Xer Recombination in Escherichia coli

Site-specific DNA topoisomerase activity of the XerC and XerD recombinases**

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Xer site-specific recombination functions in maintaining circular replicons in the monomeric state in Escherichia coli. Two recombinases of the bacteriophage λ integrase family, XerC and XerD, are required for recombination at the chromosomal site, dif, and at a range of plasmid-borne sites. Xer recombination core sites contain the 11-base pair binding sites for each recombinase separated by a 6 to 8-base pair central region. We report that both XerC and XerD act as site-specific type I topoisomerases by relaxing supercoiled plasmids containing a dif site. Relaxation by either XerC or XerD occurs in the absence of the partner recombinase and requires only a single recombination core site. XerC or XerD relaxation activities are completely inhibited by the addition of the partner recombinase, providing that the DNA recognition sequence for the inhibiting partner is present.

Site-specific recombination systems function in a wide range of microorganisms to perform programmed DNA rearrangements (1, 2). Specialized recombinases catalyze exchanges between pairs of specific short DNA sequences which share little sequence homology. DNA cleavage, strand exchange, and rejoining of the DNA strands are catalyzed by four recombinase molecules in the absence of exogenous high energy cofactors, and proceed through a series of transient recombinase-DNA covalent complexes. This differentiates site-specific recombination from other types of genetic recombination, and relates site-specific recombinases to topoisomerases, the other class of enzymes with nicking-closing activity on DNA (3, 4). Consistent with that, most of the well studied site-specific recombinases have been shown to act as topoisomerases in vitro (5–7).

Xer site-specific recombination functions in the separation of circular replicons prior to cell division by resolving multimeric forms produced by homologous recombination (8). In Escherichia coli, two recombinases, XerC and XerD, are required for recombination at the chromosomal site, dif, and at a range of plasmid-borne sites (9). Xer recombination core sites share a ~30-bp consensus DNA sequence which contains 11-bp binding sites for each recombinase, separated by a 6 to 8-bp central region (Fig. 1; Ref. 8). Recombinational exchanges between pairs of plasmid sites (e.g. the cer site of ColE1 or the psi site of pSC101) requires additional sequences and proteins whose role is to ensure that recombination is preferentially intramolecular, and converts multimers to monomers (10–12). In contrast, the chromosomal site dif consists only of a core sequence and recombination at dif occurs intermolecularly and intramolecularly, at least when assayed in dif-containing plasmids (13–17).

XerC and XerD share 37% sequence identity and display similarities with members of the bacteriophage λ integrase (λ Int) family of site-specific recombinases (9, 11, 18, 19). This family is characterized by the conservation of two motifs which contain four invariant residues (the RHRY tetrad), all of which have been implicated in catalysis (21). The tyrosine (Y) serves as the attacking nucleophile on the scissile phosphodiester bond of the DNA. This attack generates a 5’ hydroxyl end and a 3’ phosphotyrosyl-protein intermediate which is released upon ligation with the recombining partner (22, 23). The three other residues (Arg, His, and Arg) are thought to be involved in activation of phosphodiester and phosphotyrosyl bonds during cleavage and rejoining reactions (24, 25).

Despite the novelty of being catalyzed by two different recombinases, the biochemical steps of Xer recombination appear to be similar to those described for others systems of the λ integrase family (1, 2). XerC and XerD bind, respectively, to the left and right halves of the core sequence in a cooperative manner (9). Each protein specifically cleaves one of the DNA strands at the position which separates its binding arm from the central region (Fig. 1; Refs. 26 and 27). Strand exchanges at plasmid sites occur in a sequential manner. XerC catalyzes the first pair of strand exchanges to form a Holliday junction-containing intermediate which is eventually resolved by XerD to generate the recombination products (12).

In this paper, we report the characterization of a site-specific topoisomerase activity of both XerC and XerD. We show that XerC and XerD relax supercoiled plasmids containing the dif site in vitro. Relaxation occurs in the absence of the partner recombinase and appears not to require contact between distant recombinase-DNA complexes. Formation of a XerC-XerD-dif complex results in reciprocal inhibition of XerC and XerD relaxation activities.

Materials and Methods

Proteins and DNA—Plasmids used as substrates have been described previously (9, 12, 13). Purification of wild type and mutated XerC and XerD proteins used the procedure of Colloms et al. (12). Construction of the xerCY275F, xerDY279F, and xerDR274Q mutations has been described previously (9). Construction of the xerDH244L and xerDR148K mutations will be reported elsewhere.‡

General Procedures—Chemicals were from Sigma, BDH, Life Technologies, Inc., and Boehringer Mannheim. E. coli topoisomerase I was purchased from New England Biolabs. DNA purification and manipulation, gel electrophoresis, staining, and photography involved

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The abbreviation used is: bp, base pair(s).

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standard procedures (28). Agarose gels were first stained with SYBR-green and scanned in a Molecular Dynamics FluorImager 575. Quantitative analysis was performed using ImageQuaNT software. After destaining, gels were re-stained with ethidium bromide and photographed.

In Vitro Relaxation Reaction—Standard relaxation reactions contained 300 ng of supercoiled plasmid substrate in a final volume of 20 μl of “relaxation buffer” (50 mM Tris-HCl, pH 8.0, 50 mM KCl, 1.25 mM EDTA, 5 mM spermidine, 0.25 μg/ml bovine serum albumin, and 40% (v/v) glycerol) and 1 μl of protein in their respective dilution buffers (25 mM Tris-HCl, pH 8.0, 0.25 mM EDTA, 500 mM NaCl, 1 mM urea, and 50% (v/v) glycerol for XerC; and 125 mM imidazole, 5 mM NaCl, 0.5 mM EDTA, 10 mM sodium acetate, pH 5.0, and 50% glycerol (v/v) for XerD). Final concentrations of the proteins are 1.1 μM for XerC and 0.125 μM for XerD. After 16 h of incubation at 37 °C, DNA was ethanol-precipitated and analyzed by electrophoresis on 1% agarose gels (0.5 V/cm, 16 h).

Purification of Plasmid Topoisomers—Plasmid pMIN33 topoisomers were separated on a 1% agarose gel (Sea Kem GTG-agarose from Bioproducts) containing 2 μg/ml chloroquine. The gel was run for 16 h in TAE buffer containing 2 μg/ml chloroquine and stained with ethidium bromide. Bands corresponding to single topoisomers were excised from the gel on a 365-nm UV transilluminator. DNA was extracted from the gel slices by electroelution, ethanol-precipitated, and resuspended in 20 μl of water. 5 μl was used for each relaxation reaction.

RESULTS

XerC and XerD are Site-specific Topoisomerases—The E. coli chromosomal site dif exhibits no recombination activity in vitro on duplex recombination sites contained within supercoiled plasmids, under conditions where the plasmid sites psi and cer recombine efficiently (12). However, prolonged incubation of the plasmid, pMIN33, containing a single dif site, with either purified XerC or XerD led to the appearance of new DNA forms (Fig. 2, lanes 5 and 6). These new forms comigrated with relaxed topoisomers of the substrate obtained by partial treatment with E. coli topoisomerase I (Fig. 2, lane 7). Phenol extraction of the reactions, whether or not preceded by treatment with proteinase K, did not alter this pattern of bands indicating that the novel species were not DNA-protein covalent complexes (data not shown). We conclude that the new forms are relaxed topoisomers of the substrate formed by XerC- and XerD-mediated catalysis. For both pMIN33 and pSDC124, a plasmid containing two dif sites in direct repeat, the restriction enzyme digestion patterns of the substrates were not changed during incubation (data not shown). Therefore relaxation is not proceeding through recombination. Relaxation depends on the presence of the recombination site on the substrate as the cloning vector, pUC18, is not relaxed by either XerC or XerD (Fig. 2, lanes 2 and 3). We therefore conclude that XerC and XerD can act as site-specific topoisomerases on dif-containing substrates.

Both XerC and XerD relaxation activities increased with added glycerol and showed an optimal efficiency at a KCl concentration of 50 mM (data not shown). Titration of either protein in relaxation reactions showed that both are present in saturating amounts in standard reactions (see “Materials and Methods”). Even under these optimal conditions, relaxation by either XerC or XerD appears to be a slow process when compared with recombination at plasmid sites in vitro (12). Products were barely detectable after 1 h and increased up to 8 h of incubation at which time 30 to 40% of the substrate was processed (data not shown).

XerC and XerD Catalytic Residues Are Required for Relaxation Activity—Mutants in three residues of the RHRY tetrad of XerD (R148K, R247Q, and Y275F) and of the catalytic tyrosine of XerC (Y275F), are defective in the cleavage step of catalysis (9, 12, 26, 27). These mutants were also defective in relaxation (Fig. 3), suggesting that cleavage during relaxation and recombination involve the same residues and mechanism.

XerDH244L is mutated for the conserved histidine of the RHRY tetrad of XerD. Like its counterpart in the Fpl recombinase (25), XerDH244L cleaves Holliday junction-containing DNA substrates efficiently, but religiates the cleaved DNA strands poorly, resulting in the accumulation of protein-DNA covalent complexes. This failure to religate DNA leads to the accumulation of a nicked form of the substrate to which XerDH244L is covalently bound. No new DNA species or increase in the intensity of the open circle band was detectable in relaxation reactions with XerDH244L, even after release of possible protein-DNA covalent complexes by treatment of the reaction with proteinase K (Fig. 3 and data not shown).

XerDH244L is therefore defective in cleavage in relaxation reactions but not in recombination reactions, that require exchange of strands with a partner duplex within a complex containing two bound XerC molecules and two bound XerD molecules. The reasons for this difference may reside in the structure of the nucleoprotein recombination complex and/or in the conformation of the DNA substrate, since XerD resolves Holliday junction-containing DNA molecules during recombination whereas the substrates used in relaxation are unbranched. Catalysis by XerD on linear and X-shaped DNA substrates may therefore involve different catalytic residues within a given recombinase protomer.

XerC and XerD Are Type I Topoisomerases—Topoisomerases have been classified into type I or type II topoisomerases on the basis of whether they change the linking number of the DNA substrate in steps of one (type I), or two (type II) (3, 4). To investigate whether XerC and XerD are type I or II topoisomer-

![Figure 1](image1.png)

**Fig. 1.** Sequence of the 28-bp dif core site. The three blocks represent the XerC-binding site, the central region (CR), and the XerD-binding site. The black and open triangles indicate the position of XerC cleavage (top strand) and XerD cleavage (bottom strand), respectively.

![Figure 2](image2.png)

**Fig. 2.** XerC and XerD are site-specific topoisomerases. Plasmids pUC18 and pMIN33 (a pUC18 derivative containing dif; Ref. 13) were incubated with no protein (−), purified XerC (C), XerD (D), or topoisomerase 1 (T1) and analyzed on a 1% agarose gel as described under “Materials and Methods.” The different species have been confirmed by two-dimensional gel electrophoresis (data not shown). Their respective positions are indicated on the right: OC, open circle; squares, topoisomers running as negatively supercoiled forms; triangles, topoisomers running as positively supercoiled forms; and R, topoisomer fully relaxed in the gel conditions.

3. L. Arciszewska, I. Grainge, P. A. Wigge, and D. J. Sherratt, unpublished data.

4. L. K. Arciszewska, S. D. Colloms, P. A. Wigge, and D. J. Sherratt, unpublished data.
ases, relaxation reactions were performed using a purified single topoisomerase of pMIN33 as substrate (Fig. 4). XerC- and XerD-mediated relaxation of this substrate yielded the same band pattern as the substrate containing a mixture of topoisomers (Fig. 4B). Since this behavior is expected only from a type I enzyme, XerC and XerD are site-specific type I topoisomerases.

For XerD- and XerC-mediated relaxation, processed DNA molecules appear to be almost completely relaxed whereas the majority of the substrate has not been processed (Fig. 2). Absence of extensive relaxation of the species running as fully supercoiled DNA has been confirmed by two-dimensional gel electrophoresis (data not shown). In addition, the relative distribution of the relaxed topoisomers bands did not vary with varying reaction efficiency, for example, during time courses, protein titrations, or glycerol ranges (data not shown). This suggests that relaxation by XerC and XerD occurs in one of two ways. First, only one supercoil could be relaxed per cleavage event, the recombinaise remaining bound to the site after religation and being strongly prone to continue relaxing the same DNA molecule. Second, the cleaved DNA strand could be free to rotate about the intact one a large number of times before religation, leading to a large number of supercoils relaxation per cleavage event. This second mechanism seems the most plausible because it correlates better with what XerC and XerD topoisomerase activities may reflect, i.e. aberrant behavior of enzymes displaced from their normal catalytic environment (see below). Furthermore, if relaxation was processive, changes in the reaction conditions such as salt concentration might modify the behavior of the recombinaises by changing their dissociation rate from the DNA thus favoring their turnover between different DNA molecules. No change in the relative quantities of the topoisomers were observed when the KCl concentration was varied between 25 and 200 molar (data not shown). This would argue against the processive relaxation hypothesis.

**Topoisomerase Activity Does Not Appear to Require Synapsis between Distant Sites**—The relaxation activities of XerC and XerD show that either recombinaise can catalyze strand cleavage and religation in the absence of its partner. However, catalysis may occur interactions between protomers of the same recombinaise bound to two distant sites. This question was investigated by performing a series of relaxation reactions at different recombinaise/DNA-substrate ratios. If any interaction between distant sites was require for catalysis, the efficiency of the reaction should decrease as a second order function with respect to the substrate concentration under saturating protein conditions. Reaction efficiency should not vary with substrate concentration if interactions between sites are not required. Substates carrying one or two *dif* sites were also compared, reasoning that the effect of substrate dilution on relaxation efficiency should be far less dramatic for a plasmid containing two sites in the eventuality of a dependence on synapsis. For both types of substrates, no significant variation in XerD relaxation efficiency over a 5-fold dilution of the substrate was observed (Fig. 5). For XerC, a slight decrease of activity with substrate dilution was noted (Fig. 5). However, this dependence is not a second order dependence and is equally detectable on substrates containing one *dif* site (pMIN33) or two *dif* sites either in direct or in inverted orientation (pSDC124 and pGB105, respectively, Fig. 5). The observed decrease in XerC activity is therefore unlikely to reflect a requirement for an interaction between distant sites. This is supported by the observation that cleavage by XerC on linear substrates shows a linear decrease with respect to protein concentration, as would be predicted if no synapsis is required (27). Therefore, XerD and XerC relaxation activities are not likely to require synapsis between distant recombination sites.

**XerC and XerD Inhibit the Relaxation Activity of the Partner Recombinase**—Plasmid relaxation by either XerC or XerD is observed when only one of the two recombinaises is present in the reaction. Addition of the partner recombinaise results in a total inhibition of either XerC or XerD topoisomerase activity (Fig. 6). This reciprocal inhibition does not require the catalytic activity of the inhibiting partner since the catalytically inactive mutants XerCY275F and XerDY279F inhibit the wild type XerD and XerC, respectively (Fig. 6, *lanes 5 and 6*). A series of relaxation reactions on plasmids containing only the left half (XerC-binding site, pGB205) or the right half (XerD-binding site, pGB205) of the *dif* site (*lanes 8, 9, 12, and 13*) shows that inhibition depends on the binding site within a complex where both recombinases are bound to the core sequence. pGB205 is relaxed by XerC but not by XerD and vice versa for pGB206 (Fig. 6, *lanes 8, 9, 12, and 13*). Thus, relaxation does not require the presence of the binding site for the absent recombinase and a given recombinase can only mediate relaxation when it interacts with its own binding site; if there is any weak interaction with the partner recombinase-binding site it does not lead to relaxation. Reciprocal inhibition no longer occurs on pGB205 and pGB206 (Fig. 6, *lanes 10 and 14*), showing that it only occurs within a complex where both recombinases are bound to the core sequence (note the partial inhibition of XerD relaxation activity by XerC in *lane 14* when compare with *lane 13* which is consistent with the weak binding of XerC to the mutated *dif* site present in pGB205 in presence of XerD, Ref. 9). This strongly suggests that reciprocal inhibition proceeds through XerC-XerD interactions across the core sequence. No inhibition of XerD-mediated relaxation upon binding of two XerD protomers to a mutated *dif* site in which
During a complete recombination reaction, site-specific recombinases catalyze two pairs of DNA strand exchanges between two recombination sites (1, 2). In this process, recombinases catalyze both the cleavage and the rejoining steps, using covalent protein-DNA complexes as intermediates, a property shared by topoisomerases. Consistent with this, most of the well-studied recombinases have been shown to act as topoisomerases in vitro by relaxing supercoiled DNA without recombinating it (5–7). In this report, we characterize the topoisomerase activities of E. coli XerC and XerD and indicate how recombinase-recombinase interactions may be important in controlling the topoisomerase activity of site-specific recombinases.

Relaxation of supercoiled plasmids by either XerC or XerD occurs in vitro providing that the plasmid substrate contains their respective DNA recognition sequence. It occurs in the absence of the recombinase partner and is unlikely to require any contact between distant recombinase-bound sites. This supports a simple “swivel” type mechanism in which a single recombinase protomer bound to its DNA recognition sequence cleaves one of the two DNA strands which is then free to rotate about the intact one before being resealed. The pattern of topoisomers formed upon relaxation by either XerC or XerD suggests that relaxation occurs in a process in which a single cleavage event can lead to loss of a large number of supercoils before rejoining. This view is supported by the observation that a wide range of reaction conditions, which would be expected to influence recombinase binding, did not change the pattern of relaxation. The swivel mechanism has been proposed to account for relaxation by eucaryotic type I-3’ topoisomerases, that also use a 3’-phosphotyrosine DNA-protein covalent complex as a reaction intermediate (4, 22, 23).

Experimental evidence has shown that XerC and XerD cleave the phosphodiester bonds adjacent to their respective binding sites and do so independently of the catalytic activity of the partner recombinase (Fig. 1) (12, 26, 27). This eliminates models in which either recombinase would be implicated in cleavage of the phosphodiester bond activated by its partner. The fact that relaxation occurs in the absence of the partner recombinase is consistent with that. The observation that relaxation is unlikely to require contact between recombinase-DNA complexes also indicates that single protomers of either XerC or XerD do not need to interact with a second molecule of the same recombinase to catalyze cleavage and rejoining, at least in a reaction which leads to relaxation. Whether XerC and XerD are catalytically autonomous in all stages of a complete recombination reaction remains to be clarified.

Relaxation is inefficient when compared with the relatively fast rate of strand exchange observed for in vitro recombination reactions (12, 26). Under most experimental conditions, strand exchange mediated by XerC or XerD require the partner recombinase be present (9, 11, 26), although on linear nicked “suicide” substrates a low level of XerD-independent XerC-mediated cleavage was observed (27). This suggest that catalysis during a recombination reaction requires binding of both recombinase for the activation of each of their catalytic activities (a phenomenon referred to as “cross-core stimulation” in the case of λ integrase; Ref. 30). The relaxation activities we observe are probably residual low activities that may not have any biological significance. However, these activities provide a powerful tool for study the catalytic functions of the recombinases and recombinases interactions when bound on DNA.

An interesting observation is that XerD relaxes cer containing plasmids, albeit less efficiently than dif containing substrates (data not shown). This was unexpected since cleavage or strand exchange by XerD on cer containing substrates has never been detected in vitro using different recombination assays (12, 26). Consistent with that, XerD catalytic activity is not required for recombination at cer in vivo (12). Recent experiments point out that XerD-mediated strand exchange on dif containing Holliday junctions depends on the spatial configuration of the four DNA strands forming the junction (20). It is proposed that cer containing Holliday junctions adopt an inappropriate configuration for XerD catalysis to occur. XerD-mediated catalysis on unbranched substrates in the absence of XerC could be released from this topological constrains.

The reciprocal inhibition of XerC and XerD relaxation activities by the partner recombinase requires the presence of the DNA recognition sequence for the inhibiting partner and is therefore mediated by XerC-XerD interaction through the core sequence of dif. This highlights the importance of cooperative interactions between recombinases in the control of their cat-
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alytic activities. Inhibition of relaxation could occur at the level of cleavage, cleavage not being allowed until a synaptic complex competent for recombination is correctly assembled. Assembly of the synaptic complex would then couple cleavage to strand exchange. Alternatively, XerC-XerD interactions could prevent rotation of the cleaved strand about the intact one, or could stimulate the rejoining step which would then always occur without providing an opportunity for rotation.

How are XerC and XerD relaxation activities and reciprocal inhibition related to the topoisomerase activities displayed by others systems of the λ integrase family? Two other members of the family have been shown to relax supercoiled plasmids in vitro using a type I topoisomerase activity: λ integrase itself and the Cre recombinase of bacteriophage P1. In both cases relaxation is by far less efficient than strand exchange and the pattern of topoisomers obtained after relaxation resembles those obtained with XerC and XerD (5, 7). Moreover, to the best of our knowledge, Cre-mediated relaxation was only observed on a substrate containing a mutated recombination site in which 1 bp had been removed from the central region (7). This reduces the spacing between the two Cre recognition sequences and might modify or impair the interaction between the two Cre protomers resulting in reciprocal inhibition release. Relaxation by λ integrase occurs with the same efficiency on substrates with or without a recombination site (5). This has been proposed to occur upon recognition of “pseudo half-recombination sites” present on the substrates (31). The fact that relaxation is not improved by the presence of a bona fide recombination site on the substrate suggest that binding of two integrase protomers to the recombination site results in reciprocal inhibition in this system too. Taken together, these data suggest that the relaxation mechanism of XerC and XerD, and the way in which it is controlled, is similar to that of other members of the family. Perhaps significantly, the only well studied recombinase of the family for which no relaxation activity has been described so far is Flp, for which, all of the evidence suggests, needs two protomers to form a single active site capable of cleavage and rejoining (29).

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