Direct interaction of aminopeptidase A with recombination site DNA in Xer site-specific recombination

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Xer site-specific recombination at ColE1 *cer* converts plasmid multimers into monomers, thus ensuring the heritable stability of ColE1. Two related recombinase proteins, XerC and XerD, catalyse the strand exchange reaction at a 30 bp recombination core site. In addition, two accessory proteins, PepA and ArgR, are required for recombination at *cer*. These two accessory proteins are thought to act at 180 bp of accessory sequences adjacent to the *cer* recombination core to ensure that recombination only occurs between directly repeated sites on the same molecule. Here, we demonstrate that PepA and ArgR interact directly with *cer*, forming a complex in which the accessory sequences of two *cer* sites are interwrapped approximately three times in a right-handed fashion. We present a model for this synaptic complex, and propose that strand exchange can only occur after the formation of this complex.

Keywords: ArgR/*cer*/PepA/site-specific recombination/Xer

Introduction

The *Escherichia coli* Xer site-specific recombination system acts at sites found in multicopy plasmids (e.g. ColE1 *cer* and pSC101 *psi*) to monomerize plasmid multimers formed by homologous recombination. This maximizes the number of independently segregating plasmid units and helps to ensure stable plasmid inheritance (Summers and Sherratt, 1984). Four host-encoded proteins are required for recombination at the 210 bp *cer* site (Stirling *et al.*, 1988b). Two related recombinase proteins, XerC and XerD, which are members of the integrase class of site-specific recombinases, bind cooperatively and catalyse strand exchange at the 30 bp core region of *cer* (Colloms *et al.*, 1990; Blakely *et al.*, 1993). The accessory proteins ArgR (the arginine repressor) and PepA (aminopeptidase A) are absolutely required for recombination at *cer* *in vivo* and *in vitro* but are not directly involved in the strand exchange reaction (Stirling *et al.*, 1988a, 1989; Colloms *et al.*, 1996).

Recombination at *cer* is exclusively intramolecular and occurs only between directly repeated sites, so that it resolves but does not generate plasmid multimers. ArgR, PepA and the accessory sequences of *cer* have been implicated in ensuring this resolution selectivity. Evidence

for this comes from the study of a number of conditionally constrained *cer* variants which recombine exclusively intramolecularly in the presence of ArgR, PepA and accessory sequences, but recombine inter- and intramolecularly when any one of these factors is removed (Summers, 1989; Guhathakurta and Summers, 1995; Guhathakurta *et al.*, 1996). Xer recombination also acts at *dif*, in the replication terminus region of the *E. coli* chromosome, to ensure faithful segregation of newly replicated chromosomes to daughter cells at cell division (Blakely *et al.*, 1993). Recombination at *dif* does not require ArgR and PepA and does not show resolution selectivity.

ArgR is an L-arginine-dependent DNA-binding protein that acts as a transcriptional repressor of the arginine regulon. ArgR binds as a 102 kDa homo-hexamer to 18 bp ARG boxes which are usually present in two copies separated by 3 bp, in the promoter regions of the genes for arginine biosynthesis (Lim *et al.*, 1987; Glansdorff, 1996). ArgR binds to a single ARG box within *cer*, ~110 bp from the point of strand exchange, and induces a bend of ~65° (Burke *et al.*, 1994). It seems likely that during recombination at *cer*, a single ArgR hexamer binds to one ARG box from each participating *cer* site, helping to synapse two *cer* sites and/or introducing a structurally important bend within the accessory sequences.

PepA is one of the major aminopeptidases in *E. coli* and is thought to be important for metabolism of peptides supplied exogenously or produced by protein degradation within the cell (Vogt, 1970; Stirling *et al.*, 1989). PepA belongs to a widespread family of leucine aminopeptidases which are present in mammals, plants and bacteria (Cuypers *et al.*, 1982; Bartling and Weiler, 1992; Burley *et al.*, 1992; Wood *et al.*, 1993). PepA is a hexamer in solution, consisting of six identical 55 kDa subunits. It is an Mn²⁺-dependent aminopeptidase which cleaves a broad range of peptide substrates. The peptidase activity of PepA is not required for its function in recombination at *cer* (McCulloch *et al.*, 1994). In addition to its role in Xer recombination, PepA is involved in pyrimidine-specific transcriptional regulation of the *carAB* operon. This operon encodes the genes for carbamoylphosphate synthetase, which catalyses a common step in the biosynthesis of arginine and pyrimidines. The peptidase activity of PepA is not required in the pyrimidine-specific regulation of *carAB*, and it was shown that PepA binds specifically to sequences overlapping the pyrimidine-regulated P1 promoter of *carAB* (Charlier *et al.*, 1995). It therefore seems likely that the DNA-binding activity of PepA will be important for its function in Xer recombination.

Here we show that PepA binds specifically to the accessory sequences of *cer* and cooperates with ArgR to make an interwrapped complex with the accessory sequences of two *cer* sites. PepA plays a major role in

Fig. 1. Fixed topology of recombination at *cer*. Xer recombination at *cer* yields a catenated, Holliday junction-containing product with a fixed (–4A) topology. This implies that recombination at *cer* occurs only after the formation of a synapse with a fixed topology. The productive synapse is thought to contain two *cer* sites with accessory sequences (thick lines) interwrapped exactly three times in a right-handed fashion. The recombination site cores, at which XerC-mediated strand exchange takes place, are shown as open triangles.

defining the interwrapped structure of this complex and may act in a similar fashion in other systems, thereby forming a bridge between distant segments of DNA.

Results

ArgR and PepA form a nucleoprotein complex on a plasmid containing two *cer* sites

Recombination *in vitro* at *cer* gives products of fixed topology (Colloms *et al.*, 1997). This implies that the structure of the productive synapse and the topology of the strand exchange mechanism are fixed. To explain these results, it was proposed that recombination at *cer* can only occur after the formation of a productive synapse that traps three negative nodes and that strand exchange generates a Holliday junction containing a further negative node. This mechanism yields the observed four-noded (–4A) catenated Holliday junction as product (Figure 1; Colloms *et al.*, 1997). It was predicted that the productive synapse can only form readily between directly repeated sites on a supercoiled molecule, thus accounting for the topological selectivity of Xer recombination at *cer*.

ArgR and PepA are thought to be involved in formation of the productive synaptic complex (Summers, 1989; Guhathakurta and Summers, 1995; Guhathakurta *et al.*, 1996; Colloms *et al.*, 1997). In our model (Colloms *et al.*, 1997), recombination only proceeds after the formation of a complex in which the accessory sequences of two *cer* sites have been interwrapped about the accessory proteins, thereby trapping three negative plectonemic supercoils (Figure 1). If this model is correct, and assuming there are no major changes in the helical twist of the DNA within the complex, the addition of ArgR and PepA to a plasmid containing two directly repeated *cer* sites should constrain three negative supercoils. These supercoils will not be available for relaxation by topoisomerase I (Figure 2B). To test this prediction, a plasmid containing two directly repeated *cer* sites was incubated with ArgR and/or PepA and then relaxed with topoisomerase I. The resulting topoisomers were separated by electrophoresis through a chloroquine-containing gel, producing a ladder of bands each differing from its neighbour by one supercoil. The DNA is positively supercoiled by the intercalating agent and, therefore, slower migrating bands

correspond to more negatively supercoiled topoisomers. When either ArgR or PepA was added alone, there was no significant change in the distribution of topoisomers obtained (Figure 2A, lanes 2, 3 and 5). However, when PepA and ArgR were added together, the topoisomers produced were on average more negatively supercoiled than those produced in the absence of accessory proteins (Figure 2A, lane 4). This implies that ArgR and PepA, when incubated with a plasmid containing directly repeated *cer* sites, cooperate to form a nucleoprotein complex which constrains negative supercoiling.

In order to quantify the change of supercoiling, the gel shown in Figure 2A was scanned using a Fluorimager. The variation in relative intensity along a line drawn through lanes 2–5 is shown in Figure 2C. The relative amount of DNA in each topoisomer band was determined and the centre of each topoisomer distribution was calculated as the mean of the distribution. The addition of ArgR or PepA alone changed the centre of the topoisomer distribution by no more than 0.5 supercoils, whereas the addition of ArgR and PepA together changed the centre of the distribution by ~2.1 supercoils. Similar results were obtained in a number of experiments using this and other plasmids containing two *cer* sites in direct repeat. The addition of either ArgR or PepA alone had little or no effect, whereas the addition of ArgR and PepA together shifted the centre of the topoisomer distribution by between two and three supercoils.

The recombinase proteins and binding sites are not required for the formation of the nucleoprotein complex. The addition of XerC and XerD, in the presence or absence of accessory proteins, had no effect on the distribution produced (Figure 2A, lanes 6 and 7), and a plasmid carrying the accessory sequences of two *cer* sites in direct repeat, but lacking the recombinase-binding sites, was still able to form a complex that constrained 2–3 negative supercoils (data not shown).

The formation of the nucleoprotein complex does not require supercoiling

In the above experiment, negatively supercoiled DNA was incubated with PepA and ArgR before treatment with topoisomerase I. Therefore, although the nucleoprotein complex is formed on supercoiled DNA, it remains stable after relaxation with topoisomerase I. Does the formation of the nucleoprotein complex require negative supercoiling? To answer this question, a similar experiment was performed using open circle DNA as substrate. A singly nicked plasmid containing two directly repeated *cer* sites was treated with DNA ligase to resealed the nick in the presence or absence of ArgR and PepA. The resulting topoisomers were run on a chloroquine-containing agarose gel (Figure 3) and quantified as above. The addition of either ArgR or PepA alone had little effect on the distribution of topoisomers obtained (Figure 3, lanes 2–4). However, when ArgR and PepA were added together, the distribution of topoisomers produced was shifted by nearly four supercoils (Figure 3, lane 5). Thus, a nucleoprotein complex constraining negative supercoils can also be formed by PepA and ArgR on open circle DNA. It is not known why the nick ligation assay consistently gave a slightly larger shift in topoisomer

Fig. 2. Topoisomerase I relaxation of a plasmid containing two directly repeated *cer* sites. (A) Supercoiled pSDC115, containing two directly repeated *cer* sites, was mixed with PepA, ArgR, XerC and XerD, as indicated, and incubated for 15 min at 37°C to allow complex formation. The XerC protein used carried the Y275F mutation to prevent any recombination. Topoisomerase I was added and the reactions were incubated for a further 30 min to remove all free supercoiling. Reactions were stopped by the addition of SDS and proteinase K, and topoisomers were separated by electrophoresis on an agarose gel containing 0.5 µg/ml chloroquine phosphate. (B) Between two and three negative supercoils are constrained in a complex with PepA and ArgR and are not available for relaxation by topoisomerase I. (C) The gel shown in (A) was scanned using a fluorimager. The graphs show the variation in fluorescence along lines drawn through lanes 2–5. The amount of DNA in each topoisomer band was calculated as the area of each peak (or as the volume under the curve in a rectangle drawn around each band). The centres of each topoisomer distribution were calculated as the means of the distributions and are indicated by vertical arrows.

distribution than the topoisomerase I assay described earlier.

PepA and ArgR constrain supercoils only on a plasmid containing two *cer* sites in direct repeat

The supercoils constrained by PepA and ArgR on pSDC115 could be dependent on the formation of a complex that requires two *cer* sites. Alternatively, each site could wrap independently about ArgR and PepA, constraining ~1.5 supercoils at each site. To distinguish between these two possibilities, plasmids containing one *cer* site (pKS492) and no *cer* sites (pUC18) were relaxed with topoisomerase I in the presence and absence of PepA and ArgR. In both cases, the distribution of topoisomers obtained was unchanged by the addition of ArgR and

PepA, individually or in combination (Figure 4A and B). Therefore, a single *cer* site on a circular DNA molecule cannot wrap independently around ArgR and PepA, and the effect we see is specific for two *cer* sites on the same circular DNA molecule.

Recombination at *cer* requires two sites in direct repeat on a supercoiled circular molecule. Is the formation of the nucleoprotein complex at *cer* dependent on the relative orientation of the two sites? To answer this question, pSDC117, containing two *cer* sites in inverted repeat, was relaxed with topoisomerase I in the presence of ArgR and PepA (Figure 4C). No change in the distribution of topoisomers was observed. A similar result was obtained when pSDC117 was singly nicked and then religated in the presence of ArgR and PepA (Figure 3, lanes 7–10).

Fig. 3. Complex formation on nicked substrates. Plasmids containing two *cer* sites in direct (pSDC115) or inverted (pSDC117) repeat were singly nicked with DNase I to remove all supercoiling. After incubation with PepA (6 nM) and ArgR (10 nM), as indicated, the nick was re-sealed with DNA ligase, thereby trapping any supercoils caused by wrapping of the two *cer* sites about PepA and ArgR. The reactions were stopped with SDS and proteinase K, and topoisomers were separated on a chloroquine-containing agarose gel.

Either no stable complex is formed between *cer* sites in inverted repeat or a complex is formed but on average no supercoils are constrained.

The two *cer* sites are plectonemically interwrapped in the nucleoprotein complex

The above experiments show that ArgR and PepA form a complex which constrains between two and four negative supercoils on a circular DNA molecule containing two directly repeated *cer* sites. However, these experiments do not distinguish between plectonemic interwrapping and solenoidal coiling of the two sites (Figure 5A). An experiment was designed to distinguish between these two possibilities. Because supercoiling was not required for formation of the ArgR-PepA nucleoprotein complex at *cer* (Figure 3), we reasoned that the complex might form at *cer* sites carried on a linear DNA molecule. If this were the case, ligating the free ends would trap any plectonemic interwrapping as knotting. Circularization of a linear DNA molecule containing solenoidally coiled sites is expected to yield unknotted circles no matter how many solenoidal supercoils are present (Figure 5A). In contrast, circularization of a linear DNA molecule containing plectonemically interwrapped sites is likely to produce knotted products. In this case, the complexity and type of knots produced should depend on the number of plectonemic interwraps and whether the sites are in direct or inverted repeat (Figure 5A).

The plasmids pSDC115 and pSDC117, containing two *cer* sites in direct and inverted repeat respectively, were linearized with *Hind*III, incubated with ArgR and PepA, and then circularized with DNA ligase. The resulting products were singly nicked to remove any supercoiling and analysed by agarose gel electrophoresis adjacent to a ladder of nicked pSDC115 knots (Figure 5B). The mobility of nicked knots on this type of gel is determined by the number of knotting nodes (Stasiak *et al.*, 1996). When ligated in the absence of any proteins or in the presence

Fig. 4. Topoisomerase I relaxation of plasmids containing one, two or no *cer* sites. Supercoiled plasmids (A) pUC18 (containing no *cer* sites), (B) pKS492 (containing one *cer* site) and (C) pSDC117 (containing two *cer* sites in inverted repeat) were relaxed with topoisomerase I with and without PepA and ArgR, as indicated. Topoisomers were separated on chloroquine-containing agarose gels as in Figure 1.

of either ArgR or PepA alone, both plasmids yielded mainly unknotted circle. The small amount of three-noded knot (~10%) results from random tangling of the linear DNA molecule (Shaw and Wang, 1993). In contrast, when both ArgR and PepA were present, circularization yielded large amounts of knotted products. With directly repeated *cer* sites (pSDC115), ~50% of the intramolecular ligation products were unknotted circle, but a substantial amount of five-noded knot (~25% of intramolecular ligation products) and six-noded knot (~10%) was also produced. With *cer* sites in inverted repeat (pSDC117), only ~30% of the intramolecular ligation products were unknotted. The majority of the intramolecular ligation products were knotted and consisted mainly of three- (~30%), five- (~20%) and six-noded (~10%) knots. Similar results were obtained when other restriction enzymes (*Eco*RI or *Sal*I) were used to linearize pSDC115 and pSDC117. The addition of the recombinase proteins, XerC and XerD, had a small but significant effect on the distribution of knots produced: for directly repeated sites (pSDC115), the

Fig. 5. Ligation-mediated knotting of linear fragments containing two *cer* sites. **(A)** Solenoidal coiling (as found in the nucleosome core) followed by ligation is expected to yield unknotted circles, whereas plectonemic wrapping (where two segments of DNA helix are intertwined) followed by ligation is expected to yield knots. If two *cer* sites are wrapped about PepA and ArgR three times in a right-handed antiparallel plectoneme, ligation is expected to produce the knots shown. If the *cer* sites are in inverted repeat, three- (+3), five- (+5 torus) and six-noded knots are expected. If the sites are directly repeated, unknotted circle is expected as a major product, but five- and six-noded twist knots can be formed as shown. **(B)** Plasmids containing two *cer* sites in direct (pSDC115) or inverted (pSDC117) repeat were linearized with *Hind*III. Linearized DNA was incubated with PepA (17 nM), with ArgR (10 nM), with ArgR (10 nM) and increasing amounts (0.67, 3.3 and 17 nM) of PepA, or with ArgR (10 nM), PepA (17 nM), XerC (100 nM) and XerD (100 nM) as indicated. After incubation for 15 min to allow complex formation, ligase was added to circularize the DNA fragment. Supercoiling was removed by DNase I nicking, and knots were separated according to node number on a high resolution agarose gel. Lane 8 contains a nicked pSDC115 knot ladder produced by the action of topoisomerase II (knots). Lanes 1 and 9 contain linear pSDC115 and pSDC117 respectively. The positions of linear substrate (lin), nicked unknotted circle (oc) and knots with three, four, five, six and seven nodes are indicated.

amount of five-noded knot was reduced and the amount of six-noded knot was increased; for inverted repeat sites (pSDC117), the amount of six-noded knot was decreased and the amounts of five- and seven-noded knots were increased by the addition of XerC and XerD. Binding of the recombinase proteins probably changes the direction in which the free DNA arms emerge from the synaptic complex, so changing the distribution of knots obtained.

These results are inconsistent with solenoidal coiling of the DNA within the nucleoprotein complex, and strongly support a model in which two *cer* sites are plectonemically interwrapped around PepA and ArgR in a synaptic complex. The knots produced from pSDC115 and pSDC117 are broadly consistent with a synaptic complex with 3–4 plectonemic interwraps as shown in Figure 5A. The exact structure of the knots produced in these experiments cannot be determined without electron microscopy. However, the five-noded knot produced from pSDC115 migrated slightly faster than the five-noded knot produced from pSDC117 (data not shown), consistent with the former being a twist knot and the latter being a torus knot (Crisona *et al.*, 1994; Stasiak *et al.*, 1996), as predicted in Figure 5A.

DNase I protection assays of ArgR and PepA complexes with *cer*

The role of PepA in the formation of the nucleoprotein complex was examined further by DNase I protection analysis of *cer* using ArgR and PepA in combination. A 600 bp linear fragment containing two *cer* sites (Figure 6A) was used because previous results suggested that two *cer* sites on the same DNA molecule might be required to form a stable complex. This fragment was labelled with ³²P at all four ends to determine the pattern of protection of both strands of both *cer* sites in four separate experiments (Figure 6B). The pattern of protection of the two *cer* sites within the 600 bp fragment was broadly similar and is summarized in Figure 6C.

As expected, ArgR alone protected only sequences around the 18 bp ARG box within *cer* (Stirling *et al.*, 1988a). However, when PepA and ArgR were added together, the pattern of cleavages was altered dramatically over all of the 180 bp accessory sequences: protected regions and enhancements of DNase I cleavage were seen on both sides of the ARG box. The most dramatic changes occurred in a 50 bp region between the ARG box and the recombinase-binding sites. On both strands of this 50 bp region, there were five prominent enhancements of DNase I cleavage at 10–11 bp intervals, which were separated by protected regions. This altered pattern of DNase I cleavage was also apparent in the presence of PepA alone, but was strongly enhanced by the presence of ArgR. The addition of the recombinase proteins XerC and XerD did not alter the footprint other than to give protection at the recombinase-binding sites (Figure 6B, lanes 11 and 12).

A DNase I protection experiment was also carried out with a fragment carrying only one *cer* site. The same pattern of protections and enhancements was seen on fragments containing one and two *cer* sites, although the levels of enhancement and protection were lower and required higher concentrations of PepA on the fragment with one site (data not shown). This suggests that the same change in the general structure is obtained. Either one *cer* site is sufficient to form the complex detected by

changes in DNase I sensitivity or two sites can come together to form an intermolecular complex which is similar to that formed between two sites on the same molecule.

Discussion

In this study, we have shown that PepA cooperates with ArgR to form a complex with two *cer* sites. The results are consistent with a complex in which the accessory sequences of two *cer* sites are interwrapped approximately three times about PepA and ArgR. A complex with interwrapped accessory sequences was predicted previously from the topology of Xer recombination products (Colloms *et al.*, 1997). The results presented here demonstrate that PepA and ArgR are structural components of this complex.

PepA is a multifunctional protein. It was first described as an aminopeptidase (Vogt, 1970), and has homology to bovine lens leucine aminopeptidase (Stirling *et al.*, 1989). PepA is required for Xer site-specific recombination at ColE1 *cer* and pSC101 *psi* (Stirling *et al.*, 1989; Colloms *et al.*, 1996) and is also involved in pyrimidine-specific regulation of the *carAB* operon (Charlier *et al.*, 1995). Several ideas have been put forward to explain the requirement for PepA in Xer site-specific recombination. Originally, it was thought that the peptidase activity might be involved in processing one of the other proteins required for Xer site-specific recombination. However, the peptidase activity of PepA is not required for Xer site-specific recombination (McCulloch *et al.*, 1994), implying that PepA plays a structural role. This could involve direct interactions between PepA and the recombination site DNA and/or protein–protein interactions with ArgR and the recombinases (e.g. see Guhathakurta *et al.*, 1996). Charlier *et al.* (1995) demonstrated that PepA has a DNA-binding activity, and binds specifically to DNA in the *carAB* promoter region. Our results, particularly the changes in sensitivity to DNase I which occur in the presence of PepA alone, demonstrate that PepA interacts directly with the accessory sequences of *cer*.

ArgR is a homo-hexameric protein of 102 kDa (monomer size = 17 kDa). Each ArgR monomer contains one DNA-binding domain, two of which are thought to bind to a single 18 bp dyad symmetrical ARG box. Therefore, a hexamer of ArgR could potentially bind to three separate ARG boxes. The operator regions of genes regulated by ArgR all contain two adjacent ARG boxes separated by 2–3 bp to which one ArgR hexamer is thought to bind (Glansdorff, 1996). Each *cer* site contains only one ARG box, and it seems likely that in the complex formed between two *cer* sites, one hexamer of ArgR contacts and holds together one ARG box from each site.

The results of our experiments with topoisomerase I and ligation-mediated knotting show that two *cer* sites form a complex with PepA and ArgR in which the sites are interwrapped between two and four times. The topology of the *cer* recombination product strongly suggests that exactly three plectonemic supercoils are trapped in the productive synapse (Colloms *et al.*, 1997). It seems reasonable to assume that a similar complex is also present in the DNase I footprinting experiments. Two models for this interwrapped complex are shown in Figure 7. ArgR

Fig. 6. DNase I protection of a fragment containing two *cer* sites. (A) A ~600 bp fragment containing two tandemly repeated *cer* sites was 5' or 3' end labelled at *EcoRI* or *HindIII* sites and used in four separate DNase I footprinting experiments. ArgR- (R), XerC- (C) and XerD- (D) binding sites are indicated. (B) Binding reactions were carried out with end-labelled DNA fragments and ArgR, PepA, XerC and XerD as indicated. The DNA was subjected to limited cleavage by DNase I followed by electrophoresis on denaturing polyacrylamide gels. Only the *cer* site closest to the end label is shown. The organization of each site is indicated to the left of each gel. The enhancements of DNase I cleavage (filled triangles) and protection from cleavage by DNase I (vertical lines) are shown to the right of each gel. PepA concentrations used were: lanes 3, 5, 8, 10, 12, 14, 17, 22 and 24, 160 nM; lanes 6, 15, 18 and 25, 10 nM; and lane 19, 0.6 nM. (C) Summary of DNase I protection on both strands of *cer* in the presence of PepA and ArgR. Filled triangles show enhancements of DNase I cleavage. Horizontal lines indicate protected regions. The 18 bp ARG box is shown in bold and boxed, and the region protected in the presence of ArgR alone is indicated by thick horizontal lines. The XerC- and XerD-binding sites are also indicated.

binding to a single ARG box induces a bend of ~65° (Burke *et al.*, 1994). One ArgR hexamer binding to one ARG box in each of two *cer* sites could trap at most

one crossing between the sites. PepA must therefore be responsible for trapping the remaining interwappings. The fact that PepA alone can give a DNase I footprint on

Fig. 7. Models for the complex formed between two *cer* sites in the presence of PepA and ArgR. The accessory sequences of two *cer* sites are shown interwrapped about PepA and ArgR in a right-handed antiparallel plectoneme, trapping three negative supercoils. One ArgR hexamer (grey circle) contacts the ARG box from each site. The five major enhancements of DNase I cleavage, between the ARG box and the recombination core (filled arrow) of each *cer* site, are shown as small triangles. The two models differ by having either (A) two or (B) one hexamer of PepA (large filled circles) contacting various segments of the accessory sequences. The dyad axis of each complex is indicated with an arrow.

cer (Figure 6B, lane 3), similar to that produced by PepA and ArgR together, suggests that PepA is the key player in defining the structure of the complex. This conclusion is reinforced by the fact that Xer recombination at pSC101 *psi* occurs efficiently in the presence of PepA and the recombinase proteins alone and uses a productive synapse which is topologically identical to that used by *cer* (Colloms *et al.*, 1997).

The two models shown in Figure 7 both show ArgR trapping one crossing between the two *cer* sites, and PepA trapping two crossings. In Figure 7A, two hexamers of PepA are shown; each hexamer contacts one segment of DNA from each site and stabilizes one crossing between the two sites. In Figure 7B, a single hexamer of PepA is shown making contacts with four separate segments of DNA and stabilizing two crossings between the two sites.

Both of our models have a dyad axis of symmetry so that each *cer* site has an identical conformation and makes equivalent contacts with PepA and ArgR. Both models show PepA making contacts with *cer* adjacent to the recombinase-binding sites and adjacent to the ARG box distal to the recombinase-binding sites, which is consistent with the pattern of protection from DNase I. Both models also show a highly curved ~60 bp loop of DNA between the ARG box and the recombinase-binding sites which is consistent with the five enhancements of DNase I cleavage seen on both strands at 10–11 bp intervals in this region. Based on the behaviour of insertions in the *Mlu*I site of

cer, Guhathakurta *et al.* (1996) have proposed the existence of a similar loop in this region in a PepA-ArgR-XerC-XerD complex with a single *cer* site. This loop could be wrapped around the surface of PepA, with only one face of the helix exposed and available for cleavage by DNase I, as seen for DNA in the nucleosome core (Drew and Travers, 1985). Alternatively, the DNA in this loop may not interact directly with PepA, but could be held in place by contacts elsewhere on the DNA. Enhancements of DNase I cleavage would then be caused by the widened minor groove on the outside of the curve, as is seen for minicircles in solution (Drew and Travers, 1985).

There are similarities between the pattern of DNase I protection seen at *cer* and that seen in the control region of *carAB*. The binding of PepA to the control region of the *carAB* operon protected two regions of ~25 bp separated by ~65 bp (Charlier *et al.*, 1995). Between and on either side of the two protected regions covering ~200 bp, there are periodic enhancements of DNase I cleavage, suggestive of a complex in which this region is wrapped around PepA. ArgR binds to two ARG boxes in the *carAB* control region, downstream of the sequences implicated in PepA-dependent pyrimidine-specific regulation of *carAB*, bringing about L-arginine-specific control of a second *carAB* promoter. However, there is no evidence that ArgR and PepA interact with each other in this context. PepA also binds to sequences overlapping one of the *pepA* promoters, and brings about negative autoregulation of this promoter (Charlier *et al.*, 1995). It seems likely that PepA will also be involved in the regulation of other genes in *E. coli*.

Recombination only occurs between directly repeated *cer* sites on supercoiled circular DNA molecules. Supercoiling was not required for synapsis, which suggests that it is required at a later stage during the recombination reaction, perhaps during strand exchange. Synapsis was only detected with the topoisomer shift assays when two *cer* sites were present in direct repeat on the same circular molecule. It is tempting to suggest that a synapse cannot form between sites in inverted repeat or between sites on separate molecules and that this accounts for the topological selectivity of the recombination reaction. For sites in inverted repeat on a circular molecule, or on separate circular molecules, a right-handed interwrapped synapse can only form if compensating left-handed interwrapping is introduced elsewhere. This is predicted to be unfavourable on negatively supercoiled DNA. However, a synaptic complex might form more easily between sites in inverted repeat or on separate molecules on relaxed DNA. If this were the case, we might not detect these complexes because they are predicted to constrain one (inverted repeat sites) or no (intermolecular synapsis) supercoils. On linear DNA there should be no topological barrier to synapse formation between sites in inverted repeat or on separate molecules, consistent with the results of our ligase-mediated knotting experiments.

Recombination at *cer* does not occur in the absence of PepA and ArgR, implying that these two proteins somehow activate the strand exchange reaction. This could be brought about by direct protein-protein interactions between PepA and/or ArgR and the recombinases. Alternatively, the interwound complex formed between two *cer* sites, PepA and ArgR, could place the recombinase-

binding sites in an alignment or conformation that aids binding by the recombinase proteins and/or activates the first strand exchange by XerC. We expect that further investigations will reveal whether one or both of these factors are important for Xer recombination.

Materials and methods

Plasmids

Plasmid pKS492 contains *cer* as a 282 bp *HpaII*–*TaqI* fragment from ColE1 inserted into pUC18 (Stirling *et al.*, 1988b). Plasmids pKS455 and pSDC115 each contain two directly repeated *cer* sites (Stirling *et al.*, 1988; Colloms *et al.*, 1996). Plasmid pSDC117 contains two *cer* sites in inverted repeat and is identical to pSDC115, except that the copy of *cer* in the *PvuII* site of pBR322 was inserted in the opposite orientation. Plasmid pCA18 contains two 282 bp *cer* sites in direct repeat separated by 33 bp of polylinker sequences and was constructed by ligating the *NdeI*–*HindIII* *cer*-containing fragment (with the *HindIII* site filled in) from pKS492 to the *SmaI*–*NdeI* *cer*-containing fragment from pKS492. Supercoiled plasmid DNA was prepared by large-scale alkaline lysis followed by CsCl–ethidium bromide density gradient centrifugation.

Proteins

PepA was purified essentially as described by McCulloch *et al.* (1994) using a low salt precipitation step. Precipitated PepA was resuspended in 20 mM Tris–HCl, pH 8.0, 200 mM KCl, 1 mM EDTA and was purified further by gel filtration on a Superose 12 column (Pharmacia). PepA eluted at the position expected for the hexamer (mol. wt ~320 kDa). Peak fractions were made up to 50% glycerol and stored at –20°C. ArgR was purified as described by Burke *et al.* (1994). XerC and XerD were purified as described by Colloms *et al.* (1996). Protein concentrations were determined by Bradford assays and are expressed as concentration of hexamer for PepA and ArgR and of monomer for XerC and XerD.

Topoisomerase I assay

Binding reactions were set up with 500 ng of supercoiled plasmid DNA and proteins, as indicated, in 50 µl of 50 mM Tris–HCl, pH 7.5, 50 mM KCl, 10 mM MgCl₂, 0.5 mM dithiothreitol (DTT), 0.1 mM EDTA, 1 mM L-arginine, 300 µg/ml bovine serum albumin (BSA). After incubation for 15 min at 37°C, 1 U of topoisomerase I was added and the reaction was allowed to proceed for a further 30 min at 37°C. Reactions were stopped by addition of loading buffer containing 0.2 mg/ml proteinase K and 0.5% SDS. Samples were run in 1% agarose gels containing 0.5 µg/ml chloroquine phosphate.

DNase I nicking

DNase I nicking reactions were in 20 mM Tris–HCl, pH 7.5, 10 mM MgCl₂, 50 mM NaCl and 0.3 mg/ml ethidium bromide. The 30 µl reactions containing 500 ng of DNA and 1 µg/ml of DNase I were incubated at 30°C for 30 min. EDTA was added to a final concentration of 16 mM and the reactions were extracted sequentially with phenol, phenol–chloroform and chloroform.

Ligation assays

To detect changes in supercoiling on nicked substrates, plasmids were singly nicked with DNase I as described above. Binding reactions were set up with 300 ng of nicked plasmid DNA and proteins as indicated in 20 µl of 50 mM Tris–HCl, pH 7.5, 25 mM NaCl, 1.25 mM DTT, 1.25 mM spermidine, 1 mM L-arginine, 25 µg/ml BSA and incubated at 37°C for 15 min. Then 10 µl of the same buffer containing 30 mM MgCl₂, 3 mM ATP and 0.5 U of T4 DNA ligase was added and ligation was allowed to proceed at 20°C for 1 h. Reactions were stopped by addition of loading buffer containing 0.2 mg/ml proteinase K and 0.5% SDS. Samples were run in 0.7% agarose gels containing 0.25 µg/ml chloroquine phosphate. Ligation-mediated knotting was carried out in the same way except that plasmids were first linearized with *HindIII* and ligation was at 16°C for 2 h. DNA ligase was inactivated at 65°C for 10 min and knots were singly nicked with DNase I, as described above, and run on a 0.7% agarose gel.

Gel electrophoresis

All gels were 0.7 or 1% agarose in Tris–acetate running buffer [40 mM Tris–AcOH (pH 8.2), 20 mM NaOAc, 1 mM EDTA] and were run at ~3 V/cm for 16 h. Gels were stained with ethidium bromide and photographed with 260 nm UV transillumination. In addition, some gels

were stained with SYBR green I (Molecular Probes) and scanned using a Molecular Dynamics Fluorimager 575. DNA was quantified using ImageQuaNT software (Molecular Dynamics).

DNase I protection footprinting

Plasmid pCA18 was cut with *EcoRI* or *HindIII* and 3' end labelled with Klenow polymerase and [α -³²P]dATP or was dephosphorylated and 5' end labelled with T4 polynucleotide kinase and [γ -³²P]ATP according to published procedures (Sambrook *et al.*, 1989). The labelled plasmid was then cleaved with *HindIII* or *EcoRI*, and the ~600 bp fragment containing two *cer* sites was purified from a polyacrylamide gel. Binding reactions were set up in 20 µl of 10 mM Tris–HCl pH 8.0, 10% glycerol, 0.1 mM EDTA, 25 mM KCl, 1 mM L-arginine, 50 µg/ml poly(dI–dC) and ~100 c.p.s. per reaction of end-labelled fragment. Reactions were incubated at 37°C for 15 min, 2 µl of DNase I (4 µg/ml in 50 mM MgCl₂, 50 mM CaCl₂) was added and incubated for 1 min at room temperature, and the reactions were stopped by the addition of EDTA to 20 mM followed by extraction with phenol and ethanol precipitation. Reactions were resuspended in formamide loading buffer and run in a 6% polyacrylamide sequencing gel. Gels were dried, scanned with a Phosphorimager and analysed using ImageQuaNT software (Molecular Dynamics).

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