The EMBO Journal vol.12 no.4 pp.1447-1458, 1993

Protein-protein interactions directing resolvase site-specific recombination: a structure-function analysis

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Communicated by D.Finnegan

Recombination catalyzed by the γδ resolvase requires assembly of a nucleo-protein complex, the synaptosome, whose structure is determined by resolvase-*res* and resolvase-resolvase interactions. In crystals of the resolvase catalytic domain, monomers of resolvase were closely associated with one another across three different dyad axes; one of these subunit contacts was shown to be an essential inter-dimer interaction. To investigate the relevance of the remaining two interfaces, we have made site-directed mutations at positions suggested by the structure. Cysteine substitutions were designed to link the interfaces covalently, mutations to arginine were used to disrupt intersubunit contacts, and mutations to tryptophan were used to study the hydrophobicity and solvent accessibility of potential interfaces by fluorescence quenching. Characterization of the mutant proteins has allowed us to identify the dimer interface of resolvase and to assign a structural role to a second intersubunit contact. The data presented here, together with our previous results, suggest that all three of the dyad-related intersubunit interactions observed in the crystal play specific roles in synapsis and recombination.

Key words: covalent dimers/crystal packing contacts/dimer identity/protein engineering/γδ resolvase

Introduction

γδ is a member of the Tn*3* family of prokaryotic transposable DNA elements, which transpose via a replicative pathway resulting in the formation of a co-integrate molecule. The co-integrate, a fusion of donor and recipient DNA molecules with a copy of γδ at each junction, is then converted to products by site-specific recombination mediated by the resolvase protein (for a review, see Hatfull and Grindley, 1988). The resolution reaction proceeds through the formation of a highly ordered nucleo-protein complex, called the synaptosome, which is composed of the two recombination sites, called *res*, and, presumably, six resolvase dimers. Once the synaptosome is assembled, the cleavage, exchange and religation of the substrate DNA strands occur to form the products of resolution: two singly-linked catenanes, each containing a single recombinant *res* site.

The substrate for the resolution reaction is a supercoiled DNA molecule with two *res* sites in direct orientation (Reed, 1981). Each *res* site is comprised of three resolvase binding sites (called sites I, II and III) which in turn consist of imperfectly conserved 12 bp inverted repeats with variable internal spacing (Grindley *et al.*, 1982; Rimphanitchayakit and Grindley, 1990) (Figure 1). Binding of resolvase to the *res* site introduces a variety of localized DNA distortions through protein-DNA and protein-protein interactions. The single *res*-containing nucleo-protein complex, called the resolvosome, has been extensively characterized with linear DNA. It consists of ~120 bp of *res* DNA wrapped around a core of (presumably three) resolvase dimers. The DNA within each binding site is bent, the segment between sites I and II is apparently looped (Salvo and Grindley, 1989) and the DNA at the center of site I (the crossover site) has a distortion which suggests a localized unstacking of base pairs at the central dinucleotide (Hatfull *et al.*, 1987). The folding of the resolvosome requires interactions between bound resolvase dimers, and a particular protein-protein interaction has been demonstrated to be essential for this (Hughes *et al.*, 1990). The relevance of the resolvosome to synapsis is unclear; for example, it is not known whether the resolvosome is a necessary intermediate in the process. However, resolvase mutants that bind *res* but are defective in resolvosome formation are generally unable to catalyze recombination; this indicates that the structural organization of the resolvosome and the synaptosome share a similar array of inter-resolvase interactions. Little is yet known of the structural details of the synaptosome, but topological and biochemical analyses indicate that the two *res* sites are wrapped around each other, trapping three negative interdomainal DNA nodes (Wasserman *et al.*, 1985; Benjamin and Cozzarelli, 1988, 1990; Stark *et al.*, 1989).

The crystal structure of the catalytic domain (residues 1-120) of resolvase has been solved (Sanderson *et al.*, 1990). The asymmetric unit of the crystal contains three monomers of resolvase (called 1, 2 and 3) related to each other by two non-crystallographic dyad axes, thus forming two different types of dimers (1,2 and 2,3) (see Figure 2). An additional 2-fold related monomer-monomer interaction in the crystal involves subunits 2 and 3 which form a tetramer (2,3/2′,3′) with 222 (or D2) symmetry; this interaction is dominated by the 2-3′ (and identical 3-2′) interface.

Which, if any, of the three dimeric pairs seen in the crystal structure is present in solution, binds to DNA and catalyzes recombination? We can eliminate the 2-3′ interaction from consideration, since mutations at this interface inhibit an interaction between DNA bound resolvase dimers but affect neither the dimerization of resolvase nor its ability to bind the three individual sites within *res* (Hughes *et al.*, 1990). The 2-3′ interaction is essential for formation of the resolvosome and recombination activity; however, since pairs of either 2,3 dimers or 1,2 dimers could interact via 2-3′ interface, the properties of the mutant proteins with altered 2-3′ interactions do not help indicate the nature of the solution dimer.

For catalysis, a primary consideration is the distance

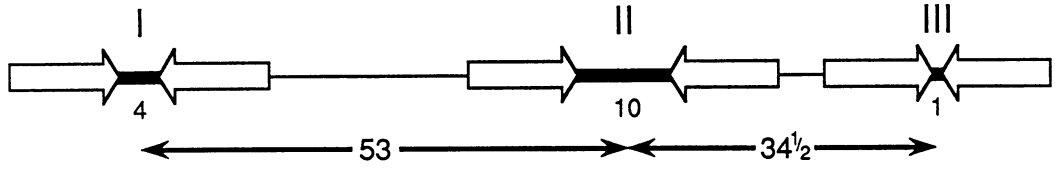

Fig. 1. The $\gamma\delta$ *res* site. Diagram of the *res* site. Open arrows represent the 12 bp inverted repeats which comprise the three resolvase binding sites, I, II and III. Numbers between inverted repeats indicate the size (in base pairs) of the spacer regions. Numbers below indicate the center to center distances between binding sites.

between the active sites of the two monomers in each dimeric configuration. During recombination, DNA cleavage at the crossover site is accompanied by transient attachment of resolvase to the recessed 5′ phosphates (Reed and Grindley, 1981). Residue serine-10 (Ser10) is the amino acid implicated in this covalent linkage (Reed and Moser, 1984; Hatfull and Grindley, 1986). The phosphodiester bonds cleaved by resolvase during recombination are separated by a two base 3′ stagger; in B-DNA this would correspond to about 13 Å. Thus, one would expect the Ser10 residues in the catalytically active dimer to be separated by a similar distance. The α-carbons of the two Ser10 residues in the 2,3 dimer are 19 Å apart (Sanderson *et al.*, 1990). This would be a reasonable distance to enable the Ser10 hydroxyls to interact with the scissile bonds, taking into account the fact that DNA distortions at the crossover site are known to accompany resolvase binding. By contrast, the 1,2 dimer (and all other dimeric pairings in the crystal) places the Ser-10 α-carbons more than 30 Å apart, a distance that seems too great to facilitate DNA cleavage without involving unprecedented distortions to the DNA at the crossover site or large conformational changes in the resolvase dimer. A further attraction of the 2,3 dimer is that, in the crystal, it forms half of a 222-symmetric tetramer (2,3/2′,3′). Such a tetramer would provide a very good way of pairing the two recombination sites (by protein–protein interactions) and had been postulated earlier on symmetry grounds; the 1,2 dimer does not participate in such a tetramer in the crystalline array.

Although the 2,3 dimer appears to be more attractive from a catalytic perspective, the 1,2 dimer appears to be potentially more robust from a physical–chemical analysis. The 1–2 interface is formed primarily of two C-terminal α helices which interact with each other and make additional contacts to the opposing monomer. The 1–2 interface is very hydrophobic, and dimerization buries 1465 Å^2 of solvent accessible surface area (Rice,P. and Steitz,T.A., in preparation). By contrast the 2–3 interface is poorly ordered in places, is not very hydrophobic and buries 1213 Å^2 of surface area. The hydrophobicity of residues at the 1–2 interface is conserved among the family of resolvases and DNA invertases, while the residues at the 2–3 interface are mostly unconserved (Sherratt, 1989). By this analysis, the 1,2 configuration would seem the more likely for a stable solution dimer. Moreover, every monomeric subunit in the crystal is related to another by a 1–2 dyad axis but only a portion participate in the 2–3 interaction (Sanderson *et al.*, 1990). This suggests that the crystal array was assembled from 1,2 dimers.

To determine which, if either, of these two interactions represents the dimeric form of resolvase in solution, we have made site-directed mutations at these interfaces and analyzed the structural and functional consequences. Position L50 at the 2–3 interface and positions M106, I110 and V114 at the 1–2 interface were selected for mutation. Each of these residues lies on the surface of the monomer and interacts with the residue at the same position in the appropriate dyad-related monomer. Three types of amino acid substitutions were made. Mutations to arginine and tryptophan were designed to disrupt monomer–monomer contacts. This strategy was successful with the arginine mutants, but in no case did a tryptophan substitution have any measurable effect on the dimeric state of resolvase. The tryptophan mutants were useful, however, for measurement of solvent accessibility (and therefore dimer interface formation) using fluorescence spectroscopy studies. The third type of mutant contained cysteine substitutions designed to form intermonomeric disulfide bonds. These mutants have allowed us to purify and assay covalent dimers and oligomers of resolvase. These studies show conclusively that the 1,2 dimer represents the solution and DNA binding dimer of resolvase; they also suggest that the 2–3 interface (like the 2–3′ interface) plays a role in interactions between DNA-bound resolvase dimers. The implications of these results for recombination are discussed.

Results

Site-specific cysteine mutants form covalent dimers and multimers via disulfide bonds

In order to prepare covalent dimers of resolvase, site-specific cysteine substitutions were made at the 1–2 and the 2–3 interfaces. The rationale for the experiment was as follows. It is known that resolvase is a dimer in solution and specifically binds DNA as a dimer (Reed, 1983; Salvo, 1990). It should therefore be possible, with the appropriate cysteine mutation, to engineer a disulfide bond across the dimer interface to produce a covalent resolvase dimer with relatively normal solution and DNA binding properties. The position of such a mutation would serve to identify the functional dimer interface. Inspection of the crystal structure and computer modeling identified position Leu50 at the 2–3, and positions Met106 and Ile110 at the 1–2 interfaces as those most likely to produce intermonomeric disulfide bonds while minimizing distortion of the interface.

The cysteine mutants were purified by standard methods under reducing conditions (Reed, 1981). In their reduced forms both L50C and M106C exhibited recombination activity *in vitro*; I110C, however, was inactive (data not shown). These three cysteine mutants were oxidized by dialysis against buffer with air bubbling through it. After 12 h approximately half of the L50C and nearly all of the M106C and I110C resolvases were in the form of covalent dimer (SS) (Figure 3). The extent to which the individual mutants are converted into the covalent form measures the relative favorability of disulfide formation at a specific position. The relative amounts of dimer formed in each case

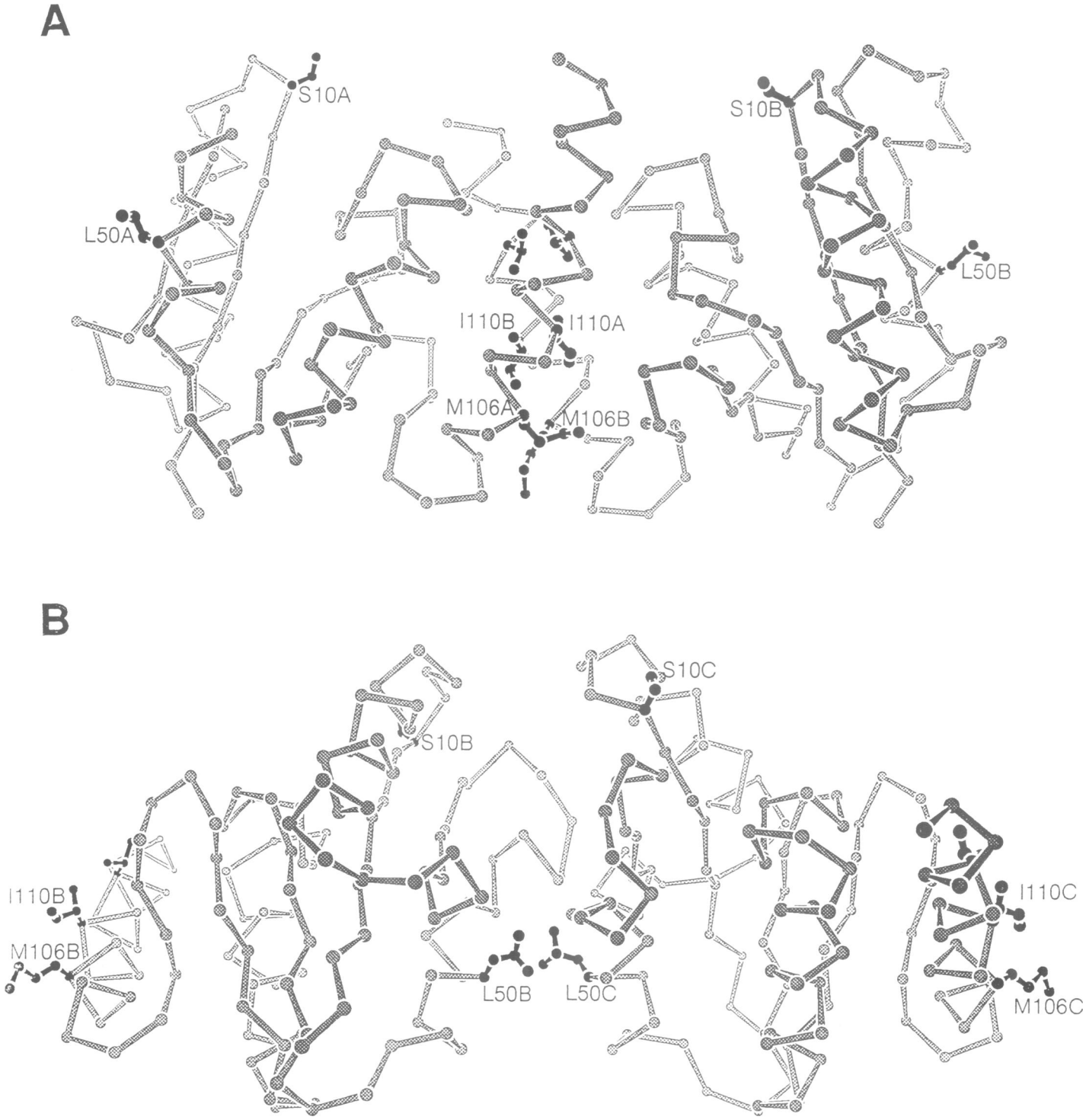

Fig. 2. α-carbon representation of the two dimers present in the asymmetric unit of the resolvase large domain crystal structure. The asymmetric unit of the crystal structure of the resolvase large domain (amino acids 1−140) contains three monomers (Sanderson *et al.*, 1990). These monomers form two alternative overlapping dimers designated 1−2 and 2−3. The plane of representation is intended to maximize representation of the 2-fold axis of symmetry at the particular dimer interface. (**A**) The 1−2 dimer. Amino acids 1−122 are included. Side chains are shown at positions S10 (active site serine), L50, M106, I110 and V114. Note the interaction between C-terminal helices (helix E) at the 1−2 interface. The distance between the S10 positions in the 1−2 dimer is greater than 30 Å. (**B**) The 2−3 dimer. The parameters are as shown for the 1−2 dimer. This arrangement places the C-terminal helices at the outside of the dimer. The distance between S10 α-carbon positions is 19.1 Å. (This figure was generated with the program MAXIM, written by Mark Rould, Yale University.)

is consistent with that expected from the computer modeling.

The apparent molecular weight of the covalent resolvases in solution was determined by gel filtration of oxidized mutant proteins along with protein markers of known molecular weight. It had been previously determined that in such an assay, wild-type resolvase behaved as a 42 kDa species indicating that it is a dimer in solution (Reed, 1983). Oxidized M106C(SS) and I110C(SS), eluted in the same volume as wild-type resolvase, between the 35 kDa and 68 kDa markers, indicating that both remained dimeric in

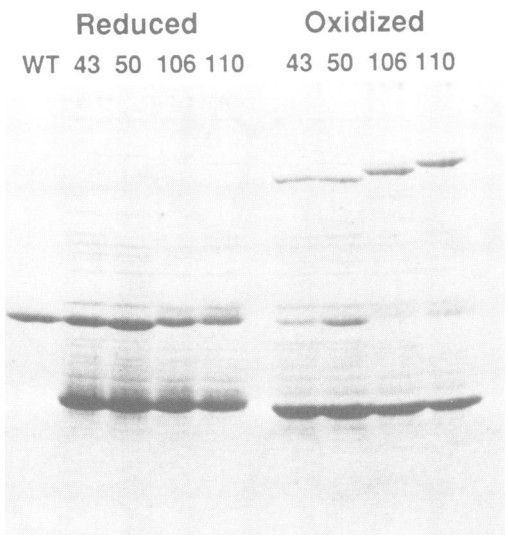

Fig. 3. SDS−polyacrylamide gel showing cysteine mutants S43C, L50C, M106C and I110C under reducing and non-reducing conditions. Crude extracts from cells overproducing resolvase cysteine mutants were oxidized, run on a 15% SDS−polyacrylamide gel and stained with Coomassie blue. Samples labeled 'reduced' were boiled in buffer containing β-mercaptoethanol before loading. Oxidized samples were boiled in non-reducing buffer. WT is wild-type resolvase. The dark band migrating below the resolvase monomer is lysozyme.

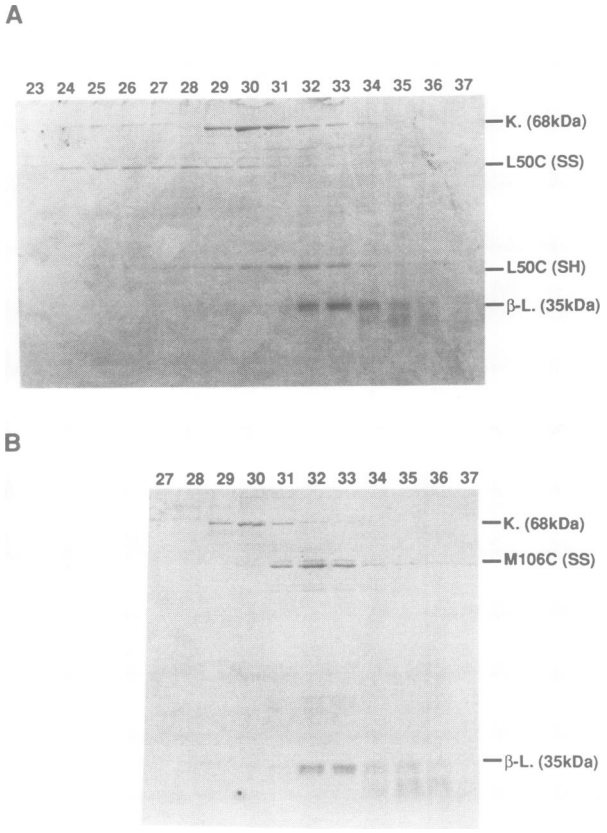

Fig. 4. Gel filtration elution profiles for L50C and M106C under non-reducing conditions. (**A**) Fractions from an FPLC Superose 12 gel filtration column showing the elution volumes of covalent and non-covalent L50C resolvase dimers relative to internal molecular weight markers. The covalent L50C(SS) dimer elutes as a range of high molecular weight species greater than 68 kDa. The non-covalent form L50C(SH) elutes mainly in the wild-type 42 kDa range. Samples from each fraction were run on 15% polyacrylamide gels under non-reducing conditions and Coomassie-stained. (**B**) The same experiment as in panel A, performed with M106C. The covalent M106C(SS) elutes at the same volume as wild-type resolvase indicating that its apparent molecular weight is 42 kDa.

solution (Figure 4B and data not shown). When this assay was performed with L50C(SS), the disulfide-linked population was excluded from the column more than the 68 kDa marker, while the non-covalent population eluted similarly to wild-type, but with some tendency toward greater exclusion (Figure 4A). These data are consistent with the following conclusion. 1,2 is the solution dimer and therefore a covalent bond across this interface (at positions 106 or 110) does not affect the apparent molecular weight in solution. L50C lies opposite the dimer interface, therefore disulfides between these positions covalently link 1,2 dimers to one another forming a mixed population of resolvase polymers across a range of relatively higher molecular weights.

A covalent 1,2 dimer retains normal DNA binding properties

The covalent dimers of resolvase were assayed for DNA binding activity using native PAGE. Assays performed with an equimolar mixture of three DNA fragments, each containing one of the individual resolvase binding sites, are shown in Figure 5. M106C in its reduced (SH) form behaved as wild-type resolvase, binding with approximately equal affinity to sites I and II and with slightly reduced affinity to site III. The complexes formed had the same mobility as their wild-type counterparts indicating similar degrees of DNA bending. M106C in its covalently linked (SS) form also behaved in a similar manner to wild-type—the only discernible difference was that binding to site I was slightly but selectively diminished. Most significantly, mobilities of the three complexes were indistinguishable from those with wild-type resolvase, strongly suggesting that the resolvase−DNA stoichiometry was unaffected by the disulfide bond across the 1−2 interface between resolvase subunits, and thus, that the covalently linked form binds to DNA as a dimer (not as a pair of covalently linked dimers). A similar conclusion can be drawn from the results with I110C since mobilities of each of the three single site complexes were unaffected by the redox state of the mutant protein. By contrast to M106C, the cysteine substitution in I110C significantly affected the properties of the mutant resolvase. As can be seen from Figure 5, I110C in both reduced and oxidized (SS) forms showed a preference for site I and bound to sites II and III with substantially reduced affinity. Other mutations that confer altered site affinity also map to this α-helix. This suggests that the conformation of these helices may be involved in the ability of the 1,2 dimer of resolvase to recognize three binding sites with differing geometries (see Figure 1).

As expected from the observations described above, M106C(SH) interacts with *res* DNA essentially like wild-type in a mobility shift gel assay (Figure 6). M106C forms single-site and fully occupied complexes with the *res* site which co-migrate with those formed with the wild-type resolvase. This indicated that the order in which the sites are filled and the conformation of the discrete complexes are not significantly affected by the cysteine mutation. When oxidized, however, M106C(SS) forms a single-site complex with anomalously slow mobility; this reflects its reduced affinity for site I. With wild-type resolvase, the highest

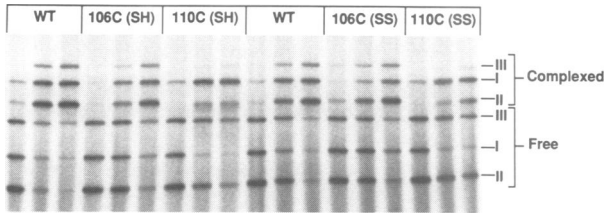

Fig. 5. Relative binding of M106C and I110C to subsites I, II and III. Binding reactions contained equimolar amounts of sites I, II and III end-labeled with ^{32}P. Binding reactions on the left and right half of the panel were identical except for the addition of 1 mM DTT in the reactions on the left half. Within each set of three reactions the amount of resolvase added increased 10-fold from left to right.

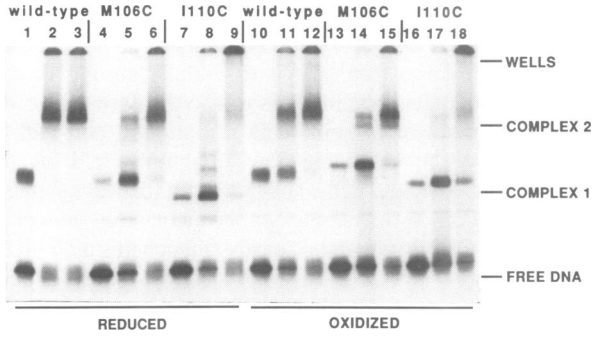

Fig. 6. Analysis of M106C and I110C resolvase/*res* interactions by native PAGE. Binding reactions contained ^{32}P-labeled *res* DNA and resolvase as indicated. Reactions in lanes 1–9 contained 1 mM DTT and the otherwise identical set of reaction in lanes 10–18 were non-reducing. Within each set of three the amount of resolvase added increased 5-fold from left to right.

mobility complex is composed of an approximately equimolar mixture of single dimer–*res* complexes with resolvase bound at either site I or II (Hatfull and Grindley, 1986). Experiments with mutant *res* sites in which either site I or II has reduced affinity for resolvase have shown that a complex with only site II bound has a lower mobility than the complex with only site I bound, and that the migration of the one-dimer complex with the wild-type protein (and the wild-type *res* site) is the average of these two mobilities (Rimphanitchayakit,V. and Grindley,N.D.F., unpublished observations). I110C, reduced or oxidized, shows a preference for site I and an inability to form efficiently a fully occupied complex.

The covalent form of L50C is unable to bind DNA (Figure 7). Early fractions from the gel filtration column, which have separated the disulfide-bonded from the non-covalent population, showed no DNA binding activity. Binding activity was recovered from these fractions upon the addition of reducing agent. L50C(SH) bound individual sites like wild-type (Figure 6 and data not shown). However, when binding to *res* was assayed, distinct differences from wild-type were observed and features similar to the cooperativity mutants (with mutations at the 2–3′ tetrameric interface), for example R2A, were seen (Figure 8). First, a complex of intermediate mobility accumulated that co-migrated with the 2-dimer complex formed by R2A. Second, two fully occupied complexes were seen, one of which co-migrated with the final complex formed by R2A, while the other co-migrated with the fully occupied wild-type complex (the resolvosome). Our interpretation of these data

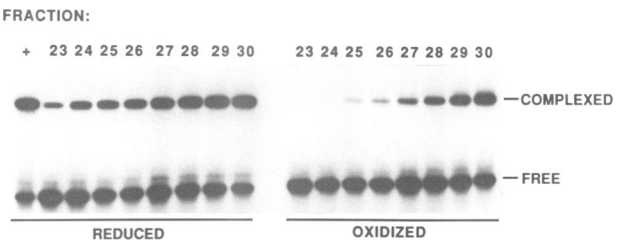

Fig. 7. DNA binding activity in L50C gel filtration fractions analyzed by native PAGE. Binding reactions contained ^{32}P-labeled site I DNA and samples from the L50C gel filtration fractions shown in Figure 4A (fraction numbers are the same as in Figure 4A). Reactions either contained 1 mM DTT (left) or were non-reducing (right); reactions were otherwise identical.

is that the mutation L50C at the 2–3 interface also (like mutations at the 2–3′ interface) interferes with the normally cooperative inter-resolvase interactions that result in formation of the wild-type resolvosome. This would mean that the resolvase dimers bound to *res* (presumably 1,2 dimers from the results described in this paper) interact via both the 2–3′ and the 2–3 interfaces. Clearly the block to resolvosome formation resulting from the L50C mutation is only partial since a portion of the fully occupied complex co-migrated with the wild-type complex. In this regard it is notable that, in contrast to the cooperativity mutant R2A (Hughes *et al.*, 1990), L50C in its reduced form retains recombination activity, albeit somewhat diminished.

Site-specific arginine mutations are able to disrupt resolvase–resolvase interactions

To develop a set of data complementary to that described above, positions at the 1–2 and 2–3 interfaces were changed to arginine and tryptophan in order to disrupt or inhibit monomer–monomer interaction at these interfaces. Leu50, Ile110 and Val114 were selected for mutation because of the close proximity of the side chains of these residues to their symmetry-related counterparts across a dimer interface. When apparent molecular weights were determinated by gel filtration none of the tryptophan substitutions was found to inhibit dimer formation. However, both I110R and V114R eluted just after the 35 kDa marker protein, suggesting that these mutant proteins were monomers in solution (data not shown). This interpretation was borne out by their behavior in DNA binding assays. A single site I complexed with either I110R or V114R had significantly higher mobility than that formed with wild-type resolvase (Figure 9A; data for V114R not shown). We interpret this as resulting from monomer binding to a single half-site in site I. This has been confirmed by the DNase I protection pattern of I110R on site I, which reveals protection of a single half-site (data not shown). Since wild-type resolvase binds cooperatively to both half-sites in a single binding site, monomer binding to half-sites is normally not observed. Independent binding of half-sites is seen, however, with the small domain alone, since the function of monomer–monomer cooperativity resides in the large domain (Abdel-Meguid *et al.*, 1983). The behavior of I110R as a monomer in solution is further illustrated by its interaction with *res* (Figure 9B). In this case we are able to discern the formation of six discrete complexes which probably represent the sequential filling of the six half-sites in *res*. At the higher concentration I110R forms a diffuse final

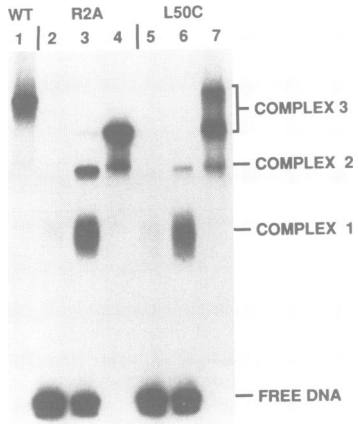

Fig. 8. Analysis of L50C−*res* interaction by native PAGE. Binding reactions contained ^{32}P-labeled *res* DNA, 1 mM DTT and resolvase as indicated. Within each set of three the amount of resolvase added increased 5-fold from left to right. Bands at the top are material that did not enter gel.

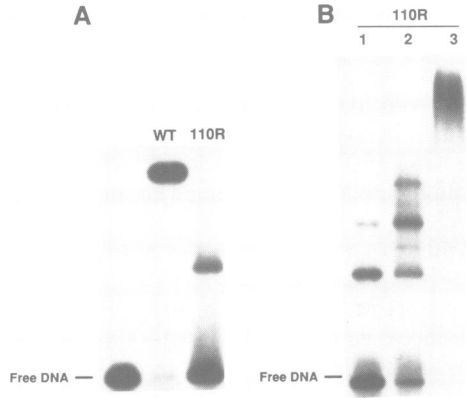

Fig. 9. Analysis of I110R−site I and I110R−*res* interactions by native PAGE. (**A**) Binding reactions contained ^{32}P-labeled site I DNA and either wild-type or I110R resolvase. (**B**) Binding reactions contained ^{32}P-labeled *res* DNA and I110R in concentrations increasing 10-fold from left to right.

complex which is presumably distorted due to perturbed monomer−monomer interactions. I110R and V114R have not been observed to catalyze recombination *in vitro*.

The L50R mutant is a dimer in solution, as assayed by gel filtration (data not shown). Although able to catalyze recombination *in vitro*, L50R has unusual DNA binding properties which suggest that dimer−dimer interactions are affected by this mutation. These properties are not fully characterized at this time but are otherwise consistent with the model of 1,2 representing the solution and DNA-binding dimer configuration.

Tryptophan fluorescence indicates that position L50 is solvent accessible while I110 is buried at an interface

Since none of the tryptophan substitutions disrupted the solution dimer, the fluorescence properties of the mutant proteins L50W and I110W were used to determine which tryptophan was buried in a dimer interface. Information about the polarity of the environment of a tryptophan can be obtained from the wavelength of its fluorescence maximum. The emission maximum of indole changes from 347 nm in water to 297 nm in cyclohexane (Sun and Song, 1977). In general, tryptophans in denatured proteins have an emission maximum at ~350 nm and folding produces a blue shift. In addition, solvent accessibility can be judged by the degree of fluorescence quenching by soluble agents such as I^- and Cs^+.

Wild-type resolvase has only one tryptophan, located in the DNA binding domain. In order to eliminate any confounding signal from this endogenous tryptophan, the fluorescence experiments were carried out with purified large N-terminal fragment of resolvase (amino acids 1−140). Figure 10A shows the emission spectra of the large fragment of L50W and I110W and the I110W intact protein. The emission maximum of L50W is at 353 nm, while that of I110W is at 328 nm. When denatured in 8 M urea both proteins have a maximum of 350 nm (data not shown). The blue shift of I110W implies that the tryptophan is shielded from solvent by a hydrophobic protein matrix. The spectrum of the I110W large fragment is essentially similar to that of the I110W intact protein. This is also the case for L50W fragment and intact protein (data not shown) and can be explained by the relatively low quantum yield of the endogenous tryptophan in the small domain (data not shown). The results of a quenching experiment are shown in Figure 10B. L50W was more easily quenched than I110W by I^- (and also by Cs^+; data not shown). These experiments show that the tryptophan at position 50 is more solvent accessible than that at position 110. These results are consistent with all those described above and with the conclusion that 1,2 is the solution dimer.

Although the tryptophan substitutions at positions 110 and 114 had no obvious effect on the dimeric state of resolvase, both the mutant proteins failed to promote recombination and exhibited altered DNA binding properties (data not shown). I110W bound with normal affinity to sites II and III but with substantially reduced affinity to site I, whereas V114W gave the opposite results, binding normally to site I but with reduced affinity to sites II and III (similar to the behavior of I110C—see Figure 5).

The M106C covalent dimer mediates synapsis and co-integrate relaxation

Despite the fact that M106C(SS) exhibits normal DNA binding properties, it does not recombine or catalyze Mg^{2+}-independent cleavage of co-integrate DNA *in vitro*. It is clear, therefore, that some feature of the covalent dimer is inhibitory to recombination. To gain a better understanding of the nature of this inhibition, we tested the ability of M106C(SS) to complement, *in vitro*, other mutant resolvases which are unable to interact productively with particular binding sites in *res*. Resolvase mutant S10L is unable to catalyze recombination because it lacks an active site Ser10. It also binds site I poorly (Hatfull and Grindley, 1986). E128K, whose active-site is functional, is unable to catalyze recombination because it fails to bind to site III. These two mutant resolvases are able to complement one another *in vitro*, since E128K can bind at site I and S10L can bind at site III (Hatfull *et al.*, 1987). M106C(SS) is able to complement the function of E128K but not S10L (Figure 11). From this we may conclude that the inhibitory effect of the disulfide does not operate at site III, and furthermore that no dissociation or re-orientation of the dimer bound at site III is required for recombination to proceed. The failure of M106C(SS) to complement S10L shows that the inhibitory

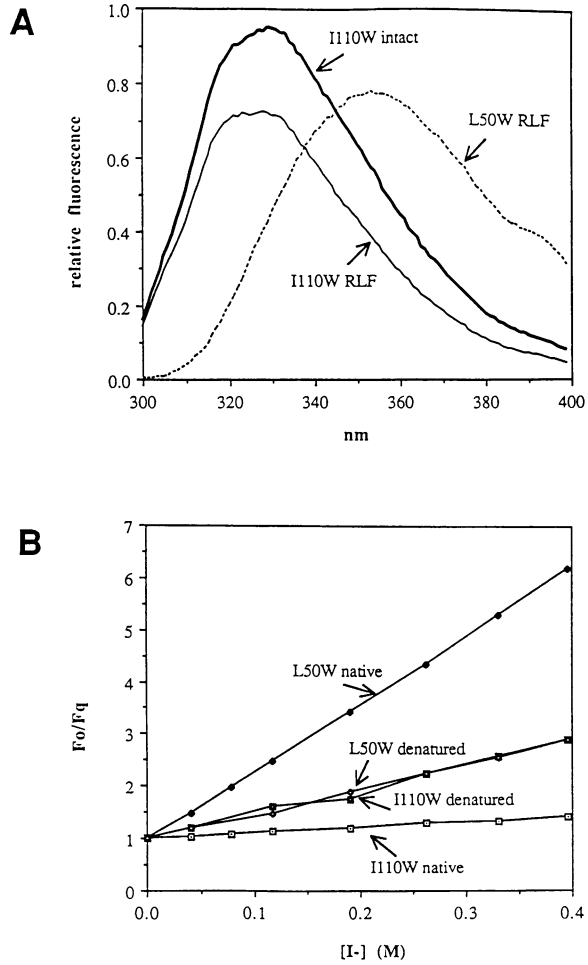

Fig. 10. Fluorescence quenching of resolvase tryptophan mutants L50W and I110W. (**A**) Fluoresence emission spectra of I110W and the large N-terminal fragment (RLF) of L50W and I110W. Fluorescence, in arbitrary units, is plotted versus wavelength. Protein concentrations for all samples were approximately equal. (**B**) Quenching of L50W and I110W (large fragment) fluorescence by KI in the native and denatured states. The ratio of fluorescence in the absence of KI to fluorescence in the presence of KI (F_o/F_q) is plotted versus concentration of KI. Non-native samples were denatured with 8.1 M urea.

effect of the disulfide operates at site I. This suggests either that recombination requires dissociation, or a specific conformational adjustment, of the dimer at site I which is prevented by disulfide linking, or that the disulfide introduces a structural perturbation in the dimer that prevents it from interacting appropriately with the crossover site.

While it is possible that recombination would require monomer−monomer dissociation to accompany the 180° strand rotation during crossover, such dissociation would not *a priori* be expected to be necessary for the initial DNA cleavage step. Wild-type resolvase efficiently cleaves supercoiled co-integrate DNA at the center of site I in the absence of Mg^{2+} and, since M106C(SS) is unable to cleave the DNA under these conditions, parsimony would favor an explanation involving distortion of the 1−2 interface. It is worth noting, however, that the interaction of M106C(SS) with site I introduces a degree of DNA bending similar to that introduced by the wild-type protein, indicating that at this relatively gross level, the protein−DNA interaction is normal (data not shown).

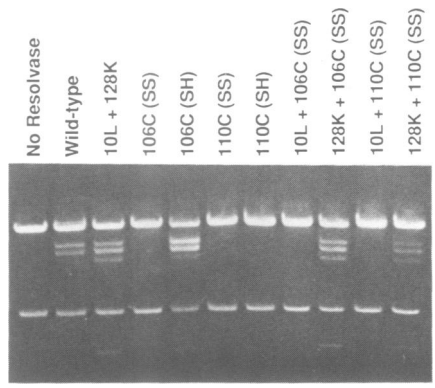

Fig. 11. Complementation between resolvase mutants *in vitro*. Reactions were standard *in vitro* resolution assays using the pRR51 co-integrate as a substrate. Resolvases were added as indicated to the reaction mixtures under non-reducing conditions. Reactions containing cysteine mutants were then divided in two and either left non-reducing (SS) or reduced with 1 mM DTT (SH). After 1 h reactions were digested with *Eco*RI, treated with proteinase K and analyzed by agarose gel electrophoresis. Doublets between the large and small bands are diagnostic of resolution.

Although we were unable to observe any recombination or Mg^{2+}-independent double strand cleavage activity of the M106C covalent dimer, we investigated whether it had any other detectable catalytic activity at the crossover site. Figure 12 shows the results of a topoisomerase assay performed with M106C covalent dimer. After a 10 h incubation with the disulfide-linked M106C under standard *in vitro* resolution conditions (but omitting reducing agent), we observed substantial relaxation of the co-integrate DNA (Figure 12, lane 2). Most of the product migrated at the position of fully relaxed or nicked circles (OC), while a small proportion migrated as linear molecules (L) or nearly relaxed topoisomers. Like resolution, this activity requires Mg^{2+} and two directly oriented *res* sites. Furthermore, the relative stoichiometry of resolvase to co-integrate DNA producing maximal relaxation (under oxidizing conditions) correlates directly with that producing maximal resolution (under reducing conditions) (data not shown). These observations support a model of M106C(SS)-mediated site-specific relaxation taking place in the context of a synaptosome, and possibly representing an intermediate step in the resolution reaction. Additional, independent evidence that disulfide-linked M106C resolvase forms synaptic complexes has been obtained using an assay for stable pairing of *res* sites designed by Parker and Halford (1992) (data not shown).

Since the oxidation of M106C to covalent dimer had not gone to completion (we estimate that ~5% remained in the free sulfhydryl form) it was important to determine whether the activity was catalyzed by the residual amount of non-covalent M106C or was an activity of the covalent dimer. One can envision a scenario where a few synaptosomes will contain a non-covalent dimer at a single site I. If the covalent dimers are catalytically inactive, these synaptosomes would presumably be limited to strand cleavages at that single crossover site (assuming action *in cis*; Dröge *et al.*, 1990), thus precluding resolution but potentially allowing relaxation or linearization. The alternative possibility is that the relaxation in a novel activity of the covalent dimer which results from inhibition of monomer−monomer dissociation, and/or distortion of the 1−2 dimer interface.

To decide between these possibilities we pursued two

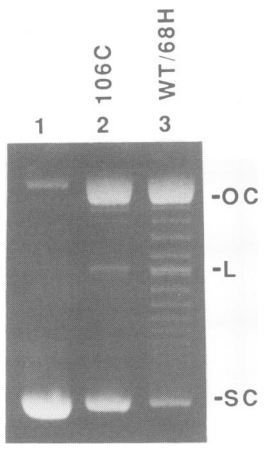

Fig. 12. Topoisomerase activity of M106C(SS) and a wild-type/R68H resolvase mixture. Lane 1 contains the co-integrate substrate pRR51 to which no resolvase has been added. The reactions were incubated with M106C(SS) (lane 2) or a 1:3 mixture of wild-type resolvase and R68H (lane 3). Positions of the supercoiled ('SC') and fully relaxed ('OC') forms are indicated; the latter included both nicked and covalently closed but relaxed species. The position of linear pRR51 is indicated ('L').

experimental approaches. In the first we reasoned that if the relaxation were due to non-covalent dimers we could enhance the relaxation activity by adding small amounts of wild-type resolvase to the reaction. Mixtures of wild-type and covalent M106C in the approximate ratios of 1:3, 1:9 and 1:27 showed no enhanced relaxation activity relative to the M106C alone (data not shown). This observation supports the conclusion that the relaxation activity is mediated by the covalent M106C dimer.

In a second approach we simulated a reaction in which catalytically active resolvase is limiting by using mixtures of wild-type protein and the mutant R68H (Hughes *et al.*, 1990). R68H is catalytically inactive, but retains the ability to synapse *res* sites in a co-integrate (Dröge *et al.*, 1990). When a mixture of wild-type and R68H (in the approximate ratios of 1:3) were incubated with a co-integrate under conditions identical to those used for M106C we observed a ladder of topoisomers, relaxed circles and some linear molecules (Figure 12, lane 3). These products presumably resulted from synaptic complexes which have a wild-type dimer at a site I and the majority of the remaining five sites occupied by R68H protein. When comparing the reaction products in lanes 2 and 3 the most notable difference is in the distribution of topoisomers. While the wild-type/R68H mixture produced a relatively even distribution of topoisomers, the M106C(SS) products were markedly biased toward the relaxed end of the distribution. Mixtures of M106C(SH) and R68H reacted under reducing conditions gave products similar to those seen with the wild-type/R68H mixture (data not shown). Examination of the kinetics of M106C(SS)-catalyzed relaxation showed the abrupt appearance of these highly relaxed topoisomers after 1 and 3 h of reaction (data not shown). Even at these early times we did not observe the more supercoiled distribution of products typical of the wild-type/R68H reaction, indicating that the bias toward full relaxation observed with M106C was not simply due to sampling at a relatively late time point in the reaction. In both reactions we verified that a fraction of the fully relaxed products were covalently closed circles (data not shown).

The situation created with the wild-type/R68H (or the M106C(SH)/R68H) mixture was intended to simulate the scenario mentioned above which places a non-covalent (and active) dimer of M106C at site I with the five remaining sites occupied by covalent (and inactive) dimers. If the latter case were responsible for the M106C-mediated relaxation we would expect to see a distribution of products similar to those of the wild-type/R68H reaction. The fact that the distribution of products in these two cases was dissimilar suggests that the relaxation we observed in each case may be mechanistically distinct. This lends further support to the conclusion that the relaxation is a novel property of the covalent M106C dimer.

Discussion

Implications of the 1,2 dimer

The crystallographic asymmetric unit contains three monomers of the resolvase catalytic domain arranged as two overlapping dimers (1,2 and 2,3) (Sanderson *et al.*, 1990). In this paper we have presented a variety of data which indicate conclusively that it is the 1,2 dimer that exists in solution and binds to the individual resolvase binding sites that constitute *res*. What are the implications of this conclusion? As described in more detail in the Introduction, our initial bias towards the 2,3 dimer as the potential catalytic unit of resolvase was driven by the suitable distance between the active site Ser10 residues, by its participation in a tetramer of 222 symmetry and by experimental evidence that interactions at this tetrameric interface were indeed relevant to recombination activity (Hughes *et al.*, 1990; Sanderson *et al.*, 1990). By contrast, the 1,2 dimer, although favored by physical–chemical considerations of the interface, had an inappropriately large distance between Ser10 residues (>30 Å) and was not seen to participate in a 222-symmetrical tetramer of the sort expected to be necessary for pairing the two crossover sites.

The apparent paradox presented by the 1,2 dimer could be resolved in either of two ways. First, the disparity of distance between the 1,2 Ser10 positions and the scissile bonds at the center of site I may simply be due to the fact that either the DNA, the resolvase or both are distorted during recombination. It is known that the DNA at the center of site I is kinked (Hatfull *et al.*, 1987) and it seems reasonable to allow that in the context of DNA binding or synaptosome formation, which involves a complex array of macromolecular interactions, the 1,2 dimer may become conformationally distinct from its crystallographic and/or solution form. DNA-induced distortion of the dimer conformation of a DNA binding protein has been inferred for the λ Cro protein from crystallographic data, which show that the quaternary structures of the free Cro dimer and Cro dimer complexed with its operator site differ significantly, DNA binding being accompanied by a large twist between the monomers (Anderson *et al.*, 1981; Brennan *et al.*, 1990). In this scenario the 1,2 dimer (or a structural variant of it) would be the catalytic unit of resolvase, although how two such 1,2 dimers interact to bring together the two crossover sites remains a mystery. The second model postulates that while the 1,2 dimer is the form that initially binds to the three sites within *res*, catalysis of strand cleavage and ligation involves the action of a different dimeric unit (presumably the 2,3 dimer) across the dyad of each site I. In this model

the catalytic dimer would be formed and stabilized in the context of the synaptosome—it could result from rearrangement of each 1,2 dimer initially bound to the two copies of site I or it could be recruited to the crossover site from other resolvase protomers bound elsewhere in the synaptic complex. An attraction of this second model is that formation of an activated transient dimer could represent a mechanism for the observed restriction of resolvase-mediated DNA cleavage to the synaptic complex (Reed and Grindley, 1981; Krasnow *et al.*, 1983).

Currently there is no experimental evidence that conclusively rules out either of the above models. However, there are data that tend to favor the hypothesis that the 1,2 dimer is also the catalytic unit of resolvase. First, coupling the hydroxyl radical generator EDTA·Fe to position 10 of resolvase promotes cleavage of site I DNA immediately adjacent to the crossover point (Mazzarelli *et al.*, 1993). This suggests, on the one hand, that in a complex with site I alone the crossover point is the region of the DNA closest to Ser10 and, on the other hand, that the two Ser10 residues of the 1,2 dimer (the form that binds site I) are much closer together when complexed with DNA, than was indicated by the crystal structure. Second, the catalytic activity of the M106C covalent dimer demonstrated here suggests that total rearrangement of the 1,2 dimer initially bound at site I cannot be essential for catalysis, since the disulfide bond would prevent dissociation. This appears to rule out the possibility that a 1,2 dimer bound at site I is converted to a 2,3 dimer within the synaptosome. Third, the direct catalytic participation of resolvase dimers bound at the accessory sites is rendered unlikely by the finding that the catalytically-defective mutant S10L can fulfil the role of wild-type resolvase at least when bound at site III (and perhaps also at site II) (Hatfull *et al.*, 1987).

Engineered intersubunit disulfide bonds have been used with the λ *c*I repressor and the λ Cro proteins to address structure−function questions (Sauer *et al.*, 1986; Hubbard *et al.*, 1990; Shirakawa *et al.*, 1991). With both of these proteins, structural information was used to select positions for mutation to cysteine in order to optimize formation of covalent dimers. Both of these mutants (*c*I repressor 88C and Cro 55C) spontaneously form covalent dimers in the absence of reducing agent as does resolvase M106C. In both cases the covalent dimers show enhanced stability to denaturation, but have ~10-fold lower affinities for their specific DNA binding sites (O_R1 for *c*I repressor; O_R3 for Cro). The observed reduction in specific binding affinity indicates that the intersubunit bonds introduce quaternary structural perturbations which disfavor dimer conformations optimal for specific binding. This could represent an overall distortion of quarternary structure in the covalent dimer and/or an inhibition of induced fit of the dimer interface during DNA binding (see Benevides *et al.*, 1991). Formation of a disulfide bond at a position unfavorable to the structure of the λ repressor dimer interface (85C) resulted in a 200-fold reduction in specific DNA binding. This observation may be similar, though not strictly analogous from a structural consideration, to the severe inhibition of DNA binding activity we observe for resolvase L50C in its covalent form.

With the M106C covalent dimer we also observe an alteration in DNA binding affinity. This is evident in binding assays either with *res* or with mixtures of individual sites on separate molecules. In either situation, we observe a decrease in the affinity of 106C(SS) for site I, in contrast to its relatively normal affinities for sites II and III. Resolvase's ability to bind sites with different spacing between half-sites is best explained by imagining that the resolvase dimer possesses considerable tertiary and/or quarternary structural flexibility which allows it to specifically bind sites with different geometries. Indeed, in the refined crystal structure of the resolvase catalytic domain it is clear that both the monomer and the interface of the 1,2 dimer undergo significant flexing (Rice, 1992). The *Escherichia coli* AraC protein and the α2 protein from yeast are both able to bind DNA sites with a range of helical geometries and, more dramatically, with both inverted and direct half-site orientations. This suggests that the DNA binding domains of these proteins have considerable freedom to rotate relative to the rest of the protein (Smith and Johnson, 1992; Carra and Schleif, 1993). The disorder of the resolvase DNA binding domain in the crystal structure of the intact protein (Abdel-Meguid *et al.*, 1984) suggests that the DNA binding domain may be flexibly attached to the catalytic domain. What role this may play in site recognition is unknown. Presumably the disulfide bond between the Cys106 residues would not directly affect the structure of the DNA binding domain, therefore its influence on quarternary structure must account for its effect on site affinity. This observation, along with the site affinity phenotypes of other mutations in the region [e.g. E102C (Mazzarelli,J., unpublished data); I110C, I110W, V114W (this paper); and E118K and E128K (Hatfull *et al.*, 1987)] support the notion that an induced conformational adjustment involving this helix of the 1,2 dimer interface accompanies DNA binding in a manner specific to the geometry of the site.

Studies on the DNA invertases Gin and Cin, which share structural and functional homology with the resolvases (Hatfull and Grindley, 1988), also implicate the 1−2 interface as playing an important role in recombination. Gin mutants F104 V and H106Y as well as Cin H106Y (corresponding to positions 107 and 109 in γδ resolvase) were isolated in genetic screens for invertases able to promote recombination independent of the enhancer protein FIS (Haffter and Bickle, 1988; Klippel *et al.*, 1988). The position of these mutations suggest that they are likely to mediate Fis independence by affecting the 1,2 dimeric interaction of the DNA invertase (Sanderson *et al.*, 1990). However, it is not yet known whether the mutations increase or reduce dimer stability or facilitate a conformational transition at the dimer interface that is essential for recombination.

Implication of the 2−3 and 2−3′ interactions

Although it is not involved in formation of the resolvase dimer, the 2−3 interface appears to mediate a relevant intersubunit interaction. The L50C mutation affects the way resolvase assembles on the *res* site, apparently reducing the cooperativity of binding. This behavior is similar to that resulting from mutations at the 2−3′ interface but is not as severe. An important difference between L50C and mutants with defective 2−3′ interactions is that the former catalyzes recombination, albeit weakly. These and our earlier results (Hughes *et al.*, 1990) now implicate both the 2−3 and 2−3′ interfaces in the formation of higher order complexes between DNA-bound resolvase dimers. The use of both 2−3 and 2−3′ interfaces for interactions between resolvase

dimers seems quite reasonable since 1,2 dimers bound to adjacent DNA sites could potentially present either one of these interfaces to one another. We do not know, however, whether either of these interactions exists independently in resolvase–DNA complexes. In the crystal structure, the combination of 2–3 and 2–3′ interfaces assemble four resolvase monomers into a tetramer with 222 symmetry (Sanderson *et al.*, 1990). Since each of these monomers is joined to a second monomer at the 1–2 interface the tetramer can actually be considered a tetramer of 1,2 dimers. We propose that this octomer may serve as the core of the synaptic complex, using the four DNA binding (1,2) dimers to link together sites II and III of one *res* to the corresponding sites of the other.

The unusual topoisomerase activity of M106C(SS)

Little is known about the mechanism of DNA relaxation by resolvase other than that it appears to require the initial formation of a synaptosome (Krasnow and Cozzarelli, 1983) and that it can proceed when only one of the two DNA strands at the crossover point is cleavable (Falvey *et al.*, 1988). This latter result suggests that topoisomerase activity may be enhanced in situations where strand scission occurs but crossover into the recombinant configuration is inhibited. Our observation of relaxation catalyzed by the R68H/wild-type resolvase mixture supports this hypothesis. In this case strand cleavage is presumed to occur at the crossover site occupied by the wild-type resolvase dimer, but crossover is prohibited due to the inability to cleave at the other site. Relaxation is a normal activity of resolvase, but is pre-empted by resolution. Situations like those described above may simply unmask this activity by specifically inhibiting resolution under conditions that allow limited modes of catalysis.

Much as is the case when considering the mechanism of recombination (see below), a key aspect of models for relaxation is whether, or to what extent, movement of protein accompanies the required movement of DNA. Since the crossover point is spanned by a resolvase dimer and relaxation must involve iterative rounds of DNA strand rotation, relaxation must be accompanied either by subunit dissociation in the dimer bound at site I, or by dissociation of protein–DNA contacts at one half of site I. Furthermore, since the wild-type topoisomerase activity is apparently controlled, giving rise to a relatively even distribution of topoisomers, transient dissociation and stepwise re-establishment of specific macromolecular contacts (be they protein–protein or protein–DNA) are likely to provide a gating mechanism for the reaction (see Champoux, 1990).

Topoisomerase activity catalyzed by the M106C covalent dimer must proceed via dissociation of protein–DNA contacts, since dimer dissociation is prevented by the disulfide. The fully relaxed state of the products of this activity indicates that the gating function is lost; this could be explained in either of two ways. It is possible that distortions of the 1,2 dimer, resulting from the disulfide linkage, cause the loss of a gating function by reducing the efficiency of either the ligation step or the re-establishment of the monomer–DNA contact. Alternatively the significant difference between M106C(SS) and wild-type resolvase may be simply the presence of the disulfide and the consequent inability of the 1,2 dimer to dissociate. Under this scenario, loss of gating by the covalent dimer would be interpreted as a direct consequence of its requirement for dissociation from one half of site I; the ability of wild-type resolvase to give gated relaxation would then imply that it maintains the DNA contact throughout and, thus, dissociates the catalytic dimer instead.

Concluding remarks

Topological analyses of the resolvase-mediated recombination indicate that the crossover of DNA strands proceeds through a simple right hand rotation of cleaved duplex ends; it has been proposed that this is accompanied by the exchange *in trans* of subunits between resolvase dimers bound at site I (Stark *et al.*, 1989; Kanaar *et al.*, 1990). This so called 'subunit exchange' model provides a mechanism for the orderly movement of the covalent protein–DNA adducts and the maintenance of phosphoester bond energy through the final ligation of the recombinant joints. A less attractive feature of the subunit exchange model is its requirement for relatively gross perturbations of the quaternary structure at the dimer interface. The structure of the 1,2 interface would not seem to lend itself easily to such perturbations. If our surmise regarding dimer dissociation during wild-type topoisomerase activity is correct, however, we may have found an indication of the 1,2 dimer's ability to dissociate during catalysis. Since our reticence in modeling 1,2 dimer dissociations (and/or rotations) is founded on the structure of the interface, as observed by crystallography, we must remain mindful of potential differences between this and the nature of the solution interface. Studies of the Tn*3* resolvase and the Gin invertase suggest these recombinases may exhibit significant monomeric behavior in solution (Bednarz *et al.*, 1990; Kahmann,R., personal communication). Finally, we have no information about the nature of the 1,2 interface once resolvase in bound to DNA or after synapsis has occurred. Nevertheless, the interlocked nature or the 1,2 interface, and the resulting resistance to rotation, may make it necessary to consider strand exchange mechanisms that do not involve concomitant exchange of catalytic subunits.

Work reported in this study, as well as in a previous one (Hughes *et al.*, 1990), has shown that the contacts between resolvase monomers apparent in the crystal structure have proven to be relevant to some aspect of the protein's function. It is gratifying to observe that these physical molecular arrangements are reflective of higher order biological processes. This correspondence between protein–protein interactions in the crystalline state and *in vivo* has also been observed with the RecA protein whose crystal packing in a six-fold symmetrical helix is strikingly similar to observations of RecA polymerized onto DNA as observed by electron microscopy (Egelman and Stasiak, 1986; Story *et al.*, 1992). Our analysis of the crystal structure of the resolvase large domain has shown that crystallographic information can be very informative about not only tertiary but quaternary structure and function.

Materials and methods

Design of disulphide bonds

Single cysteine substitutions were chosen so as to allow formation of disulfide bonds between symmetry-related cysteine residues across an intermonomeric two-fold axis. L50 is the only residue at the 2–3 interface where the symmetry related α-carbons come within 10 Å of one another. The 1–2 interface includes two long α-helices that interact along their length, providing positions M103, M106, I110 and V114 as candidates for disulfide bonds.

Disulfide bonds at these positions were inserted into a model of the 1,2 dimer using the interactive graphics program FRODO. Using stereochemical terms only (with no X-ray terms included in the energy function), these models and a wild-type model were then subjected to 60 cycles of energy minimization with the program XPLOR (Brünger *et al.*, 1987). The candidates were then judged based on the severity of distortions induced in the minimized model and on the geometry of the disulfide. Based on this procedure, M106C was judged to be the best and M103C the worst.

Plasmids and mutagenesis

Oligonucleotide-directed mutagenesis was done as described by Kunkel *et al.* (1987). The presence of the complete *tnpR* gene in M13 resulted in low yields of uridine-containing template DNA. We therefore made a truncated version of *tnpR* (*tnpR-ΔEag*I) which includes the N-terminal domain of *tnpR* up to an *Eag*I site which was introduced by oligonucleotide-directed mutagenesis, overlapping codons 140–142 at the junction of the N- and C-terminal domains. pNG247, which contains the *tnpR-ΔEag*I truncated gene cloned into M13mp11, was used to make U-containing template. The desired mutant *tnpR-ΔEag*I genes were subcloned as *Bst*XI–*Eag*I fragments into pNG250, to reconstruct the complete *tnpR-Eag*I gene. *Bst*XI–*Sal*I fragments containing the *tnpR-Eag*I mutant genes were subcloned from the pNG250 derivatives into pAS1 (Rosenberg *et al.*, 1983) to create the resolvase overproducer plasmids.

Purification of resolvases

With the exceptions of I110R and V114R, resolvases were purified essentially as described by Reed (1981). I110R and V114R could not be extracted from the cell pellet with 2 M NaCl (the normal procedure). These mutants were extracted with a solution of 8 M urea, 20 mM Tris (pH 8.0), 10 mM $MgCl_2$ and subsequently exchanged into a solution of 2 M NaCl, 20 mM Tris (pH 8.0), 10 mM $MgCl_2$ by dialysis. A similar denaturation/renaturation procedure is part of the standard resolvase purification and does not significantly affect the activity of the wild-type protein.

Formation of intermonomeric disulfide bonds was promoted by dialysis of each cysteine-substituted resolvase against buffer containing 20 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$ and 2 M NaCl with air bubbling through it. Samples were dialyzed for 12 h at 4°C. It was determined that this duration of air oxidation produced maximal disulfide formation.

The solution molecular weight of resolvase derivatives was determined by gel filtration chromatography. Samples (200 μl) containing oxidized resolvase and the internal molecular weight markers, Klenow fragment of DNA polymerase I (68 kDa) and β-lactoglobulin (35 kDa) in 20 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$ and 2 M NaCl, were loaded onto an FPLC Superose 12 column (Pharmacia) and eluted with the same buffer. Fractions (0.5 ml) were collected and samples from these were analyzed by SDS–PAGE; proteins were visualized by Coomassie blue staining.

Fluorescence quenching of tryptophan mutants

An SML 8000C spectrofluorimeter (SML Aminco) was used for the fluorescence measurements. Standard conditions for the experiment were: 5.6–6.5 μM protein in 5% saturated $(NH_4)_2SO_4$, 20 mM Tris (pH 7.5) at 20°C. The excitation wavelength was 290 nm. Emission spectra of large fragment I110W and L50W taken at 2× and 0.1× this protein concentration were essentially unchanged in shape, as were spectra taken in 2 M NaCl rather than 5% saturated $(NH_4)_2SO_4$. For denatured samples 8.1 M urea was added to the standard buffer. Quenching experiments were done by sequentially adding 20 ml aliquots of 4 M KI or Cs_2SO_4 to 2 ml protein samples in standard buffer (to a final $[I^-]$ or $[Cs^+]$ of 0.4 M). Fluorescence was monitored at the λ_{max} for emission of the particular sample: 328 nm for I110W, 353 nm for L50W and 350 nm for denatured samples. The overall shape of the emission curve was checked after the last addition of quenching agent to verify that the protein was not perturbed.

Binding and reactions in vitro

Conditions for native PAGE of resolvase–DNA complexes and resolution *in vitro* have been described previously (Reed, 1981; Hatfull and Grindley, 1986). Unless specifically indicated, all resolvase reactions were done in the absence of reducing agent. Reactions done under reducing conditions included 1 mM DTT. DNA substrates for gel retardation assays shown in this paper are as follows: Site I shown in Figure 7 is a 92 bp *Sal*I–*Eco*RI fragment of pRW109 (Wells and Grindley, 1984); *res* site-containing DNA shown in Figures 6, 8 and 9 is a 203 bp *Sal*I–*Hin*dIII fragment from pRW80 the co-integrate analog with two copies of *resΔ101R* (Wells and Grindley, 1984); site I-G4C shown in Figure 9A is a 63 bp *Eco*RI–*Bgl*II fragment from pVR42. This site I has a C substituted for G at the 4 position of site I-R (Rimphanitchayakit and Grindley, 1990) which enhances the affinity of this half-site. All the DNAs mentioned above were uniquely 3′ end-labeled with α-^{32}P-labeled nucleotides using the Klenow fragment of DNA polymerase I (a gift of Cathy Joyce) and gel purified. The mixture of sites I, II and III was generated by digestion of pVR90 with *Bam*HI and *Hin*dIII, 3′ end-labeling as above and subsequent digestion with *Asp*718 and *Sal*I. These labeled fragments were separated from unincorporated labeled nucleotides by passing the reaction over a Sephadex G-50 (Pharmacia) spun column.

The assay for synapsis by inhibition of Tn*21* resolution and pCP10 have been described previously (Parker and Halford, 1991). The assay for topoisomerase activity was essentially an *in vitro* resolution reaction under non-reducing conditions. These reactions were incubated at 37°C for 10 h, treated with proteinase K and run on a 1% agarose (Sigma) TAE gel at 2 V/cm for 12 h. Gels were stained with ethidium bromide and visualized by UV transillumination.

Acknowledgements

We thank Vicky Derbyshire for help with the gel filtration analysis. We thank Christian Parker and Joan Mazzarelli for helpful discussions and comments on the manuscript. We thank Kate Tatham for endless patience with the manuscript preparation. The research was supported by NIH grants GM22778 to T.A.S. and GM28470 to N.D.F.G. P.A.R. was supported by an NSF pre-doctoral fellowship.

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Received on November 16, 1992