

Cleavage of the site-specific recombination protein $\gamma\delta$ resolvase: The smaller of two fragments binds DNA specifically

(transposition/protein crystallography/protein–DNA recognition)

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ABSTRACT The 20,500-dalton $\gamma\delta$ resolvase monomer can be cleaved by chymotrypsin into a 5000-dalton COOH-terminal fragment and a 15,500-dalton NH₂-terminal fragment that have been purified. Two crystal forms of the large fragment have been obtained, one of which is isomorphous with crystals of the native protein, showing that the large fragment makes the protein–protein contacts in the crystal and that the small fragment is segmentally disordered relative to the large fragment. Nuclease protection demonstrates that the small fragment binds specifically to all three DNA binding sites protected by resolvase. However, unlike native resolvase, which binds to all three complete sites with equal affinity, the small fragment binds to each of the six half sites with a different affinity. It has not been possible to demonstrate specific DNA binding of the larger fragment. Thus, resolvase has a modular construction analogous to that found for some repressors and activators; its COOH-terminal domain recognizes specific sequences in the DNA and its NH₂-terminal domain mediates protein–protein interactions and probably has the enzymatic activity.

The $\gamma\delta$ resolvase is a 20,500-dalton protein encoded by the transposon $\gamma\delta$, a member of the Tn3 family of prokaryotic transposable elements (for reviews, see refs. 1 and 2). The primary function of resolvase is to catalyze an intramolecular site-specific recombination between two identically oriented copies of $\gamma\delta$. The natural substrate is a cointegrate molecule, an intermediate formed during $\gamma\delta$ transposition, that consists of donor and target replicons fused together with a copy of $\gamma\delta$ at each junction. In addition, resolvase is a transcriptional repressor that regulates expression of the divergently transcribed *tnpA* (transposase) and *tnpR* (resolvase) genes.

Both the recombination reaction and site have been characterized *in vitro* (3–6). During recombination, resolvase acts as a site-specific topoisomerase whose activity is only triggered by interaction of two resolvase-bound recombination sites. The recombination occurs at a fixed position within the *tnpA*–*tnpR* intercistronic region. Resolvase protects from nuclease digestion a 35-base-pair (bp) region (site I) centered on the crossover point. Two additional binding sites lie between site I and the start of the *tnpR* coding region. The three sites span \approx 120 bp of the intergenic region and, since all three are required for maximal recombination efficiency, these sites define the recombination site, *res*. The promoters for *tnpA* and *tnpR* transcription lie within site I and site II, respectively; these two sites are, therefore, the operators for regulation of leftward and rightward transcription (7) (unpublished observations).

High-resolution crystallographic studies of the $\gamma\delta$ resolvase have been hampered by the poor diffracting quality of the crystals at hand (8). These crystals diffract anisotropical-

ly to 7 Å and 4 Å along the *a** and *c** axes, respectively. In an attempt to overcome this problem and to identify the activity domains of resolvase, we have subjected the protein to mild proteolysis. In this paper we show that such treatment yields two fragments, a result similar to that obtained with several other repressors and activators. The smaller COOH-terminal fragment binds specifically but independently to both halves of all three DNA sites to which resolvase binds. In addition, one crystal form of the large NH₂-terminal fragment is isomorphous with crystals of intact resolvase, showing that it contains the protein–protein contacts and that the small domain is flexible relative to the large domain.

MATERIALS AND METHODS

Enzymatic Digestion of Resolvase and Isolation of Fragments. Resolvase was purified using minor modifications of the procedure of Reed (3). A sample of purified resolvase (3 mg/ml) was dialyzed at 4°C against 20 mM Tris-HCl, pH 7.5/1 M NaCl/2.5 mM EDTA/30% (vol/vol) glycerol. After dialysis, the protein concentration was adjusted to 1 mg/ml with the above buffer, and the solution was incubated in a water bath at 25°C. Trypsin treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone (Worthington) or α -chymotrypsin treated with *N*- α -tosyllysine chloromethyl ketone (Sigma) were added to the protein solution, and incubation continued for 40 min. The final protease concentration in the mixture was 0.5 μ g/ml. Freshly prepared phenylmethylsulfonyl fluoride (PhMeSO₂F) solution was added to stop the digestion, and the sample was immediately passed through a Sephadex G-50 column (2.5-cm diameter; 18 cm long) pre-equilibrated with the eluting TEB buffer (20 mM Tris-HCl, pH 7.5/2.5 mM EDTA/25 mM 2-mercaptoethanol/0.1 mM PhMeSO₂F/0.02% NaN₃) containing 1 M NaCl. Fractions were monitored by absorbance at 280 nm to locate the protein. The progress of digestion and purity of samples were then examined by electrophoresis on NaDodSO₄/polyacrylamide gels.

Amino Acid Sequence Analysis. The amino acid composition and yields of purified large fragment were determined by acid hydrolysis of a sample in 6 M HCl for 16 hr at 115°C *in vacuo*. The hydrolysate was analyzed on a Beckman Model 121 M amino acid analyzer. A second sample was dissolved in 0.3 ml of 4 M guanidine-HCl containing 1.7% triethylamine, in order to determine the NH₂-terminal sequence. The protein was then immobilized to 40 mg of *p*-phenylene diisothiocyanate-activated aminopropyl glass, and the sequence of the first 14 residues was determined (9).

Nuclease Protection Experiments. End-labeled fragments of *res* site DNA used for DNase protection experiments were obtained from the plasmid pRR51 as described (3, 5) or from pRW80 (unpublished data). pRW80 is a cointegrate analog in which both *res* sites are terminated at the right end (at position 100) by an *EcoRI* linker; this *EcoRI* site is joined to

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Abbreviations: bp, base pair(s); CAP, catabolite gene activator protein; PhMeSO₂F, phenylmethylsulfonyl fluoride.

the *EcoRI* site of pBR322 (10) with the *HindIII* site of pBR322 positioned 29 bp beyond the fusion. Construction of pRW80, which retains full *res* activity (unpublished observations), will be detailed elsewhere. DNA fragments were labeled at their 5' ends with ^{32}P as described by Maxam and Gilbert (11). DNA from pRR51 was labeled at the *Sal I* site 70 bp to the *tnpA* side of the crossover point, and DNA from pRW80 was labeled at the *HindIII* site 135 bp to the *tnpR* side of the crossover point.

Resolvase or its amino- or carboxyl-terminal fragments were mixed with *res* site DNA (<10 nM) in 10- μl reactions containing 20 mM Tris-HCl, pH 7.5/1 mM dithiothreitol/40 mM NaCl/5 mM MgCl₂/1 μg of sonicated calf thymus DNA. After 10 min at 37°C to allow binding of protein to DNA, the reactions were shifted to 20°C for 5 min, then DNase I (Worthington) was added to 1 $\mu\text{g}/\text{ml}$. Nuclease digestion was stopped after 2 min by addition of 2 μl of 100 mM EDTA/NaDodSO₄. Samples were mixed with 2 vol of formamide/10 mM EDTA/0.1% xylene cyanol and bromophenol blue, and were analyzed by electrophoresis on 0.5-mm thick 8% polyacrylamide gels (acrylamide:bis-acrylamide, 30:1) in 50 mM Tris borate, pH 8.3/1 mM EDTA/8 M urea.

To measure the equilibrium dissociation constant, K_d , of intact resolvase, protection experiments were done in 200- μl reactions containing 20 mM Tris-HCl, pH 7.5/1 mM dithiothreitol/40 mM NaCl/5 mM MgCl₂/1 μg of sonicated calf thymus DNA. End-labeled *res* site DNA fragments were included at a concentration of 0.2 nM or less. Resolvase dilutions were freshly prepared in 20 mM Tris-HCl, pH 7.5/400 mM NaCl/bovine serum albumin (100 $\mu\text{g}/\text{ml}$). After 10 min at 37°C and 5 min at 20°C, DNase I was added to 1 $\mu\text{g}/\text{ml}$. Nuclease digestion was stopped by addition of EDTA to 7.5 mM and ammonium acetate to 0.75 M. Twenty micrograms of tRNA carrier was added and the nucleic acid was precipitated by addition of 3 vol of ethanol. The precipitate was washed with 70% ethanol, dried, and dissolved in 80% formamide/10 mM NaOH/1 mM EDTA/0.1% xylene cyanol and bromophenol blue for electrophoretic analysis.

RESULTS

Enzymatic Cleavage of Resolvase. Chymotryptic cleavage of resolvase produces directly only a large and small fragment of 15,500 and 5,000 daltons (Fig. 1). This pattern does not change even at a 5 times higher concentration of chymotrypsin. Digestion using trypsin produces exactly the same pattern, except that the small fragment disappears after a few minutes of digestion, and at 10-fold higher concentrations of trypsin the large fragment is further cleaved to produce an additional fragment of 10,000 daltons (data not shown). High yields of the small fragment of resolvase are only obtained using chymotrypsin digestion. The large and small fragments that result from chymotrypsin cleavage were easily separated by gel filtration on Sephadex G-50 as shown by electrophoretic analysis of column fractions (Fig. 1b).

Amino acid composition and NH₂-terminal sequence analysis of the large fragment establishes that it consists of residues 1–140. The sequence of the first 14 amino acid residues of the purified large fragment was determined using a solid-phase sequencer and was found to be identical to the known amino acid sequence of the first 14 residues of the intact molecule (7). Furthermore, the amino acid composition of the large fragment is in agreement with the composition of the first 140 amino acids of resolvase. The amino acid composition of the large fragment, the known specificity of chymotrypsin, and the molecular weights determined for both fragments are consistent with a cleavage between phenylalanine-140 and residue 141. Thus, the large fragment of resolvase comprises residues 1–140, and by difference the small fragment probably contains residues 141–183.

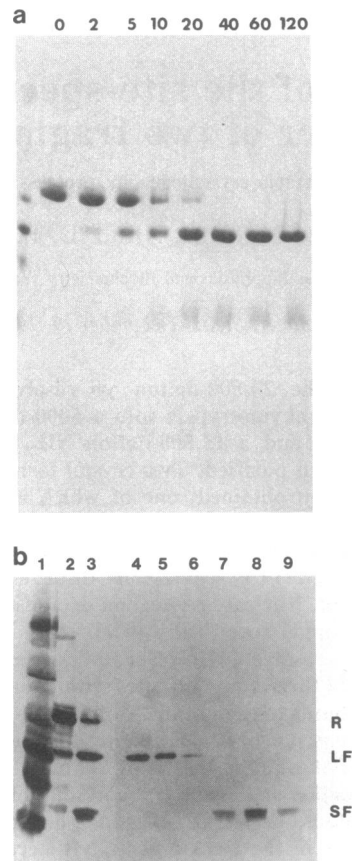


FIG. 1. (a) Time points from digestion of $\gamma\delta$ resolvase with chymotrypsin. A sample containing 0.4 mg of intact resolvase in 0.2 ml of a 20 mM Tris-HCl (pH 7.5) buffer containing 1 M NaCl/2.5 mM EDTA/30% (vol/vol) glycerol was digested with 0.1 μg of α -chymotrypsin at 25°C. Samples of 15 μl were removed at times indicated above lanes (minutes) and added to 10 μl of a solution containing PhMeSO₂F/2% NaDodSO₄/12% glycerol/5 mM dithiothreitol/bromophenol blue (tracking dye), and immediately put in boiling water for 5 min. These samples were then examined by electrophoresis on a NaDodSO₄/15% polyacrylamide gel. Molecular size markers are in left lane. (b) A silver-stained (12) NaDodSO₄/15% polyacrylamide gel showing the purity and resolution of alternating fractions from a resolvase digestion after being excluded from a Sephadex G-50 column. Lane 1 contains molecular size standards. From top to bottom: 43.0, 25.7, 18.4, 13.3, 6.2, and 3.0 kilodaltons. Lane 2, starting native resolvase; lane 3, the digestion pattern of native resolvase after incubation for 30 min with chymotrypsin; lanes 4–9, alternating fractions from a Sephadex G-50 column. R is resolvase, LF is large fragment, and SF is small fragment.

Fragment Binding to DNA. Interactions of each of the fragments with DNA were examined by nuclease protection experiments (13) to ascertain whether the large or small fragment retained the specific DNA-binding activity shown by native resolvase. In this technique, the susceptibility of an end-labeled duplex DNA fragment to DNase I is tested in the presence and absence of each fragment. An example of a DNase I protection experiment using a DNA fragment from pRW80 is shown in Fig. 2a. As can be seen, 2.5 μM small fragment protects *res* DNA (lane 4), while even at 33 μM the large fragment does not protect (lane 2). A mixture of both small and large fragments (lane 3) gives the identical pattern of protection as the small fragment alone. To determine the sites of protection on the other strand of the DNA duplex, we have performed similar experiments with pRR51 DNA and have obtained similar results (Fig. 2b). A compilation of all the data is shown in Fig. 3. Although the small fragment gives a very similar protection pattern to that of intact resolvase, there are some notable differences. While the external

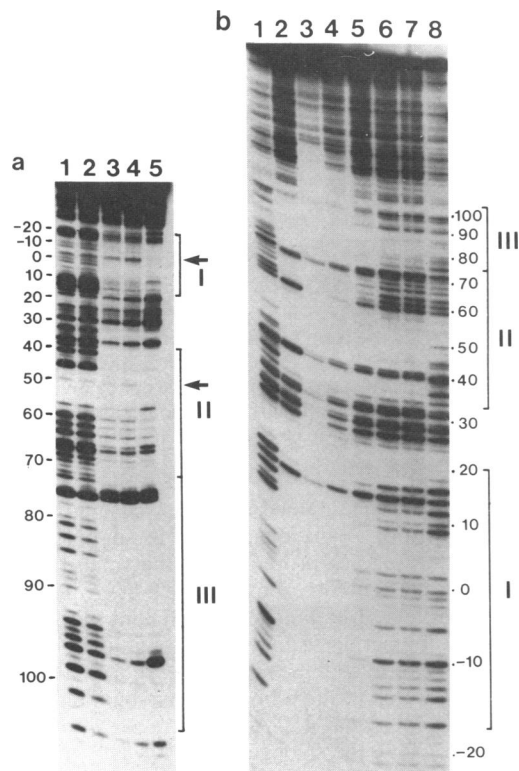


FIG. 2. (a) DNase I protection of resolvase fragments bound to *res* DNA. All lanes contain pRW80 DNA that has been 5'-end-labeled at the *Hind*III site. Lane 1 shows the DNase I digestion pattern obtained with unprotected DNA. The reactions in lanes 2-5 were incubated with protein additions before DNase I digestion as follows: lane 2, the large fragment of resolvase at 33 μ M; lane 3, a mixture of the large (33 μ M) and small (2.5 μ M) fragments of resolvase; lane 4 the small fragment of resolvase at 2.5 μ M; lane 5, intact resolvase at 1 μ M. The arrows mark a pair of bands at positions -2 and -3 in site I and one band at position 50 in site II that show cleavage sites that are not protected by small fragment but are protected by intact resolvase. (b) Measurement of the equilibrium dissociation constant, K_d , of small fragment and *res* DNA. Reactions contained pRR51 DNA 5'-end-labeled at the *Sal*I site. Lanes 1 and 8 are control reactions showing the DNase I digestion pattern in the absence of resolvase or small fragment. Before DNase I digestion, the remaining reactions were incubated with added protein as follows: lane 2, intact resolvase at 1 μ M; lanes 3-7, small fragment of resolvase at 11 μ M, 5.4 μ M, 2.7 μ M, 1.3 μ M, and 0.7 μ M, respectively. Numbers indicate the position of DNase I cleavage sites in the *res* sequence relative to the center of the crossover point (0); a numbered band results from DNase I cleavage of the phosphodiester bond 3' to the corresponding base. Positive numbers are *tnpR* proximal; negative numbers are *tnpA* proximal. The same numbering system is shown in Fig. 3.

boundaries of the three sites remain the same, the small fragment fails to protect the center of site I in the region of the crossover point. In addition, the small fragment leaves unprotected a substantially larger part in the center of site II than is found with intact resolvase.

Initial experiments showed that the concentration of small fragment necessary to give complete protection was higher than that of intact resolvase. We have used DNase I protection to estimate the equilibrium dissociation constants of both resolvase and its small fragment with *res* site DNA. The data in Fig. 2b show that the small fragment binds to different sites within *res* with different affinities. A clear example of these differences can be found in site II; the left half of site II (II-L) is well protected at a small fragment concentration of 0.7 μ M (Fig. 2b, lane 7, positions 36 and 49) but the right half (II-R) is unprotected at 1.3 μ M and only partially protected at 2.5 μ M (lanes 3 and 6, positions 61 and 63). The

data obtained both with pRR51 (Fig. 2b) and with pRW80 DNA show that the small fragment recognizes and binds to the left or right halves of each resolvase binding site independently. The affinities for each "half site" appear to follow the order II-L \approx III-L $>$ I-R $>$ III-R \approx I-L $>$ II-R. If we assume that the equilibrium dissociation constant, K_d , is equal to the concentration of resolvase fragment that gives 50% protection, then the K_d ranges from $\approx 5 \times 10^{-7}$ M for the strongest interactions to $\approx 2 \times 10^{-6}$ M for the weakest interactions. Similar experiments were done using intact resolvase and pRR51 DNA, and contrasting results were obtained (data not shown). With the DNA fragments at $< 2 \times 10^{-10}$ M, virtually complete protection of all three sites was observed at concentrations as low as 2×10^{-8} M resolvase monomer. At 1×10^{-8} M, both halves of all three binding sites were $\approx 50\%$ protected; at 5×10^{-9} M, essentially no protection was observed. The K_d for the interaction between intact resolvase with the entire *res* site is therefore about 10^{-8} M, assuming that all resolvase molecules bind. This is not an unreasonable assumption, because a molar ratio of 20 monomers per cointegrate effects resolution and the studies reported here would require 12. The dramatic change in degree of protection over only a 4-fold concentration range suggests that resolvase binds cooperatively to the three sites.

Two observations establish that the DNase I protection afforded by addition of the small fragment is due to the binding of the small fragment and not to contaminating native protein. Most convincing is the fact that small fragment binds independently, with differing affinities to each of the half-sites, whereas native protein does not. Furthermore, silver staining of a heavily loaded NaDodSO₄/polyacrylamide gel of small fragment shows no evidence of native protein (Fig. 1b).

Crystals of the Large Fragment of Resolvase. Two crystal forms of resolvase large fragment have been obtained, one of which is isomorphous with crystals of the native protein and the other of which is suitable for high resolution structure analysis. The former are large hexagonal-bipyramid crystals grown by vapor diffusion against about 30% saturated ammonium sulfate solution, exactly the same conditions used to crystallize intact resolvase (8). Comparison of diffraction photographs of the *h0l* zone of these crystals with those of intact resolvase (data not shown) indicates that not only are the two crystals isomorphous, but also that there are no obvious intensity differences (in this zone). Since the large fragment comprises only 76% of the resolvase molecule, the similarity of the diffraction patterns is rather surprising and must arise from the small fragment being disordered in the native crystals. Also, it indicates that in crystals of native resolvase, protein-protein interactions are maintained by the large domain and do not involve the smaller domain.

Three-dimensional data to 7 Å resolution were measured on the hexagonal crystals of the large fragment using a modified Picker diffractometer and a Rigaku Ru-200 rotating anode as an x-ray source. The 392 independent reflections measured gave a symmetry R-factor

$$R = \sum_h \sum_i \frac{(|I_{i,h} - \langle I_h \rangle|)}{\langle I_h \rangle}$$

(where $I_{i,h}$ is the i th observation of the reflection h while $\langle I_h \rangle$ is the mean intensity of the i th reflection) of 0.023 when merged with their Friedel mates. The R-factor between this fragment data set and a native resolvase data set was 0.116, showing some significant intensity differences but not nearly that expected from removal of 24% of the protein scattering matter. These modest intensity differences between the two data sets might become interpretable when a good set of phases becomes available, but probably most of the small fragment is disordered.

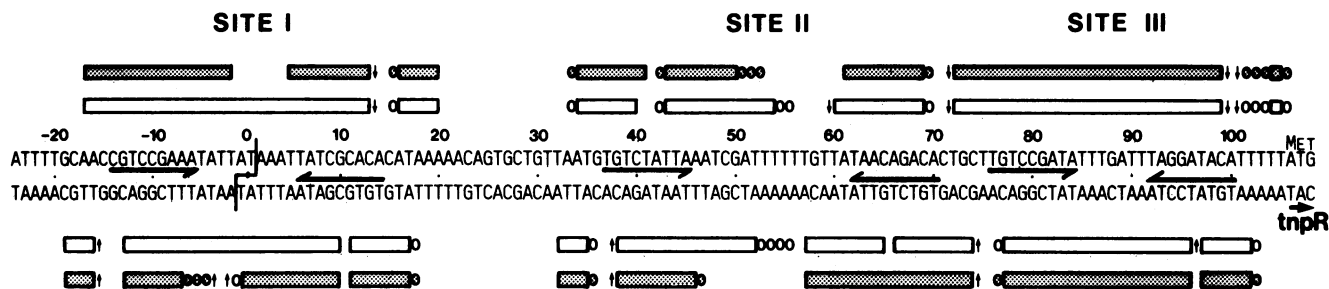


FIG. 3. The *res* site of $\gamma\delta$ showing protected regions. The DNA sequence of the $\gamma\delta$ *res* site is shown with numbering from the center of the crossover point. Open bars show the protection against DNase I digestion afforded by intact resolvase; shaded bars show that afforded by the small fragment. Circles mark phosphodiester bonds at the junctions between protected and unprotected regions that are not cleaved detectably by DNase I even in the absence of resolvase; protection at these sites cannot be determined. Arrows mark phosphodiester bonds at which DNase I cleavage is enhanced. In each half-site the nine base sequences that are related to the nine base resolvase-binding consensus sequence (5) are marked by the horizontal lines with half arrowheads indicating orientation.

To establish that the crystals of native resolvase were not large fragment produced by endogenous protease or that fragment crystals were not of native contaminant, single crystals of native and fragment resolvase were analyzed by NaDodSO₄/polyacrylamide gel electrophoresis (data not shown). The results clearly indicated that native crystals have only slight contamination with large fragment and that large fragment crystals contain no intact resolvase.

At lower protein and higher ammonium sulfate concentrations, a second crystal form of large fragment is obtained that diffracts to better than 2.7 Å resolution (unpublished results). These orthorhombic crystals are very suitable for high-resolution crystal structure analysis, in contrast to the hexagonal crystal form, which like the native crystal form diffracts only to about 7 Å along the a*-b* directions.

DISCUSSION

The resolvase monomer has a modular construction that is analogous to the structures of *E. coli lac* repressor (14–16), λ cI repressor (17), and catabolite gene activator protein (18) (CAP). It can be cleaved by chymotrypsin into a large NH₂-terminal fragment (15,500 daltons) and a small COOH-terminal fragment (5000 daltons). As was first demonstrated for *lac* repressor (19) and subsequently for λ cI repressor (17, 20) and CAP (21), the small fragment of resolvase binds specifically to DNA, whereas the large fragment forms the oligomer interactions. While the DNA binding fragment is NH₂-terminal for *lac* and λ cI repressors, it is COOH-terminal for CAP and resolvase.

It is likely that much of the small fragment has a structure that is very similar to that observed for the DNA binding domain of CAP. The amino acid sequence of the last 60% of this 43-residue polypeptide shows a striking homology (22) to that of the two-helix (E and F) motif (23) in CAP and *cro* that is postulated to be involved in DNA sequence recognition (18, 24) (Fig. 4). Thus, resolvase is likely to have an identical two-helix motif. As is proposed for CAP (18, 22), *cro* (30), and λ cI repressor (32), the second helix of this two-helix structure may impart specificity by penetrating into the major groove of B DNA so that side chains emanating from it may form hydrogen bonds with the edges of the bases (Fig. 5). Since Tn3 and $\gamma\delta$ resolvase recognize each others' DNA binding sites, it is perhaps unexpected that the sequence of the first 140 residues of these proteins is 91% identical while the homology for the last 43 residues is significantly lower (47% identity). However, of the 10 resolvase residues that are in positions corresponding to CAP and *cro* side chains proposed (22, 30) to interact with DNA (Fig. 4), 7 are identical and 3 are functionally similar between Tn3 and $\gamma\delta$ resolvase.

Previous studies (5) of the interaction of intact resolvase with *res* suggested that a 9-bp segment of DNA near the ends

of each binding site contained the major determinants of resolvase recognition. This conclusion is supported by our finding that the regions protected by the small fragment of resolvase are centered on each of these 9-bp sequences. The size of the protected half-sites is consistent with one small fragment binding per half-site, although no direct data on the stoichiometry are available. In general, those half-sites that have the best match to the 9-bp resolvase-binding consensus sequence T-G-T-C-Y-N-N-T-A (where Y is a pyrimidine and N is any base) have the strongest affinity. However, site II-R shows an anomalously poor binding (possibly binding at III-L and II-R interfere with one another).

The nuclease protection studies presented here show that unlike the results obtained for the binding of the small fragments of *lac* (19) and λ cI (20) repressors to their operators, the small fragment of resolvase binds independently with a different affinity to each of the six half-sites in *res*. This is presumably due to the smaller size of the resolvase small fragment and the larger distances between the small fragment binding sites within each of the three resolvase sites. The variable distances between sequences protected by small fragment within and between resolvase sites (Fig. 3) makes the apparent cooperative binding of intact resolvase particularly interesting. Any cooperativity must be due primarily, possibly completely, to protein-protein interactions between the large domains. This is supported by the observation that the native resolvase and large fragment can crystallize isomorphously, demonstrating that the large fragment provides the protein-protein contacts.

Comparison of DNase I protection data on the native protein and small fragment (Fig. 2) shows that the presence of the large fragment in the native protein provides protection

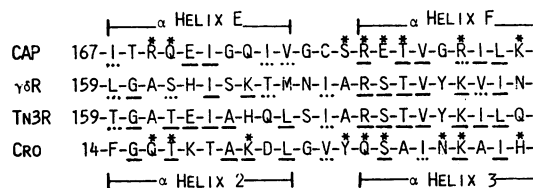


FIG. 4. Amino acid sequences in $\gamma\delta$ resolvase (4) and Tn3 resolvase (25) that are homologous to those of the E and F helices of CAP and the α_2 and α_3 helices of *cro* as has been noted earlier (22). Residues that are identical to either CAP or *cro* are underlined with a solid line, and similar residues are underlined with a dotted line. The homologies shown here are stronger than the homologies of CAP (26), *cro* (27, 28), and λ cI (29) to other phage and bacterial repressors. Asterisks denote residues that are proposed to make contact with DNA in CAP (22) and *cro* (30) (and by homology, presumably resolvase). Similar homologies to Hin, Gin, Pin (31), Xis, and the resolvase of Tn501 and Tn21 (33) have also been found (unpublished results).

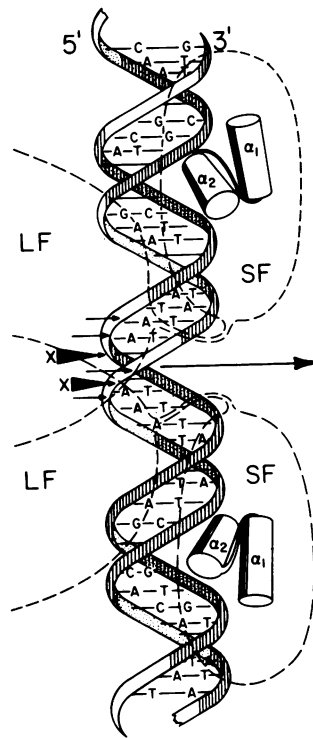


FIG. 5. A schematic drawing of a resolvase dimer (dashed lines) interacting with site I DNA derived from the DNase I protection data and sequence homologies to CAP and *cro*. Small fragment (SF) protects largely one side of the DNA from DNase I (striped regions), while the large fragment (LF) when in native resolvase protects the other side of the DNA (arrows) at the middle of the site. The upper SF lies slightly behind and the lower SF lies slightly forward of the DNA in this view. Side chains from a two-helix structure (α_1 and α_2) homologous to the E and F helices of CAP are assumed to make specific interactions with bases in the major groove. The crossover reaction occurs (thick arrows) near the large fragment.

against cleavages in the middle of site I, implying that the large fragment lies in the middle of site I where the recombination occurs. Furthermore, both the additional DNase I protection provided by the native protein and the recombination cleavages lie on the side of the DNA opposite that protected by small fragment. This leads to a model for resolvase interacting with site I (Fig. 5), which places the small fragments on one side of the DNA at the outer parts of the binding site and the large fragment on the other side at the middle of the site. Several independent mutants of resolvase that are defective in resolution but retain repressor function have been isolated (unpublished results). All of these mutations lie within the large fragment, consistent with the NH_2 -terminal domain catalyzing the recombination reaction.

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