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Lilley, David M. J.

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Holliday junction-resolving enzymes - structures and mechanisms

David M. J. Lilley

Cancer Research UK Nucleic Acid Structure Research Group,

MSI/WTB Complex,

The University of Dundee,

Dow Street,

Dundee DD1 5EH,

U.K.

* To whom correspondence should be addressed

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Editorial enquiries to Professor Lilley:

Tel: (+44)-1382-384243

FAX: (+44)-1382-385893

Email: d.m.j.lilley@dundee.ac.uk

ABSTRACT

Holliday junction-resolving enzymes are nucleases that are highly specific for the structure of the junction, to which they bind in dimeric form. Two symmetrically-disposed cleavages are made. These are not simultaneous, but the second cleavage is accelerated relative to the first, so ensuring that bilateral cleavage occurs during the lifetime of the DNA-protein complex. In eukaryotic cells there are two known junction-resolving activities. GEN1 is similar to enzymes from lower organisms. A crystallographic structure of a fungal GEN1 bound to the product of resolution has been determined. These complexes are dimerized within the crystal lattice such that the strands of the products may be simply reconnected to form a junction. These structures suggest a trajectory for the resolution process.

TEXT

Holliday junctions and their processing

Holliday junctions (1) are DNA branchpoints in which four helices are covalently connected by the exchange of strands. They can be generated by strand invasion into a double helix, and by the reversal of a fork, and thus are central intermediates in genetic recombination, the repair of double-strand DNA breaks and the processing of blocked replication forks. They must be processed back into duplex DNA species, and this can occur in a variety of ways. The most widespread is by the action of junction-resolving enzymes, nucleases that are selective for branched DNA. Such resolution activities have been isolated from a wide range of organisms and viruses. In eukaryotes there is an important alternative to resolution, called dissolution, in which junctions are pushed along by the Bloom's helicase and then decatenated by a topoisomerase.

In the presence of metal ions but the absence of proteins, the four-way junction folds by pairwise coaxial stacking of helical arms to adopt the right-handed stacked-X structure (2,3) in one of two stacking conformers (4) that interconvert in solution (5,6). The stacked-X structure has been confirmed by X-ray crystallography (7). However, the binding of junction-resolving enzymes invariably result in a major distortion of the structure (8-12), and that this is probably very important in the resolution process.

The occurrence of junction-resolving enzymes in lower organisms

Until relatively recently, the only known junction-resolving enzymes had been found in lower organisms. All the characteristic properties of such enzymes were elucidated the study of these proteins, and their investigation also stimulated the development of many of the now-standard tools used in their analysis, such as the supercoiled cruciform assay for studying bilateral cleavage kinetics (13,14).

Resolving enzymes have been isolated from bacteria and their phages, archaea and yeast mitochondria (15,16). These are relatively small (typically around 150 amino acids, except for the mitochondrial enzymes that are twice that) and basic (pI = 8.8 to 9.8) proteins, and the majority can be placed into one of two superfamilies (17,18). The first group comprises RuvC of *E. coli*, the yeast mitochondrial enzymes Cce1 and Ydc2 and the vaccinia virus enzyme A22, all of which fall into the superfamily that includes RNaseH and the integrases. By contrast, phage T7 endonuclease I, gram positive bacterial RecU and the archaeal Hjc and Hje enzymes are grouped in the family of nuclease enzymes that includes the restriction enzymes and MutH. The first group (predominantly cellular resolving enzymes) are characterized by a significant sequence specificity for cleavage (19-21). Although they will bind to junctions of indiscriminate sequence, they exhibit marked preferences for cleavage, implying sequence-specific interactions in the transition state (22).

X-ray crystallographic structures have been obtained for most of the these junction-resolving enzymes. In general the shape of the dimeric proteins is prolate, based upon mixed α□β folds. Over the group, significantly different architectures are adopted. RuvC structure (23) is similar to that of RNaseH (24), and Ydc2 of *S. pombe* (25) appears to be an elaborated version of RuvC. By contrast, the structures of T7 endonuclease I (26), the archaeal junction-resolving enzymes Hjc (27,28) and Hje (29), and RecU of the gram positive bacteria (30,31) are evidently related to those of the restriction enzymes. In all cases the active sites of these enzymes contain divalent metal ions coordinated by the acidic amino acid side chains. Single metal ions have been shown to be bound in the active sites of T4

endonuclease VII (32), Ydc2 (25) and RecU (30,31), while two ions have been observed in that of T7 endonuclease I (33,34). The structures of two enzymes of phage origins have been determined for the complex of a dimer bound to a DNA junction (35,36). These structures are quite unalike. T4 endonuclease VII presents a flat S-shaped surface to which the junction binds in a flat, open H-shaped conformation (35). In the complex with T7 endonuclease I the junction retains a pairwise coaxial alignment of arms, although base stacking is interrupted at the centre. The enzyme binds the junction on the major groove side, interacting with each phosphate of both continuous strands over 8 nucleotides. Formation of the endonuclease I dimer creates two hemi-cylindrical clefts each 30 Å long that are mutually perpendicular, and the DNA arms are bound along the length of these basic channels. The enzyme selects for a DNA structure that can adopt the geometry of the junction where the two axes are almost perpendicular to each other.

The key properties that a Holliday junction-resolving enzyme should exhibit

It is self evident that a junction-resolving enzyme must be able to bind highly selectively to a four-way helical junction, and introduce symmetrically-paired bilateral cleavages (Figure 1). It has been long known that the phage enzymes bind to DNA junctions in dimeric form with at least a 1000-fold greater affinity than for a duplex of the same sequence (10). The affinity for junctions is typically around nM for most of these enzymes (10-12,37). While these properties are clearly essential, we know nothing about how these enzymes find DNA junctions in the crowded and complex milieu of the cell. It seems probable that the d would bind to duplex DNA and slide or hop along it until it finds a junction in order to reduce the dimensionality of the search. Upon encountering a junction the greater affinity would keep it in place, but there are no experimental data that currently address this.

Although it has been demonstrated unequivocally that the active form of a resolving enzyme bound to a four-way junction is a dimer (10,19,37-39), the monomer-dimer equilibrium in solution in the absence of the junction varies widely in affinity and rate of association (10,19,38,40)

In order to resolve a junction into duplex species two phosphodiester linkages must be hydrolyzed. Upon binding to the junction, DNA strands must enter the active sites of the dimer, and be cleaved. This can occur 5' or 3' to the point of strand exchange, and the distance is not critical, but it must be the same for both sides, i.e. the cleavage sites must be symmetrically disposed. Moreover it is important that the cleavages are coordinated in some manner such that both are made before the enzyme dissociates. Otherwise a unilaterally cleaved junction will result that is likely to be toxic to the cell. This is discussed further in the following section.

How resolving enzymes achieve a productive resolution of a Holliday junction

It is clearly critical that junction-resolving enzymes generate bilateral cleavage of the junction, but how is this achieved ? The key to the study of this was the use of supercoiled cruciform substrates (Figure 2) (13,41,42). The cruciform structure is an opposed pair of hairpin loops extruded from duplex DNA where the sequence has two-fold symmetry, i.e. an inverted repeat (43). The junction between the two stem-loops and the duplex DNA is exactly equivalent to a four-way junction, and this has long been known to be a substrate for junction-resolving enzymes (44,45). The extruded cruciform structure is in equilibrium with the unextruded duplex form, but in linear DNA the equilibrium is heavily biased towards the duplex because the cruciform is less stable than the duplex by ≥ 55 kJ mol^{-1}. However, if the inverted repeat is contained within a supercoiled circular DNA molecule the cruciform can become more stable than the duplex because its formation is accompanied by an unwinding (a negative change of twist) and this generates a relaxation of negatively supercoiled DNA. Thus above a threshold level of negative superhelix density the cruciform is stable in a circular DNA molecule, but this continues only as long as the DNA remains supercoiled (46). If the covalent continuity of the circle is broken at any point the cruciform is rapidly reabsorbed. Thus if an enzyme makes a single cleavage in the DNA, the cruciform can only remain extruded if it is preserved by the DNA-protein interactions in the complex. If the protein dissociates before cleavage of the second strand occurs, the supercoiling will be released and the cruciform is reabsorbed. At that point no substrate remains for a second cleavage reaction. What remains then is a circular DNA molecule containing a nick. On the other hand, if the enzyme makes bilateral cleavages within the lifetime of the complex, the circular DNA becomes linearized. Nicked, linear and supercoiled DNA forms of a plasmid DNA are well separated by electrophoresis in an agarose gel, so it is a simple matter to distinguish unilateral and bilateral cleavage.

It was found that resolving enzymes convert supercoiled plasmid DNA containing a cruciform into linear product, indicative of bilateral cleavage (13). However, careful analysis of the products of cruciform cleavage by yeast mitochondrial CCE1 and bacterial RuvC at early times revealed that nicked DNA was formed as a transient intermediate, that was then converted into linear product by a second cleavage (14,42). This indicated that the two cleavages were not necessarily simultaneous, but that the second cleavage must occur within the lifetime of the complex so that the cruciform was not reabsorbed by release of negative supercoiling. The data were fitted to a kinetic model of sequential cleavage, whereupon it emerged that the second cleavage was accelerated by ten or one hundred fold relative to the first for CCE1(14) and RuvC (42) respectively. It is this acceleration that ensures productive resolution because the second cleavage follows hard on the heels of the first, and its likely to result from the release of strain in the complex following the cleavage of the first strand. Similar results were found for other junction-resolving enzymes (37,38), including the eukaryotic GEN1 (40) as we shall see shortly.

Processing of Holliday junctions in eukaryotic cells

In eukaryotic cells Holliday junctions are processed by two rather distinct mechanisms, that can be differentiated by whether or not they involve hydrolytic cleavage. Dissolution requires the BLM helicase to translocate two adjacent junctions towards each other so that they may be unlinked by the action of topoisomerase IIIα (47-49). This mechanism is likely to be the primary response to the existence of DNA junctions in mitotically-dividing cells, and defects in the pathway lead to Bloom syndrome (50) that results in genomic instability (51). Any junctions that resist dissolution are processed resolution, i.e. the action of nucleases that are selective for the structure of a four-way DNA junction as discussed above.

Despite heroic efforts to identify and purify a cellular resolution activity (i.e. not mitochondrial) from eukaryotes over a long period (52-54), such enzymes were only relatively recently identified. The first was GEN1 (Yen1 in yeast) (55-57), a member of the FEN1/XPG superfamily of 5' nucleases that includes EXO1, FEN1 and XPG (58-60). It emerged that the properties of GEN1 were closely similar to those of the long-studied resolving enzymes of lower organisms in most respects (see below).

The second activity identified in eukaryotes was a considerably more elaborate apparatus, comprising a number of different proteins that includes two nucleases acting together as a complex. The nuclease making the initial cleavage within the junction is the nuclease SLX1. This is a member of the UvrC family of endonucleases that contains a GIY-YIG motif that creates a metal ion-binding active center in a number of nucleases(61-63). SLX1 is bound to a much larger protein SLX4, that binds a whole series of enzymes involved in DNA repair (64-68). It then emerged that SLX1 only introduces a single cleavage into the junction, and the second cleavage is generated by the MUS81-EME1 nuclease that is also bound as part of the SLX4 complex (69,70). Mus81 had been studied earlier, and had biochemical properties like those of a flap endonuclease (71-73). But the functional junction-resolving enzyme is the SLX1-SLX4-MUS81-EME1 complex.

GEN1 and SLX4 are synthetically lethal in human cells due to dysfunctional mitosis resulting from unprocessed junctions(74). Therefore it seems there is some redundancy in resolution capacity, but it is essential that one of the two systems is functional. Expression of human GEN1 restores the meiotic phenotype of *mus81Δ* fission yeast (75), and persistent DNA junctions in meiotic yeast can be resolved by Yen1 (76). West and coworkers have shown that GEN1 activity is regulated through its compartmentalization in the cell (76,77).

Properties of a fungal GEN1 enzyme

Human GEN1 was first identified, along with the yeast ortholog Yen1 (55), followed a little later by an ortholog from *Caenorhabditis elegans* (56). West and coworkers characterized the cleavage of junction species by human GEN1 (57). However the human GEN1 was subject to aggregation on DNA such that no discrete complex of GEN1 bound to a DNA junction could be observed in the absence of competitor DNA, although more discrete binding was demonstrated in the presence of poly dI.polydC (78). We also found that human GEN1 formed multiple complexes with four-way DNA junctions so that a proper biophysical or structural study of the interaction was not possible (unpublished). We therefore searched for a GEN1 ortholog with properties more amenable to quantitative biophysical analysis, and turned to the thermophilic fungi as a possible source. Thermostable proteins are frequently better behaved than their mesophilic equivalents. Using bioinformatic analysis we identified all the FEN1/XPG superfamily orthologs in these species, including GEN1. Alignment of the protein sequence of the putative GEN1 from *Chaetomium thermophilum* with that of the flap endonuclease FEN1 showed that there were seven strongly conserved acidic amino acids in common that corresponded to metal ion-binding residues in the active site of FEN1. We expressed and purified GEN1 from *C. thermophilum* as an N-terminal 1-487 amino acids fragment (hereafter termed CtGEN1) by construction of a synthetic gene with optimized codon usage for *E. coli* (40).

CtGEN1 was shown to be a nuclease that is highly selective for four-way DNA junctions, cleaving 1 nucleotide 3' to the point of strand exchange on two strands symmetrically disposed about a diagonal axis (40) (Figure 3A). Importantly, we showed that it bound to DNA junctions as a discrete homodimer with nanomolar affinity (Figure 3B). However, in contrast to the enzymes of phage and lower organisms it was found to be predominantly monomeric in solution. Binding is strongly cooperative and thus it is likely that GEN1 dimerizes on the DNA junction; this is currently under investigation using single molecule methods. Using supercoiled cruciform substrates we showed that CtGEN1 dimer makes sequential cuts, with a ten-fold acceleration of the second cleavage.

Aside from the oligomeric state of the free protein, the properties of CtGEN1 are closely similar to those of the resolving enzymes from bacterial, phage and mitochondrial origins discussed above. All the basic principles of the *modus operandi* of the enzymes of the lower organisms apply very well to GEN1.

The structure of CtGEN1

Wild-type CtGEN1 was mixed with a DNA junction in the presence of 2 mM $MgCl_2$ and allowed to crystalize (79). Crystals of CtGEN1 in complex with a DNA junction were obtained, and diffraction phased by single-wavelength anomalous dispersion using selenomethionine-substituted CtGEN1. The structure was solved to a resolution of 2.5 Å (PDB 5CO8). In addition, the structure was solved after soaking with Mn^{2+} ions (PDB 5CNQ).

Under the conditions of crystallization the CtGEN1 was active and thus a product complex resulted as the functional unit in the crystal (though not the asymmetric unit). This was one of the two possible products corresponding to just two of the arms of the junction (Figure 4A), comprising one full length strand of 30 nt, and two short strands of 14 and 16 nt. The axes of two arms of the product are approximately perpendicular, and the trajectory of the DNA is closely similar to that of a DNA flap bound to FEN1 (60).

The CtGEN1 monomer approximates to a hemi-ellipsoid of 80 x 30 x 30 Å, broadened at one end to 40 Å (Figure 4B). The main section of the protein centers around a central seven-strand mixed β sheet that is flanked on both sides by α helices. The connectivity of helices and sheet is generally very similar to that observed in the FEN1 family members (59,60,80). However, CtGEN1 lacks the helical arch that selects the single-stranded flap of the DNA substrate in FEN1. The C-terminal, wider end of CtGEN1 contains a three-strand antiparallel β sheet and four α helices, with no counterpart in the other FEN1-XPG family members. The structure is similar to a series of chromodomain proteins. This has also been noted in human GEN1, studied as a non-specific complex bound to DNA (81).

The structure of the CtGEN1 monomer provides a basic flat face on which the DNA binds. Examination of the electrostatic surface reveals a striking DNA-binding path whereby each point of contact with the ribose-phosphate backbone of DNA has an electropositive patch. There are no base-specific contacts, consistent with binding that lacks any sequence specificity. More than one turn of helix from both arms is bound to the protein, with the extra C-terminal domain of CtGEN1 allowing an increased length of DNA to be bound compared to FEN1. Altogether the bound DNA buries a surface area of almost 1,400 $Å^2$.

The active site of CtGEN1 is strongly electronegative, containing six conserved acidic amino acids (colored magenta in Figure 4B) that bind two divalent cations. The 5' end of the 3' strand generated by nucleolytic cleavage is directed into the active center. The active site conforms to the standard two-metal ion model of phosphoryl transfer reactions (82) where the metal ions position the reactants, activate the nucleophilic water molecule and stabilize the anionic transition state.

A complex of CtGEN1 bound to a DNA junction

In addition to the product of resolution, the crystal of CtGEN1 also provided a very strong indication of the likely structure of structure of a CtGEN1 dimer bound to a four-way junction (79). Careful examination of the crystal lattice showed that two product complexes were dimerized through contact between three alpha helices from each monomer arranged as a kind of tripod (Figure 5). The dimer interface is relatively small (a buried surface area of 530 $Å^2$), consistent with the bias to the monomer in free solution. This dimeric unit has a coaxial alignment of the uncleaved DNA helices (top left and bottom right in Figure 5), while the helical arms containing the cleavage sites (these are effectively marked by the metal ions, shown as magenta spheres in Figure 5) are rotated towards each other on the major groove side to include an angle of close to 90°. This is very different from the stacked X-formation of protein-free junction in the presence of divalent metal ions, but the global geometry in this complex is identical with that deduced by comparative gel electrophoretic analysis (79), so providing complete agreement between structure in the crystal and in solution. It is not known at what point the structural change occurs; this might occur by conformational selection or induced fit.

Computationally it was possible to reconnect the strands to generate a covalently-intact four-way junction, requiring only that the base pair located at the junction-proximal end of each uncleaved helix be unpaired and unstacked. This local distortion was confirmed in solution by permanganate probing and unquenching of 2-aminopurine fluorescence (79). Importantly, the spectroscopic study further revealed that the region around the point of strand exchange was significantly more unstacked in the intact junction compared to that in the product, indicating a degree of refolding of the central region following strand cleavage.

Progress towards the trajectory of the resolution process

We are now in a position to fill in some structural detail for the scheme shown in Figure 1 as it applies to GEN1. First, we have found out that in free solution CtGEN1 exists mainly in monomeric form. It therefore most probably assembles as a dimer in the course of binding to a four-way DNA junction. GEN1 binds selectively to DNA junctions with nM affinity, and in doing so both radically changes

the global disposition of the arms and opens the center, leading to a strained conformation. After binding, sequential cleavages are introduced on two diametrically-opposite strands 1 nt 3' to the point of strand exchange, with a ten-fold acceleration of the second cleavage. Although this requires more in-depth study, particularly using experiments at the single-molecule level, it seems quite probable that relaxation of a strained structure in the intact junction complex is relieved upon introducing the first cleavage, and this accelerates the cleavage at the second site. The net effect is that the second cleavage rapidly follows the first, ensuring that two cleavages are made before the complex dissociates and hence a productive resolution event results.

In conclusion

We see that as a class the Holliday junction-resolving enzymes have fundamental characteristics in common. They must bind selectively with high affinity to four-way DNA junctions in dimeric form, and introduce two symmetrically-disposed hydrolytic cleavages of the phosphodiester backbone. Although the resolving enzymes are strongly selective for the structure of the junction, paradoxically in all cases binding brings about major changes to the DNA structure. Thus these enzymes recognize the structure of the distorted junction. This is likely to be critical in their function. It is important that bilateral cleavage is completed before dissociation of the protein-junction complex, and this is achieved by acceleration of the second cleavage. That increase in the rate of strand cleavage is likely achieved due to the release in structural strain after the first cleavage. Eukaryotic GEN1 exhibits all these properties, and we see the structural basis for much of this. At present rather less is known about the SLX1-SLX4 resolving enzyme complex, but it is likely that similar mechanisms must operate.

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FIGURE LEGENDS

Figure 1. Scheme showing binding and cleavage events in the action of a junction-resolving enzyme. The resolving enzymes undergo a monomer-dimer equilibrium in solution, the position of which differs widely between enzymes. Binding occurs in dimeric form so that symmetrical bilateral cleavages are made. Dissociation of the protein leaves a resolved junction, i.e. two nicked duplex species.

Figure 2. Scheme showing the principle of using a cruciform-containing supercoiled DNA to study uni- and bilateral resolution cleavage of a four-way junction. The cruciform structure is inherently unstable in duplex DNA, but enjoys a stable existence in negatively supercoiled circular DNA. If the resolving enzyme dissociates after a single cleavage is made, nicked circular DNA results, and no further cleavage is possible because reabsorption of the cruciform removes the substrate. Bilateral cleavage generates a linear DNA product. Supercoiled, nicked and linear DNA are readily separated by agarose gel electrophoresis.

Figure 3. Cleavage and binding processes of a fungal GEN1 junction-resolving enzyme.

A. Cleavage of a four-way junction by CtGEN1. A four-way junction was separately radioactively [5'-^{32}P]-labeled on one single strand (named b, h, r and x). Each of the four resulting species was incubated with (+) or without (-) CtGEN1 and the products separated by electrophoresis in a polyacrylamide gel. The junction is strongly cleaved on two diametrically opposite strands (h and x), and weakly on the other two (b and r). Both cleavages are 1 nt 3' to the point of strand exchange.

B. A fixed concentration of four-way junction (82 pM) was incubated with increasing concentrations of CtGEN1 in the presence of 1 mM Ca^{2+} ions. Under these conditions CtGEN1 is not active, so the DNA junction remains intact. DNA-protein complexes migrate more slowly than the free junction (arrowed right). Other experiments show that the strong upper band comprises a dimer of CtGEN1 bound to the junction. Fitting of these data show the binding to occur in a strongly cooperative manner.

Figure 4. The crystal structure of a CtGEN1 monomer bound to the product of resolution cleavage.

A. Scheme showing the cleavage of a four-way junction and the formation of a product complex. Cleavage of the junction occurs at the arrowed sites, generating products with uncleaved red and yellow strands. Each product has two approximately half-length strands. Only the first product crystallized as a complex with CtGEN1, shown as the lower schematic.

B. The structure of the functional unit of the product complex in the crystal. The two arms of the junction are approximately perpendicular, and bound to the enzyme at multiple sites. The protein structure is shown in cartoon form, with alpha helices and coil regions colored yellow, and sheet cyan. The active site resides are shown in stick form colored magenta, with one Mg^{2+} ion shown as a green sphere.

Figure 5. A dimer of CtGEN1 observed in the crystal lattice. Two product complexes are juxtaposed within the lattice mediated by a protein-protein interface. One Mg^{2+} ion is shown for each enzyme monomer (green sphere), showing the position of the active sites. The uncleaved arms are coaxial (although not stacked) and the two cleaved arms are mutually perpendicular to the uncleaved arms and to each other. The two complexes are distinguished by color; one complex is shown with green protein bound to blue DNA while the other is straw protein and salmon DNA.