoted resolution of both *res*×site I and site I×site I substrates *in vivo* (Figure 3).

In vitro properties of resolvase mutants

The three Tn*3* resolvase mutants, D102Y, E124Q and D102Y/E124Q, were overexpressed and purified. All three mutants were unimpaired in their ability to resolve a standard substrate with directly repeated *res* sites (pMA21; Figure 4B). Tn*3* E124Q resolvase had some tendency to give enhanced levels of products with double-strand breaks at site I, but this property was much less marked than in the case of the γδ resolvase E124Q mutant (M.R.Boocock, X.Zhu and N.D.F.Grindley, manuscript in preparation). However, only D102Y and D102Y/E124Q were active *in vitro* on a *res*×site I substrate (pAL265) under standard conditions (Figure 4C), and only the double mutant showed any recombination activity under any conditions on a site I×site I substrate (pAL225; Figure 4D; data not shown). Recombination by the D102Y and D102Y/E124Q mutant resolvases is most efficient with the 'standard' substrate pMA21, and least efficient with pAL225 which has no accessory sites (Figure 4), showing that although the accessory sites are no longer required, they still stimulate recombination.

The topologies of the reaction products were analysed by nicking the products with DNase I, followed by agarose gel electrophoresis (Figure 4). The major or exclusive product from treatment of the *res*×*res* substrate pMA21 with each of the four proteins was a simple (2-noded) catenane (Figure 4B); restriction digests confirmed that resolution products were predominant. Both D102Y and D102Y/E124Q recombined the *res*×site I substrate pAL265. Again, the predominant restriction pattern was that of resolution, and the products were mainly 2-noded catenanes, although there was more evidence of products with alternative topologies (3- and 4-noded; Figure 4C). The behaviour of D102Y/E124Q with the site I×site I substrate pAL225 was quite different; approximately equal amounts of resolution and inversion products were

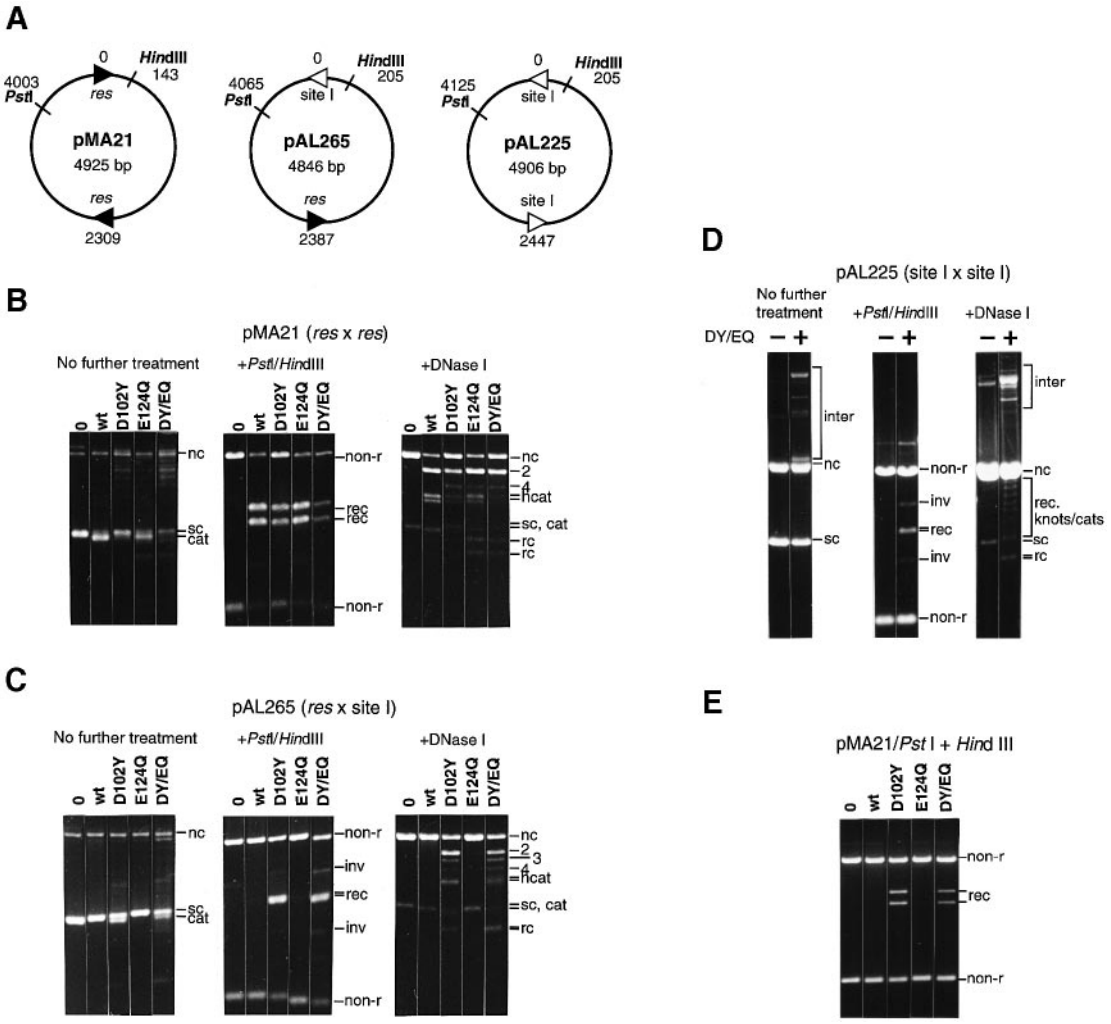

Fig. 4. Recombination activities of mutant resolvases *in vitro*. (**A**) The plasmids used in the *in vitro* experiments. *res* is represented as a black arrowhead, and site I as an unfilled arrowhead. The numbers indicate the positions of cleavage by restriction enzymes and by resolvase (i.e. centre of binding site I of *res*). (**B**) Reactions of pMA21 with Tn*3* resolvase and mutants. Following incubation with resolvase for 1 h, each sample was divided into three equal aliquots and treated as indicated (see Materials and methods). nc, nicked circular substrate (pMA21); sc, supercoiled plasmid substrate; cat, supercoiled catenane resolution product; non-r, non-recombinant *Pst*I + *Hin*dIII digestion products; rec, recombinant *Pst*I + *Hin*dIII digestion products; hcat, catenane resolution product, with one supercoiled and one nicked DNA circle; rc, free circular resolution product; 2, 4, products with two topological nodes (i.e. fully nicked catenane) or four nodes (probably knot iteration product). (**C**) Reactions of pAL265 with Tn*3* resolvase and mutants. Following incubation with resolvase for 5 h, samples were treated as in the legend to (B). The annotation of the gels is as in (B); inv, bands from *Pst*I + *Hin*dIII digestion of inversion recombination products. (**D**) Reactions of pAL225 with D102Y/E124Q resolvase. Following incubation for 5 h, samples were treated as in (B). The annotation is as in (B and C); inter, products of intermolecular reactions. (**E**) Reactions of pMA21, linearized prior to reaction with *Pst*I + *Hin*dIII, with Tn*3* resolvase and mutants. After reaction with resolvase for 16 h, samples were loaded directly onto the gel. The annotation is as in (D).

observed in restriction digests, and the products were of varying topologies, as evidenced by the 'ladder' of topologically complex monomeric products, and free circular resolution products, observed by gel electrophoresis after nicking (Figure 4D). Both intra- and intermolecular recombination had occurred. The monomeric products with greater mobility than nicked substrate circle in Figure 4D are the result of intramolecular reactions, whereas the species running more slowly than nicked circle are products of intermolecular reactions.

D102Y and D102Y/E124Q had the ability to recombine *res* sites on linear DNA (Figure 4E), indicating loss of the normal requirement for supercoiling in the substrate. These two mutants also promoted recombination between *res* sites in inverted repeat in a supercoiled plasmid (data not shown); this reaction is not observed with wild-type resolvase.

We carried out assays for binding of resolvase to *res* and binding site I of *res*, and for synapsis of full or partial *res* sites, to determine how, if at all, the mutants were affected in these properties. Binding of D102Y to *res* (Figure 5) was essentially identical to binding of the wild-type protein; a pattern of six bands corresponds to binding of six resolvase monomers, and the three alternate more intense bands correspond to cooperative formation of one, two or three on-site dimers (Blake *et al.*, 1995; D.G.Blake, unpublished results). Similarly, binding to site I gave the characteristic weak monomer and strong dimer complexes. E124Q showed an aberrant binding pattern, consistent with less stable complex formation, and increased representation of the complexes of resolvase monomers. The double mutant D102Y/E124Q resembled E124Q in its binding properties. Synapsis of a pair of *res* sites can be detected by gel electrophoresis after protein–protein

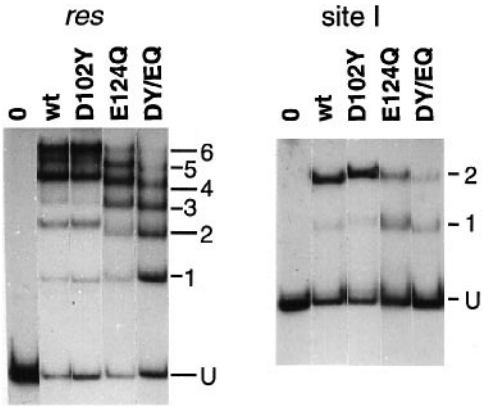

Fig. 5. Binding of Tn*3* resolvase and mutants to *res* and site I. U, unbound DNA fragment. Numbers indicate the predicted numbers of resolvase monomers in the complexes.

crosslinking (Watson *et al.*, 1996); the three mutant resolvases and wild-type resolvase gave similar yields of synaptic complex in this assay (data not shown), despite the differences in binding noted above. No synapsis by any of the proteins was detectable in a plasmid containing two isolated site Is.

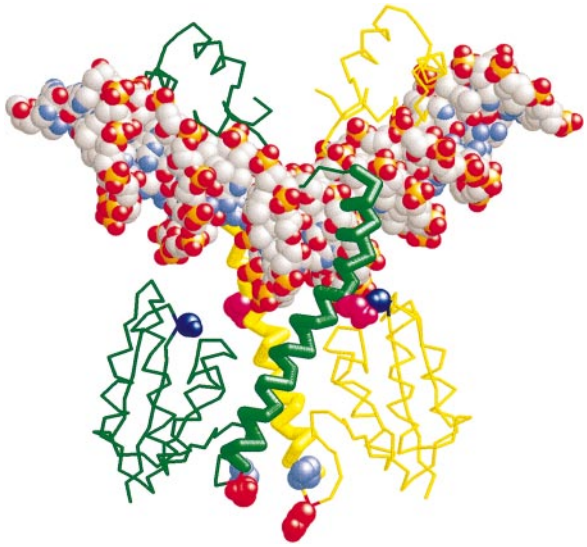

Fig. 6. The structure of γδ resolvase bound to a 34 bp site I fragment (Yang and Steitz, 1995) is shown (peptide backbone only; the subunits of the dimer are coloured green and yellow), with the sidechains of residues relevant to this work in spacefill representation. S10 is dark blue; E102 is red; M103 is pale blue; E124 is magenta. The DNA is in spacefill representation (CPK colours; nitrogen, blue; oxygen, red; phosphorous, yellow; carbon, grey). The peptide backbone of the E-helices is shown thicker. The image was created with RasMol2.

Discussion

We have shown that mutation of just two amino acid residues confers on Tn*3* resolvase the ability to promote recombination in the complete absence of the accessory binding sites II and III. Below, we consider what aspects of resolvase enzymology might have been changed by these mutations.

Recombination between a complete *res* and site I, catalysed by wild-type resolvase, has been observed previously (Bednarz *et al.*, 1990). The reaction was much slower than the equivalent *res*×*res* reaction, but surprisingly, the specificity of the natural reaction was maintained; only resolution products, and not inversion products, were seen, and the resolution product had the normal 2-noded catenane topology. It was suggested that non-specific DNA adjacent to site I was recruited into a synaptic complex with architecture similar to the normal one. The properties of the D102Y mutant suggest a similar scenario, since the reaction of the *res*×site I substrate gives a predominantly 2-noded catenane resolution product. The enhanced activity of the mutant on this substrate could be explained by enhanced ability to form a 'normal' synaptic complex using surrogate accessory site DNA, or the catalytic activity of the mutant could be less demanding of a specific synaptic complex structure. We favour the latter alternative, given the properties of the D102Y/E124Q double mutant, and the apparent lack of enhancement of synapsis by D102Y (see Results). The behaviour of the double mutant on *res*×*res* and *res*×site I substrates (predominance of resolution and 2-noded catenane products) also indicates a preference for reaction in a 'normal' synaptic complex, although the larger amounts of non-standard product topologies and evidence of inversion products in the restriction digests suggest reduced selectivity.

In the site I×site I reactions catalysed by the double mutant resolvase, equal amounts of resolution and inversion products were observed, and product topology was variable with no clear bias towards 2-noded catenane. The 'ladder' of topologically complex monomer-sized products strongly suggests recombination following synapsis by random collision (for a discussion of the interpretation of product topology see Stark and Boocock, 1995). These observations clearly indicate that the standard synaptic complex has been dispensed with. Furthermore, as expected from the results of Bednarz *et al.* (1990), the left–right asymmetry of site I is apparently not detected by the recombination machinery; the inversion products are the result of joining of two left half-sites and two right half-sites, whereas only left-right junctions are observed in normal *res*×*res* recombination. A simple hypothesis is that site I×site I recombination follows synapsis by interaction of dimers bound at each site I. However, it is possible that the reaction is still stimulated by further resolvase subunits (perhaps bound at DNA adjacent to the site Is).

We selected the sequence segment from amino acid residues 94–120 for saturation mutagenesis for the following reasons. First, several activating mutations in the invertases Gin and Cin, which confer FIS/enhancer-independent strand exchange activity, were in the homologous region of these proteins (Haffter and Bickle, 1988; Klippel *et al.*, 1988). Secondly, it was predicted that this region of resolvase might have important functions during strand exchange. In the crystal structure of a γδ resolvase dimer bound to site I of *res*, an important component of the dimer interface is contributed by residues of the E-helix, the sidechains of which interact with the E-helix and other surfaces of the partner subunit (Figure 6). The invertase-activating mutations are in residues corresponding in resolvase to residues of the E-helix which make contacts with the other subunit. Also, a chemical modification of a γδ resolvase cysteine mutant (M106C) on the interface

caused hyperactive behaviour *in vitro* (M.R.Boocock and N.D.F.Grindley, unpublished results). Some models for strand exchange by resolvase require disruption of the dimer interface; mutations of residues contributing to the interface might make it easier to separate the subunits, or reduce the degree of control on the disruption.

However, the activating mutations at D102 and M103, identified in the *in vivo* screen, were not predicted by this reasoning. In γδ resolvase, E102 (the residue equivalent to Tn*3* resolvase D102) does not contribute to the crystallographic dimer interface in any of the structures solved (Sanderson *et al.*, 1990; Rice and Steitz, 1994; Yang and Steitz, 1995). E102 has very different conformations in the two subunits of the resolvase dimer–site I complex (Figure 6). In one, the glutamate sidechain extends out from the subunit surface, and does not contact any other parts of the protein. The other glutamate sidechain is folded back to interact with a lysine residue (K105). In Tn*3* resolvase, residue 105 is a glutamine. Both E102 sidechains are very distant from the DNA of site I (Figure 6). In the structures of γδ resolvase without DNA, the conformations of E102 are quite similar to those seen in the co-crystal. In no case does E102 interact directly with part of another subunit. M103 is the N-terminal residue of the E-helix, but also makes no *trans*-interactions. In one subunit, the M103 sidechain makes a hydrophobic interaction with the M106 sidechain of the same E-helix. The region around E102 is apparently quite mobile, the residues having high B-coefficients and showing somewhat different conformations in five different observed crystallographic forms of γδ resolvase (Rice and Steitz, 1994; Yang and Steitz, 1995).

Note that since our method of mutagenesis created random point mutations, not all possible amino acid changes at D102 and M103 will have been screened. Single point mutations of the codon for D102 (GAT) can cause substitution by E, N, H, Y, V, A or G; M103 (ATG) can be substituted by I, L, V, T, K or R. The hyperactive mutant sidechains (Y, V, I) are large and rather hydrophobic; the significance of this is not yet clear.

Currently we have two hypotheses for the mode of stimulation of activity by the D102 and M103 mutants. One is that this region of the resolvase surface is involved in dimer–dimer contacts required for formation of the productive catalytic assembly at site I. The mutations might have disrupted a control feature, which prevents productive interaction unless preceded by formation of a full synaptic complex involving sites II/III. A second hypothesis is that these mutants change the properties of a 'hinge' between the E-helix and the main part of the N-terminal domain. The globular part of the N-terminal domain (residues 1–100) is held in place by interactions with residues of the E-helix of its own subunit, and residues of the E-helix of the partner subunit. We speculate that a significant movement of the 1–100 region relative to the E-helix of the same subunit might be integral to the catalytic mechanism. Mutations in other parts of the interface between the 1–100 region and the rest of the dimer (for example, at E124) could increase its freedom of movement. This hypothesis is therefore consistent with the increased activation in the double mutant resolvase. We note that some activating mutations of Gin invertase are at residues predicted to be contributing to the equivalent interface (Klippel *et al.*, 1993).

The mutation at E124 was introduced by design, because in one subunit of the co-crystal structure this residue hydrogen bonds to S10 (the active site nucleophile) and R68 (another residue at the catalytic site) of the partner subunit (Yang and Steitz, 1995). Maybe this interaction sequesters the sidechains of these active site residues, thus preventing catalytic activity in inappropriate situations. The γδ resolvase E124Q mutant showed activated behaviour, consistent with this idea (Figure 3; M.R.Boocock, X.Zhu and N.D.F.Grindley, manuscript in preparation). The Tn*3* E124Q mutant is less hyperactive than its γδ counterpart, suggesting that other residues have a modulatory effect on the behaviour of the mutants.

We propose that the combined mutations at D102 and E124 have somehow released resolvase at site I from dependence for activity on a signal from the correctly formed complex. Activation is by altering the behaviour of resolvase at site I, and not by affecting any property of the subunits at the accessory sites, since wild-type resolvase is completely inactive on a site I×site I substrate, whereas D102Y/E124Q is active. Our evidence does not suggest that the mutants are enhanced in their ability to promote synapsis of two copies of site I.

At present, we can only speculate on the nature of the activation. As stated in the Introduction, either a special geometry of juxtaposition of the site Is provided by the synaptic complex, or direct contacts between subunits bound at site I and those bound at sites II and/or III must normally stimulate catalytic activity. The mutants no longer require this stimulation for activity, but the fact that their recombination activity is still highest on substrates with full *res* sites indicates that the mechanism of stimulation has not been eliminated by the mutations.

It has become clear that a primary function of the elaborate synaptic intermediates in site-specific recombination and transposition systems is the imposition of control over the catalytic machinery. These complexes act as sensors, which cause recombination to be 'programmed'; only when specified conditions are fulfilled is the architecture required for activation of catalysis achieved. Major roles of the *res*–resolvase synaptic complex are to sense the topological relationship of the paired sites (ensuring only intramolecular recombination of direct repeats), and the supercoiling status of the substrate. The work presented here demonstrates that the uncontrolled catalytic potential of resolvase is only thinly veiled in the natural system; by changing just two amino acid residues, the regulatory power of the synaptic complex has been abolished. We can now begin to analyse the details of the control mechanisms which have been disrupted by these mutations, and which in their natural form lead to the exquisite specificity of resolvase-mediated recombination.

Materials and methods

Plasmids and DNA

The pBR322 derivatives pMA21, pAL225 and pAL265, used for *in vitro* reactions, have been described previously (Bednarz *et al.*, 1990). The plasmids used for the *in vivo* screens, pDB34, pDB35 and pDB37, are derived from pMA21, pAL225 and pAL265, respectively, by inserting a GalK-encoding DNA fragment into the tetracycline resistance gene (thereby inactivating it), and a kanamycin resistance-encoding gene into

the β-lactamase gene (thereby inactivating it), then replacing the pBR322 origin of replication with the origin of the low copy number plasmid pSC101 (Manen and Caro, 1991). pOG5 has been described (M^{c}Ilwraith *et al.*, 1997). pCO1 is similar to pOG5, except that the *Sst*I–*Eco*RI insert in the pMTL23 polylinker is a 44 bp site I fragment (sequence as in M^{c}Ilwraith *et al.*, 1997). pAT5, the expression plasmid used in the *in vivo* screens, is derived from pALTER-1 (Promega), and carries a functional β-lactamase gene (Figure 2A). The low level of resolvase expression in DS941 cells containing pAT5 was found to be sufficient for resolution of pDB34 (see Results for further details). The resolvase reading frame is bounded by unique *Nde*I and *Asp*718 restriction sites. The reading frame from the *Nde*I site at the start codon to the unique *Bam*HI site at the codon of residue 121 was reconstructed from synthetic oligonucleotides in several steps, thereby introducing unique *Mlu*I, *Hin*dIII, *Age*I, *Cla*I and *Pst*I sites which do not change the amino acid translation, but allow the dissection of the first 430 bp of the reading frame at ~80 bp intervals. Another site, *Eag*I, at codon 142 was introduced by site-directed mutagenesis, and the sequence corresponding to the resolvase C-terminus was modified with oligonucleotides to create the *Asp*718 site (S.J.Rowland, unpublished; Figure 2B). The plasmids used for overexpression of resolvase and mutants thereof are derived from pET11a (Dubendorff and Studier, 1991). pSA1101, which expresses wild-type resolvase, was created in several steps from pET11a. Besides the insertion of the resolvase reading frame, the β-lactamase gene has been disabled in pSA1101, and a kanamycin resistance-encoding cassette has been inserted (S.J.Rowland, unpublished). The resolvase reading frame from pAT5 or its mutants may be exchanged with that of pSA1101 on an *Xba*I–*Xba*I fragment. Full sequences of these plasmids and details of their construction are available on request from W.M.Stark.

Supercoiled plasmid DNA was purified from transformed DS941 (Summers and Sherratt, 1988), using an alkaline lysis method followed by caesium chloride–ethidium bromide density gradient ultracentrifugation. DNA concentrations were estimated by measuring absorbance at 260 nm. The fragments used in the binding assays were made by *Mlu*I + *Asp*718 digestion of pOG5 (170 bp *res* fragment), or pCO1 (74 bp site I fragment), followed by labelling and filling of the ends with [α-^{32}P]dCTP and Klenow fragment DNA polymerase.

In vivo screen for resolvase mutants

DS941 is a *recF*$^{-}$, *galK*$^{-}$, F^{-} strain. Colonies of DS941 harbouring one of the GalK-expressing plasmids pDB34, pDB35 or pDB37 are red on MacConkey agar (Difco Ltd) indicator plates containing galactose. Resolution of these plasmids by resolvase expressed from pAT5 or its mutants separates the *galK* gene from the plasmid replication origin and β-lactamase gene. The resulting colonies are *galK*$^{-}$, and pale yellow ('white') on MacConkey plates. Libraries of plasmid DNA with mutations in the *Cla*I–*Bam*HI segment of the resolvase reading frame (corresponding to amino acid residues 94–120) were created by ligation of double-stranded oligonucleotides to the purified pAT5 *Cla*I–*Bam*HI vector fragment. Both oligonucleotides were synthesized with ~1% of each 'wrong' base at each position (that is ~3% of all bases were mutant). The plasmid DNA libraries (~5000 mutant pAT5 plasmids in total) were then used to transform competent DS941 cells containing the test substrates pDB34, pDB35 and pDB37; the colour of the colony indicating whether or not resolution of the test plasmid was complete. 'Hyperactive' mutants were picked as white colonies observed when DS941/pDB37 (*res*×site I) or DS941/pDB35 (site I×site I) was transformed with pAT5 mutant libraries. The sequence of the full resolvase reading frame was confirmed for each mutant pAT5 isolated. Sequences of randomly chosen pAT5 mutants indicated that ~50% of the library plasmids contained small insertions or deletions (mainly 1–4 bp) within the *Cla*I–*Bam*HI segment, but the remainder contained only random point mutations of the predicted number and types (data not shown). We are thus confident that all or nearly all possible single point mutations within the *Bam*HI–*Cla*I segment were screened.

Purification of resolvase and mutants

Resolvase overexpression was induced by adding isopropylthio-β-galactoside (IPTG) to exponentially growing cultures of BL21(DE3)-pLysS (Studier *et al.*, 1990) transformed with pSA1101 (or derivatives having mutant reading frames). The following method was used for purification of the D102Y and E124Q resolvases. After sonication of the cells, resolvase remained in the insoluble fraction, and was redissolved in a buffer containing 7 M urea. A crude separation on a short column containing S-Sepharose cation exchanger (Pharmacia) was followed by precipitation of resolvase by dialysis against non-denaturing buffers. The precipitate was redissolved in a 7 M urea-containing buffer, and separated on a Mono-S cation exchange column (Pharmacia); resolvase was eluted at ~300 mM NaCl. Fractions containing resolvase were dialysed against a buffer containing 2 M NaCl, 20 mM Tris–HCl pH 7.5, 0.2 mM dithiothreitol (DTT), 0.1 mM EDTA. Purified resolvase was stored at –20°C following addition of glycerol (final 50% v/v) to the dialysed solution. The double mutant resolvase D102Y/E124Q was purified by a similar method. Wild-type resolvase purification was as previously reported (Stark *et al.*, 1989). Its activity was indistinguishable from resolvase purified by a denaturing method (as above).

Reactions with resolvases

A typical reaction was started by adding 6 µl of resolvase, diluted to ~2 µM in a buffer containing 20 mM Tris–HCl pH 7.5, 0.1 mM DTT, 0.1 mM EDTA, 1 M NaCl, 50% v/v glycerol, to 60 µl of a solution of plasmid DNA (20 µg/ml) in a buffer containing 50 mM Tris–HCl pH 8.2, 10 mM $MgCl_2$, 0.1 mM EDTA. The mixture was incubated at 37°C for 1 h, unless stated otherwise, and the reaction was stopped by heating at 70°C for 5 min. The sample was then divided into 20 µl aliquots for further treatment or gel electrophoresis. Before loading onto 0.7% agarose gels, 5 µl of loading buffer (50% v/v glycerol, 1% sodium dodecyl sulfate, 1 mg/ml protease K, 0.1 mg/ml bromophenol blue) was added to each 20 µl sample. DNase I nicking, gel electrophoresis and visualization of bands on gels were as described (Stark *et al.*, 1991). Synapsis assays were as described by Watson *et al.* (1996).

Gel binding assays

Resolvase was diluted as in the section above. Dilutions were stored at –20°C. The DNA fragment to be bound was dissolved in a buffer containing 50 mM Tris–10 mM glycine pH 9.4, 0.1 mM EDTA, 10% v/v glycerol, and 50 µg/ml supercoiled pUC4K (Taylor and Rose, 1988). Radiolabelled binding site-containing fragments were at very low concentration (≪1 nM). To each 10 µl sample, 0.5 µl of diluted resolvase was added, giving a final NaCl concentration of 48 mM. The samples were mixed and incubated at 37°C for 10 min, then cooled in ice and loaded immediately onto a 6% polyacrylamide gel (30:0.8, acrylamide: bisacrylamide). The buffer in the gel and tanks was 50 mM Tris–glycine pH 9.4, 0.1 mM EDTA. The gels were run at 4°C with recirculation of the buffer, and were pre-run at ~6 V/cm for ~1 h before loading the samples. The gels were run at ~6 V/cm for ~4 h, then dried, and the bands were visualized by autoradiography.

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