Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 5429–5433, August 1986
Biochemistry

Analysis of γδ resolvase mutants *in vitro*: Evidence for an interaction between serine-10 of resolvase and site I of *res*

(site-specific recombination/transposon γδ/protein–DNA complexes)

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Communicated by Melvin I. Simon, April 10, 1986

ABSTRACT **The resolvase encoded by the transposon γδ mediates a site-specific recombination between the two copies of γδ in a cointegrate molecule to yield the final products of transposition. Several mutants of resolvase that lack recombinational activity have been isolated previously, and one of these, which has a serine-to-leucine change at position 10, we have characterized *in vitro*. We also have constructed a serine-to-cysteine change at position 10 by *in vitro* mutagenesis and have analyzed this mutant protein *in vitro*. We find that the cysteine-10 mutant is defective in recombinational activity but binds to the recombinational site, *res*, similarly to wild-type, as assayed by gel electrophoresis of the protein–DNA complexes. In contrast, the leucine-10 mutant has a specific defect in complex formation with site I, which contains the recombinational crossover point, although it can bind and provide the ancillary functions of resolvase at sites II and III. It has been shown that a phosphoserine linkage is made to the DNA during recombination; because serine-10 is absolutely conserved amongst the family of homologous site-specific recombination proteins, it is a good candidate for the active-site serine. The properties of these resolvase mutants with substitutions at position 10 are consistent with this hypothesis and suggest that serine-10 is in close contact with the DNA at site I.**

Transposition of γδ, one of a family of prokaryotic transposable elements related to Tn*3*, occurs in two distinct stages (for reviews, see refs. 1 and 2). The first step, mediated by the *tnpA* gene product, is the fusion of donor and target replicons to form a cointegrate molecule. During this process the transposon is duplicated so that there are two copies of the element in the same orientation, one at each junction of the donor and target sequences. In the second stage, the product of the *tnpR* gene, resolvase, mediates a conservative site-specific recombination between the two transposons to yield the final products of transposition: donor and target molecules, each containing one copy of the transposon. In this second step, resolvase acts at a site, *res*, within the *tnpA–tnpR* intercistronic region (3, 4). Promoters for both the *tnpA* and *tnpR* genes lie within *res*, and resolvase acts both as a recombination mediator and as a transcriptional regulator (5, 6).

The 115-base-pair (bp) *res* site (Fig. 1) contains three binding sites for resolvase (6, 7). Each consists of two imperfectly conserved 9-bp inverted repeats (consensus sequence 5′ TGTCYNNTA, where Y = pyrimidine and N = any base) separated by a short spacer of variable length (10 bp in site I, 16 bp in site II, and 7 bp in site III). The crossover point lies at the center of the 10-bp spacer in site I (8), although all three sites must be present for efficient recombination (6, 7). The requirements for the site-specific recombination reaction *in vitro* are simple, and efficient cointegrate resolution is observed by using a negatively supercoiled DNA substrate with two directly repeated copies of *res*, purified resolvase protein, Mg^{2+}, and a simple buffer (9).

γδ resolvase has a modular construction and can be proteolytically cleaved into two domains: a 43-amino acid C-terminal domain that has DNA binding activity and recognizes the 9-bp binding sequence and a 140-amino acid N-terminal domain that provides protein–protein interactions and the catalytic functions (10). Resolvase probably binds as a dimer, since each binding site has dyad symmetry, and resolvase binds cooperatively to the two halves. The active form of the related resolvase of Tn*21* has been shown to be a dimer (11). The N-terminal domain has no detectable DNA binding activity, although it is presumably in close proximity to the crossover point at the center of site I in order to perform its catalytic functions. This idea is supported by the observation that intact resolvase but not the C-terminal fragment protects the center of site I from DNase I digestion (10).

Reed and Moser (12) have shown that, in an apparent intermediate in recombination, resolvase is covalently attached to the DNA via a phosphoserine linkage. The precise serine involved has proved difficult to determine biochemically, but as only two serines (positions 10 and 39 in γδ resolvase) are highly conserved amongst related site-specific recombination proteins (residue 39 is a threonine in the Tn*917* resolvase; ref. 13), it is likely to be one of these serines (see ref. 14).

tnpR1 encodes an altered resolvase (serine-10 to leucine) that is one of several resolvase mutants that have lost recombinational functions but retained DNA binding activity (15). The regulatory properties of this mutant gene product suggested that it binds less well to site I than to sites II and III, and we decided to analyze this mutant *in vitro*. For comparison, we also have constructed a mutant resolvase with the more conservative change to cysteine at position 10, using oligonucleotide-directed mutagenesis. The DNA binding properties of several proteins have been studied by polyacrylamide gel electrophoresis (16–18), and we have used this technique to compare the binding properties of the Leu-10 (*tnpR1*), Cys-10, and wild-type resolvases. We find that the Leu-10 resolvase has a specific defect in binding to site I, regardless of whether sites II and III are present on the same fragment. Cys-10 has no recombinational activity but binds to all three sites in a way similar to that of wild-type. These results suggest that serine-10 is in close proximity to the DNA at site I and are consistent with the hypothesis that serine-10 is an active-site residue.

MATERIALS AND METHODS

Oligonucleotide-directed mutagenesis was used to introduce a cysteine codon at position 10 by using methods described by Kunkel (19). A phage M13 clone carrying the resolvase

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Abbreviation: bp, base pair(s).

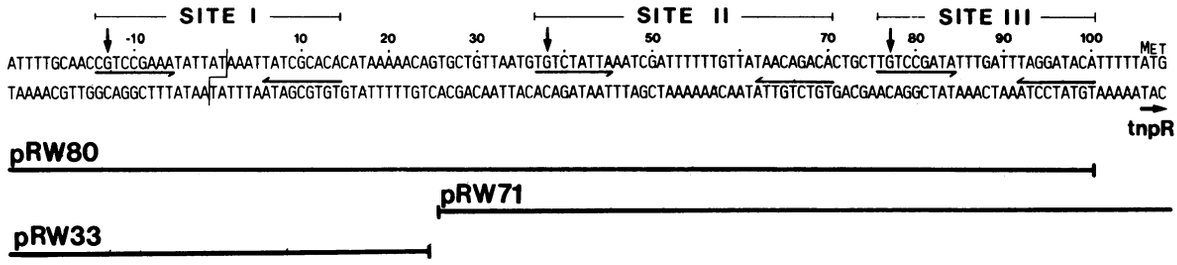

FIG. 1. Sequence of the γδ transposon *res* site showing the three resolvase-binding sites. The numbering scheme is the same as that used previously (6) and runs from the center of the crossover point in site I (marked). The horizontal arrows show the 9-bp binding sequences within each site that are related to the consensus sequence 5′ TGTCYNNTA. Methylation of several purines by dimethyl sulfate strongly inhibits binding (see Fig. 4); these include the guanine residues on the top strand at −13, +38, and +77 (marked by vertical arrows), which we have used as indicators of resolvase binding to sites I, II, and III, respectively. The lines below the sequence indicate the *res* site sequences contained in the DNA fragments used in the binding studies.

gene (mGH-157) was used as a substrate for the mutagenesis, and the mutant gene was sequenced by using appropriate primers. All oligonucleotides were synthesized on a Biosearch SAM One. The mutant gene was cloned into the plasmid pAS-1 for overproduction and purification of the mutant protein (21). In both the initial M13 clone and the Cys-10 mutant clone, there is an additional threonine residue at position 2 in the protein as a result of previous cloning steps to increase the efficiency of overproduction. The addition of the threonine to wild-type resolvase has no effect in any of the functional assays we have tried.

The standard resolution reaction (10 μl) was similar to that described by Reed (9) and contained 20 mM Tris (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 μg of bovine serum albumin per ml, 100 mM NaCl, 0.25 μg of pRR51 DNA, and resolvase. Reactions were incubated at 37°C for 30 min, followed by 10 min at 65°C, and then were digested with *Eco*RI in a final volume of 20 μl. Proteinase K was added to a final concentration of 1 mg/ml, and incubation was continued at 37°C for 5 min before loading the entire reaction onto a 1% agarose gel.

DNA restriction fragments were labeled at their 5′ ends as described by Maxam and Gilbert (20). DNA fragments used were the 204-bp *Sal* I–*Hin*dIII fragment from plasmid pRW80 (with an intact *res* site), the 128-bp *Sal* I–*Hin*dIII fragment from pRW33 (with site I only), and the 210-bp *Eco*RI–*Pvu* I fragment from pRW71 (contains sites II and III but not site I) (see Fig. 1). These fragments originate from the *res* site deletions Δ101R, Δ24R, and Δ25L, which are described elsewhere (6). Wild-type and mutant resolvases were purified as described by Reed (9) with minor modifications, notably the use of low-salt precipitation as a purification step.

Resolvase binding reactions were similar to those used for previous DNase I cleavage-inhibition experiments (7). Resolvase was mixed with labeled DNA fragments (<10 nM) in 10-μl reactions containing 20 mM Tris (pH 7.5), 5 mM $MgCl_2$, 100 mM NaCl, 0.1 mM dithiothreitol, 100 μg of bovine serum albumin per ml, and 100 μg of sonicated calf thymus DNA per ml. After incubation for binding at 37°C for 5 min and equilibration to room temperature for 5 min, 3 μl of loading buffer (5% Ficoll in 20 mM Tris, pH 7.5/5 mM $MgCl_2$/100 mM NaCl/0.1% xylene cyanol FF) was added, and the entire reaction was loaded onto a 1.5-mm 5% polyacrylamide gel (polyacrylamide/methylenebisacrylamide, 30:1; 100 mM Tris borate, pH 8.3/1 mM EDTA). The gel was prerun for 10 min at 120 V and left running while the samples were loaded. Preliminary experiments showed that the reactions were at equilibrium. On completion of the electrophoresis, the gels were dried down and autoradiographed.

DNA fragments were chemically modified by using procedures similar to those of Maxam and Gilbert (20). DNA fragments were partially methylated by incubation with 1 μl of dimethyl sulfate at 20°C in 200 μl of 50 mM cacodylate/10 mM $MgCl_2$/0.1 mM EDTA for 1 min; the reaction was stopped by adding 50 μl of 1 M Tris acetate, pH 7.5/1 M 2-mercaptoethanol/1.5 M NaOAc/50 mM $Mg(OAc)_2$/1 mM EDTA. The DNA was precipitated with ethanol and resuspended in 10 mM Tris, pH 7.5/1 mM EDTA.

Protein–DNA complexes formed with modified DNA were separated from uncomplexed DNA by polyacrylamide gel electrophoresis as described above. Bands corresponding to complexed and uncomplexed DNA were identified by autoradiography and excised, and the DNA was eluted. The DNA was cleaved at the modified positions by incubation at 90°C for 10 min in 10 mM phosphate (pH 7.5) followed by incubation at 90°C for 30 min in 10 mM phosphate/150 mM NaOH, and the products were analyzed on 6% sequencing gels.

RESULTS

***In Vitro* Resolution Activity of Leu-10 and Cys-10 Resolvases.** Leu-10 resolvase is totally defective in resolution activity *in vivo* (15). We assayed the resolution activity of Leu-10 resolvase in a standard *in vitro* assay and could not detect any activity (Fig. 2, lane 3). However, we found that Leu-10 resolvase could provide some of the functions necessary for resolution *in vitro*. The extent of the *in vitro* resolution reaction with wild-type resolvase could be limited by reducing the amount of resolvase below that necessary to

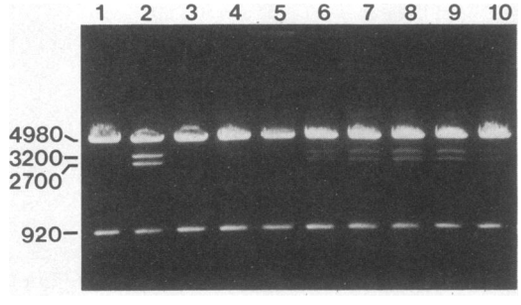

FIG. 2. Resolution activity of Leu-10 resolvase *in vitro*. Resolution reactions were performed under standard conditions, and the DNA was cleaved with *Eco*RI before electrophoresis. The 4980-bp and 920-bp fragments represent unreacted starting material, and the 3200-bp and 2700-bp fragments are the products of resolution. Protein additions were as follows: no resolvase (lane 1), 10 pmol of wild-type resolvase (lane 2), and 16.7 pmol of Leu-10 resolvase (lane 3). The remaining reactions (lanes 4–10) each had 0.5 pmol of wild-type resolvase and the following amounts of Leu-10 resolvase: none (lane 4), 50 pmol (lane 5), 16.7 pmol (lane 6), 5 pmol (lane 7), 1.67 pmol (lane 8), 0.5 pmol (lane 9), and 0.17 pmol (lane 10). The amount of resolution seen in lane 4 is less than that expected for 0.5 pmol of wild-type resolvase and 0.06 pmol of substrate DNA and implies that the amount of active protein is only about 0.25 pmol.

occupy the six binding sites on each cointegrate molecule (Fig. 2, lane 4). Addition of Leu-10 resolvase to this limited reaction increased the yield of resolved products (Fig. 2, lanes 5–10), even though the Leu-10 resolvase alone had no catalytic activity. This enhancement occurred over a broad range of amounts of Leu-10 resolvase added (0.5–16.7 pmol). Addition of more than this (50 pmol) inhibited the activity of the wild-type protein (Fig. 2, lane 5), and less (0.17 pmol) resulted in little or no enhancement (Fig. 2, lane 10).

Cys-10 resolvase also had no recombinational activity *in vivo* or *in vitro*, although it showed a similar pattern of enhancement to that seen with Leu-10 resolvase (data not shown).

Binding of Wild-Type and Mutant Resolvases to Sites I, II, and III. Increasing concentrations of protein were added to a mixture of two labeled DNA fragments, one that contained only site I and one that contained sites II and III, and the products were analyzed by polyacrylamide gel electrophoresis (Fig. 3). With wild-type resolvase, both fragments formed complexes with the same efficiency, with an apparent equilibrium dissociation constant (K_d) of about 10^{-8} M, in good agreement with the K_d for *res* DNA determined by DNase cleavage-inhibition studies (10). The site I DNA (which has only a single site) formed only one complex with resolvase, whereas site II/III DNA (which has two sites) formed two separable complexes.

The genetic characterization of *tnpR1* suggested that the Leu-10 resolvase may have a reduced affinity for site I relative to sites II and III (15). To test this hypothesis, we compared the ability of Leu-10 and wild-type resolvases to form complexes with the different binding sites. Fig. 3 *Upper* shows a comparison of the complexes formed by wild-type and Leu-10 resolvases. Leu-10 resolvase was clearly defective in complex formation with site I DNA but bound to site II/III DNA similarly to wild-type resolvase, although the complexes with both DNA fragments had slightly altered mobilities. Even at high protein concentrations of Leu-10 resolvase, only a small amount of site I DNA was complexed.

In contrast, Cys-10 resolvase regained most of the binding activity lost in Leu-10 resolvase (Fig. 3 *Lower*), and bound site I DNA almost as well as did wild type. The affinity for site I DNA was slightly less than that for site II/III DNA as judged by the disappearance of unbound DNA with increasing protein concentration.

We also compared complex formation of wild-type and Leu-10 resolvases with sites I, II, and III, each on separate DNA fragments (data not shown). The affinity of wild-type resolvase was somewhat lower for site III than for sites I and II in this assay. Leu-10 resolvase also bound site III poorly, the ratio of site III to site II complex being lower than that with wild-type resolvase. However, the defect in binding of Leu-10 resolvase to site I was substantially greater than the defect in binding to site III as compared with wild-type resolvase.

Interaction of Wild-Type and Leu-10 Resolvases with *res*. The natural substrate for resolvase, *res*, has three binding sites. Having established a defect in the binding of Leu-10 resolvase to site I alone, we wished to determine if its binding to site I is influenced by the presence of sites II and III on the same fragment. The products formed by binding wild-type or Leu-10 resolvase to *res* DNA are shown in Fig. 4. As increasing amounts of wild-type protein were added, several complexes were observed. We tentatively assign these as DNA complexed with one dimer (the fastest moving complex), two dimers, and finally, with the slowest mobility, three dimers of resolvase. Cys-10 resolvase had an identical pattern of complex formation with *res* DNA as wild-type resolvase had (data not shown).

Leu-10 resolvase formed similar complexes with *res*, but the pattern of complex formation was different. The one-

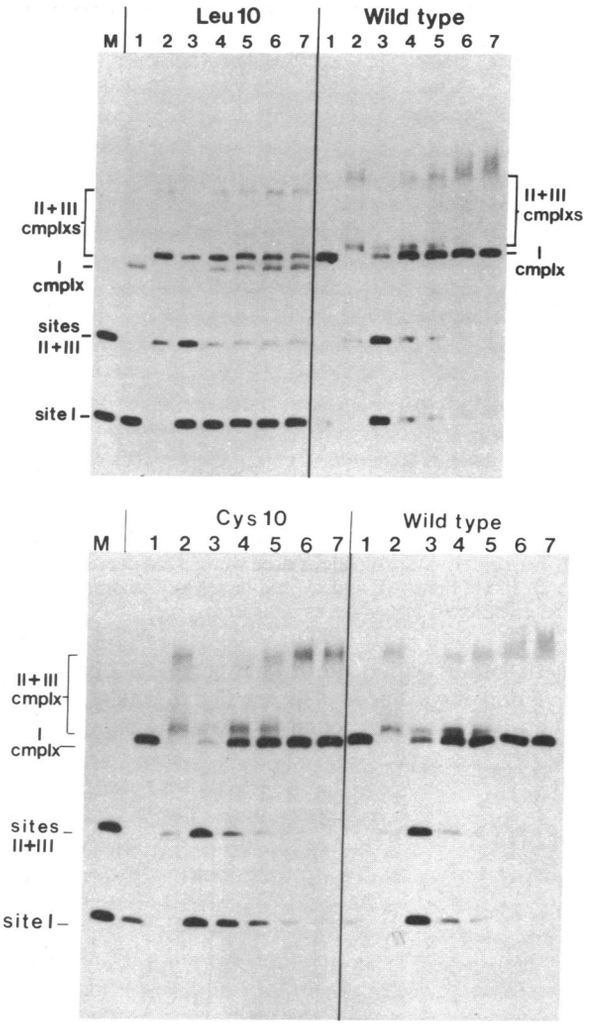

FIG. 3. (*Upper*) Electrophoretic analysis of complex (cmplx) formation by Leu-10 and wild-type resolvases. Lanes: M, mixture of 5′-labeled DNA fragments, one with site I and one with sites II and III as indicated; 1 and 2, controls that show the effect of adding either Leu-10 or wild-type resolvase (50 nM) to the individual DNA fragments; 3–7, effect of adding increasing concentrations of resolvase (either Leu-10 or wild-type) to the mixture of DNA fragments. The resolvase concentrations used were: 5 nM (lanes 3), 16.7 nM (lanes 4), 50 nM (lanes 5), 167 nM (lanes 6), and 500 nM (lanes 7). (*Lower*) Electrophoretic analysis of complex formation by Cys-10 and wild-type resolvases. The concentration of resolvases and the labeled DNAs are as described in *Upper*.

dimer complex formed a sharp band, as opposed to the diffuse band formed with wild-type resolvase, and had reduced electrophoretic mobility. In addition, less of this complex was chased into two- and three-dimer complexes as protein concentration increased.

To determine if these differences resulted from defective interaction of Leu-10 resolvase with site I, we analyzed the nature of the one-dimer complexes by methylation interference. Methylation by dimethyl sulfate of the N-7 position of guanine within the conserved binding sequence 5′ TGTCYNNTA strongly inhibited binding of resolvase at that site (this manuscript; E. E. Falvey and N.D.F.G., unpublished data). Thus, the distribution of methylated guanine residues on the upper strand at positions −13, +38, and +77 (sites I, II, and III, respectively) indicates the sites to which resolvase is bound. Fig. 5 shows the methylation patterns of the one-dimer complexes formed with either wild-type or Leu-10 resolvase. Binding of the mutant resolvase was inhibited only

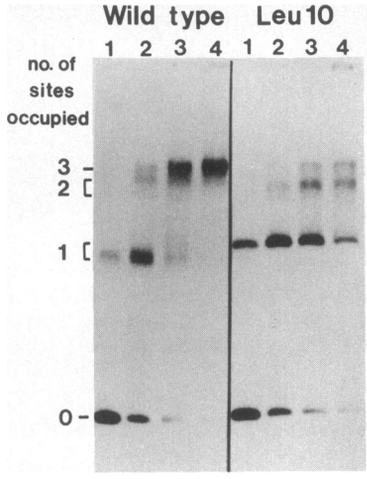

FIG. 4. Comparison of the DNA–protein complexes formed between *res* DNA and either wild-type or Leu-10 resolvase. All lanes contain 5'-labeled pRW80 DNA (which contains all three binding sites). The protein concentrations used were: 5 nM (lanes 1), 16.7 nM (lanes 2), 50 nM (lanes 3), and 167 nM (lanes 4). Uncomplexed DNA and the complexed forms are as indicated.

by methylations within site II (and completely inhibited by methylation of guanine at +38; see Fig. 5, lane 4), indicating that Leu-10 resolvase is bound only to site II in the one-dimer

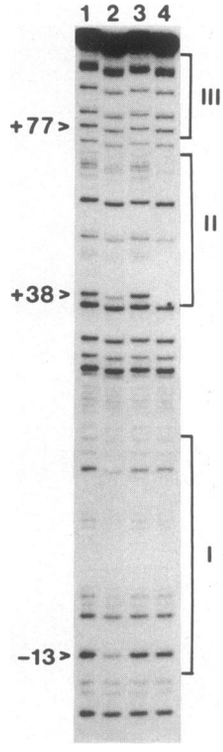

FIG. 5. Analysis of the one dimer–*res* DNA complexes formed with Leu-10 and wild-type resolvase. Resolvase (50 nM) was added to reactions containing partially methylated pRW80 DNA that was 5'-labeled at the *Sal* I site (site I proximal), and the complexes were separated by gel electrophoresis as in Fig. 4. Complexes with one site occupied and uncomplexed DNA were purified, the DNA was cleaved at the methylated positions, and the products were separated on a 6% sequencing gel. Lanes: 1 and 2, uncomplexed and complexed DNA, respectively, from the reaction with wild-type resolvase; 3 and 4, uncomplexed and complexed DNA from the reaction with Leu-10 resolvase. The positions of the guanine residues used to indicate site-specific binding are shown. Numbers are relative to the cross-over point as shown in Fig. 1.

complex. By contrast, binding of wild-type resolvase was inhibited about 50% by methylation of guanine at −13 and about 50% at +38 (see Fig. 5, lane 2), suggesting that this one-dimer complex is an equal mixture of resolvase bound to site I and resolvase bound to site II. Thus, by comparison with wild-type resolvase, the Leu-10 resolvase shows defective binding to site I when it interacts with *res* as well as when site I is separated from sites II and III. This experiment also confirmed our initial assignment of the fastest moving complex as a complex with a single site occupied by resolvase.

DISCUSSION

We have made use of polyacrylamide gel electrophoresis to study the interactions of the wild-type and two mutant γδ resolvases with *res* DNA. The gel assay is based on that described by others to study a variety of DNA–protein interactions (16–18). It is a simple, versatile technique, provides excellent separation of the various forms of complexed and noncomplexed DNAs, and is proving to be a useful tool in the analysis of the interaction between resolvase and its DNA substrate. In general, the number of complexes formed with wild-type γδ resolvase is equal to the number of binding sites on the DNA fragment. This permits a tentative assignment of the complexes as having one, two, or three sites filled with resolvase, the mobility of the DNA being reduced as the number of bound resolvase active units (presumably dimers) increases. This assignment seems to be reliable, since analysis of the presumed one-dimer and three-dimer complexes by methylation inhibition has shown that one and three binding sites, respectively, are occupied by resolvase (Fig. 5; E. E. Falvey and N.D.F.G., unpublished data). We do not fully understand why some complexes run as sharp bands while others run as diffuse bands. Possible factors include exchange of protein dimers between binding sites during electrophoresis, ability of a protein–DNA complex to adopt alternative configurations, and differences in the mobility of the components within a complex (for example, one dimer-*res* complexes may have different mobilities depending on whether site I or site II is occupied). Most complexes with a unique composition appear to run as sharp bands (for example, site I alone with resolvase, the Leu-10 resolvase–*res* DNA complex with a single site occupied, and the resolvase-*res* complex with all three sites occupied). However, the complex of resolvase with DNA containing sites II and III with both sites filled is always diffuse.

Using the gel assay, we have demonstrated that the Leu-10 mutant resolvase has a defect in its binding to site I, although its interaction with site II appears to be indistinguishable from the wild-type protein. The defect in site I binding is observed whether site I is alone on a DNA fragment or is accompanied by the rest of *res*. However, the loss of recombinational activity is not a result simply of the weaker binding to site I, since the Cys-10 resolvase binds almost as well as wild-type yet is also recombinationally inactive.

The ability of the Leu-10 resolvase to enhance cointegrate resolution by a suboptimal amount of wild-type resolvase indicates that, although recombinationally inactive, the mutant can carry out the ancillary functions of resolvase (those performed by the wild-type protein at sites II and III) necessary for recombination. This result, and the normal binding to site II, strongly suggest that the Leu-10 mutant protein is essentially correctly folded and carries out the protein–protein interactions that may be required for binding *res* and for the productive interaction of two *res* sites in a synaptic complex.

Taken together, these results suggest that serine-10 is playing an important role in recombination, for which even the rather similar cysteine cannot substitute. What, then, can

we infer from the observation that the more drastic change to a leucine causes an additional defect in binding to site I? There are two major distinctions between site I and the other two binding sites: site I contains the recombinational crossover point and has its own characteristic geometry (the spacing between the 9-bp recognition sequences). The primary interactions in binding resolvase to *res* involve the C-terminal domain of resolvase and the conserved 9-bp binding sequences present at each site; these interactions are common to all three sites (10). In addition to these, there must be a specific interaction between the recombinational active site located in the N-terminal domain of resolvase and the DNA at the center of site I (see ref. 15), in which the two serine residues (presumably contributed by each monomeric unit of a dimer) are located in close proximity to the two phosphates at the cleavage positions. Presumably the productive interaction of the resolvase active site and the DNA at sites II and III is precluded either by the geometry of these sites or by the nucleotide sequence of the spacers. Replacing the active-site serine with a bulky hydrophobic amino acid would be expected to disrupt the interaction with site I either through loss of hydrogen bonds between the serine and phosphates or through steric interference (or both). If, as seems likely, this secondary protein–DNA interaction is exclusive to the crossover region, such an amino acid substitution should result in a site I-specific DNA binding defect—exactly the properties of the Leu-10 resolvase.

From these results we can conclude that serine-10 is an important residue for the recombinational activity of $\gamma\delta$ resolvase. The conservative change to a cysteine residue at position 10 results in loss of recombinational activity, whilst the more drastic change to a leucine results both in a loss of the recombination function and in a defective interaction with site I. These data, together with the absolute conservation of serine-10 amongst related site-specific recombination proteins, are consistent with the hypothesis that this residue is the serine that is linked to *res* DNA in a recombinational intermediate (12). Additional support for this is provided by the observation that substitution of the other highly conserved serine at residue 39 with a cysteine has no effect on resolvase-mediated recombination *in vivo* (V. Rimphanitchayakit, G.F.H., and N.D.F.G., unpublished observations).

We thank Barbara Newman for providing purified Leu-10 resolvase and Joe Salvo for providing wild-type resolvase. We also thank Joe Salvo and Eileen Falvey for critical reading of the manuscript and Don Crothers for suggesting the methylation inhibition experiment to identify DNA sites occupied. This work was supported by National Institutes of Health Grant GM28470.

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