Interaction of the resolving enzyme YDC2 with the four-way DNA junction

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Received September 3, 1998; Revised and Accepted October 30, 1998

ABSTRACT

Holliday junctions (four-way DNA junctions), formed during homologous recombination, are bound and resolved by junction-specific endonucleases to yield recombinant duplex DNA products. The junction-resolving enzymes are a structurally diverse class of proteins that nevertheless have many properties in common; in particular a high structure specificity for binding and metal-dependent, (frequently) sequence-specific cleavage activity. In Saccharomyces cerevisiae, the enzyme CCE1 is necessary for the resolution of recombining mitochondrial genomes, and in Schizosaccharomyces pombe the homologous protein YDC2 is thought to have a similar function. We have generated an inactive mutant of YDC2, D226N, that retains structure-specific junction binding and have analysed the interaction of this protein with the four-way DNA junction. YDC2 binds the four-way junction in two specific complexes (I and II), unfolding the stacked X-structure into a conformation where the arms extend to the four corners of a square. This structure is reminiscent of that of the free junction in the absence of metal ions and of the structures imposed on the Holliday junction by CCE1 and RuvA. DNase I probing reveals footprints specific for complexes I and II which extend from the junction centre on all four arms. No protection is observed with the small, hydrophobic probe DMS.

INTRODUCTION

The Holliday junction (four-way DNA junction), formed at the point of strand exchange between two duplex DNA molecules, is a central intermediate of both homologous and some site-specific recombination events. The final steps in the pathway of homologous recombination, which serves both to rearrange and repair DNA, occur when a junction-specific endonuclease resolves the Holliday junction, giving rise to two recombinant DNA duplexes. Holliday junction endonucleases have been detected in many organisms, ranging from eubacteria and bacteriophage to eukaryotes and pox viruses (reviewed in 1). To date, the best characterised enzyme of this class is RuvC, which acts in concert with the RuvAB complex to catalyse branch migration and resolution of the Holliday junction in Escherichia coli (reviewed in 2). RuvC is present in most (though not all) eubacteria, but is absent from the eukaryote Saccharomyces cerevisiae and from the archaeal species for which genome sequences have been completed. The equivalent enzymes involved in genomic DNA recombination in the eucaryal and archaeal domains thus remain unidentified.

Homologous recombination is a highly active process in the mitochondria of S. cerevisiae, where the protein CCE1 (MGT1) has been identified as the mitochondrial Holliday junction endonuclease (3–7). CCE1 has been expressed heterologously in E.coli and both the nature of its interaction with the four-way junction and its sequence-specific endonuclease activity have been characterised (8–11). The enzyme binds four-way DNA junctions as a dimer, unfolding the junction so that the DNA arms adopt an extended, square configuration (9). CCE1 is highly sequence-specific for cleavage of Holliday junctions (though not binding), with cleavage after a 5'-CT dinucleotide favoured by at least a factor of 600 over any other dinucleotide sequence (10).

In the past year, three groups have independently identified a homologue of CCE1 from Schizosaccharomyces pombe, YDC2 (SpCCE1) (12–14). YDC2 shares 28% sequence identity with CCE1 and is 25% smaller. We have previously reported the over-expression, purification and initial characterisation of YDC2 (12). In common with all other junction-resolving enzymes studied it binds four-way DNA junctions as a dimer with a high degree of structural specificity and exhibits sequence-specific cleavage activity. As with CCE1, gel electrophoretic retardation experiments with radioactively labelled four-way junctions have demonstrated the existence of two specific YDC2–junction complexes, complex I and complex II, which probably correspond to binding of one and two dimers of YDC2, respectively, to each DNA junction. The biological significance of the second complex is unclear. This paper reports the further characterisation of the YDC2 enzyme, the generation of a catalytically inactive mutant and the investigation of its interaction with the four-way junction in complexes I and II.

MATERIALS AND METHODS

Expression and purification of YDC2

The YDC2 gene was amplified by PCR and cloned into plasmid pUC119 previously (12). The PCR primers introduced several restriction sites to allow subsequent subcloning, in particular a NcoI site was introduced at the start codon and a BamHI site was introduced after the stop codon. The YDC2 gene was removed from pUC119-YDC2 by digesting with NcoI and BamHI and was

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inserted into the NcoI and BamHI sites of expression plasmid pET19b (Novagen). This construct allows expression of the YDC2 protein in E. coli strain BL21 (DE3) under the control of the T7 polymerase promoter. The recombinant protein expressed is identical in sequence to the native yeast protein. Cells were grown in LB medium containing carbencicin (100 μg/ml) in shaker flasks at 28°C to an OD600 of 0.8. Isopropyl β-D-thiogalactoside was added to a final concentration of 0.1 mM and the cells were incubated for an additional 2.5 h under the same growth conditions. Cells were harvested by centrifugation and resuspended in 5 ml/g (wet wt of cells) lysis buffer (50 mM Tris–HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA, 0.2 mM DTT). Cells were lysed by sonication (three 60 s bursts on ice) and the lysate was cleared by centrifugation (4°C, 20 min, 40 000 g). The cleared lysate was subjected to ammonium sulphate fractionation. Protein precipitating between 30 and 50% saturation was resuspended in buffer A (20 mM Tris, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.2 mM DTT) and applied to a POROS HS 20/100 column (Applied Biosystems). Protein was eluted with a gradient of 0.05–1.0 M NaCl in buffer A. Fractions were collected and analysed by SDS–PAGE. Fractions containing YDC2 at a purity >75% were pooled, concentrated and applied to a Superose-12 column (30 × 1 cm internal diameter; Pharmacia) pre-equilibrated with buffer A plus 0.2 M NaCl and eluted isocratically. After gel filtration, YDC2 was analysed by SDS–PAGE and densitometric quantification and was found to be ~95% homogeneous. The concentration of pure YDC2 was estimated from the absorbance of the protein at 280 nm, using the estimated extinction coefficient of ε280nm = 1.5 (12). All YDC2 protein concentrations have been calculated for a dimer of the enzyme.

Site-directed mutagenesis

Single-stranded pUC19-YDC2 DNA for sequencing and mutagenesis was produced with the helper phage MK407 (Promega). Site-directed mutagenesis of YDC2 was carried out using uracil-laden template DNA isolated from strain CJ236 and mutants were selected in strain DH5α (15). After mutagenesis, DNA sequencing was used to confirm that no spurious mutations had been introduced. The YDC2 D226N mutant was subcloned into pET19b, expressed and purified as described above for wild-type YDC2.

Oligonucleotide synthesis

Oligonucleotides were synthesised on a 394 DNA/RNA synthesiser (Applied Biosystems), purified by gel electrophoresis in 12% polyacrylamide containing 7 M urea, the bands excised and DNA eluted and recovered by ethanol precipitation. Oligonucleotides were radioactively labelled at their 5′-termini using [γ-32P]ATP and T4 polynucleotide kinase.

Assembly of four-way DNA junctions

Stoichiometric quantities of three unlabelled and one 5′-32P-labelled strands were annealed by incubation in 50 mM Tris–HCl, pH 7.6, 10 mM MgCl2, 5 mM DTT, 0.1 mM spermidine and 0.1 mM EDTA for 3 min at 85°C followed by slow cooling. Assembled junctions were purified by gel electrophoresis in 5% polyacrylamide gels. Bands were excised and DNA recovered by electroelution.

Junction 1. This is a fixed junction with 20 bp in each arm, assembled from four oligonucleotides each of 40 nt in length as described (8). Tethered junctions based on the sequence of junction 1 were constructed from three oligonucleotides, generating arms of 22, 25 and 15 bp with tethers of eight T residues as described (8).

Junction 3. Comparative gel electrophoresis experiments utilised a version of junction 3 with four arms of 40 bp in length (16). Six forms of junction with two long and two short arms were derived from this junction as described previously (17).

Gel electrophoretic retardation analysis of YDC2–DNA junction interactions

Samples of purified YDC2 protein were incubated with radioactively 5′-32P-labelled four-way DNA junctions in binding buffer (20 mM Tris–HCl, pH 8.0, 200 mM NaCl, 0.2 mM DTT, 1 mM EDTA, 0.1 mg/ml BSA and calf thymus duplex competitor DNA where stated) in 10 μl total volume for 5 min at 20°C. Prior to addition of 1/6th vol loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll type 400). Samples were loaded onto 5% polyacrylamide gels and electrophoresed in TBE buffer. After electrophoresis, gels were dried on Whatmann 3MM paper and exposed to X-ray film for documentation or storage phosphor screens for quantification on a Fuji BAS-1500 phosphorimager. In experiments to measure dissociation constants, data were analysed as fraction DNA bound versus protein concentration and were fitted by non-linear regression analysis as described previously (9). At higher concentrations of YDC2, where a discrete super-retarded band was visible (complex II), it was counted as bound DNA. For the purposes of the calculation, YDC2 was considered to be an undissociable dimer in solution.

Single turnover kinetic analysis

Determination of the first order rate constants of junction cleavage were carried out by first incubating 8 × 10^-8 M 5′-32P-labelled junction with 5 × 10^-7 M YDC2 dimer in binding buffer. Under these conditions, the dissociation constant for junctions varies from 1.3 to 6.5 × 10^-9 M, so all junctions will be bound. Samples were pre-equilibrated at 37°C and reactions were initiated by the addition of MgCl2 to a final concentration of 15 mM. At set time points an aliquot of the reaction mix was removed and the reaction was stopped by the addition of an equal volume of formamide loading buffer (95% v/v formamide, 50 mM EDTA, pH 8.0, 0.1% bromophenol blue, 0.1% xylene cyanol FF) and heating at 90°C for 2 min, followed by storage on ice. Reaction products were analysed by denaturing gel electrophoresis on a 4% nN thick polyacrylamide gels in TBE buffer containing 7 M urea, run at 50°C. After electrophoresis gels were dried on Whatmann 3MM paper and exposed to X-ray film for documentation or phosphor screens for quantification on a Fuji BAS-1500 phosphorimager.

DNA sequencing

Cloned sequences were verified using primer extension and dideoxynucleotide triphosphates (18). For sequence markers used to analyse cleavage positions, chemical degradation sequencing
was employed using standard reactions followed by piperidine cleavage (19).

**DNase I and DMS probing**

Junction 1 (8 × 10^−8 M), 5′,32P-labelled on one strand, was incubated with the required concentration of YDC2 D226N mutant, in binding buffer supplemented with 5 mM MgCl2 and 2.5 mM CaCl2, 10 µl total volume. In some experiments, calf thymus duplex competitor DNA was added, as indicated. After 5 min, either 0.1 U DNase I or 0.5 µl dimethylsulphate (DMS) was added and the reaction was incubated for 2 min at 20°C. Reactions were stopped by the addition of 1 vol formamide loading buffer and heating to 90°C for 2 min. Samples were analysed by denaturing gel electrophoresis and autoradiography as described above.

**Phosphorimages**

The gel images presented in this paper are high quality reproductions of phosphorimages, with a linear relationship between signal and image intensity and have not been edited.

**RESULTS**

**Cleavage of tethered and unconstrained four-way junctions by YDC2**

We have examined the rates of cleavage of a variety of four-way junction sequences by CCE1 using a single turnover kinetic assay, (10). CCE1 cleaves almost exclusively after 5′-CT sequences, with any other dinucleotide sequence being cleaved at least 600-fold more slowly. In a variety of branch-migrating junctions, CCE1 cuts at all possible 5′-CT sites with approximately equal efficiency (M.J.Schofield, D.M.J.Lilley and M.F.White, unpublished observations). In contrast, YDC2 cleaves the branch-migrating junction J1m4 at a subset of 5′-CT and 5′-TT sequences, leaving other such sequences uncut (12). There may therefore be a factor other than local DNA sequence that has an effect on YDC2 cleavage efficiency. One possibility is that YDC2 has a strong preference for cleaving either continuous or exchanging strands in the stacked X structure of the junction that exists in the presence of magnesium ions. Such a selectivity appears to be the rule for junction-resolving enzymes: CCE1 (8), RuvC (20) and RusA (21) prefer to cut continuous strands, whilst T4 endonuclease VII prefers exchanging strands (16). YDC2 introduces a single nick in junction 1 by cleavage of the r strand (which is a continuous strand in the folded form of this junction) at the point of strand exchange (12).

In order to study the effect of junction stacking on cleavage by YDC2, we synthesised two forms of junction 1 tethered to adopt a specific stacking conformer. In J1T1, the b and r strands are joined by an eight thymidine (T8) tether, forcing the junction to adopt the conformer in which strands b and r are continuous, while h and x are exchanging. In J1T2, the tether links the h and x strands, thus limiting the junction to the conformer where the h and x strands are continuous and b and r are exchanging (Fig. 1). Cleavage rates of the r strand of junction 1 by YDC2 were measured under single turnover conditions for both of the tethered species, as well as the untethered junction. In each case YDC2 introduced a single cleavage in the r strand of junction 1 at the point of strand exchange, with rates of 2.9 × 10^−3/s for the unconstrained junction, 1.0 × 10^−3/s for J1T1 and 0.34 × 10^−3/s for J1T2. Thus YDC2 cuts the same sequence 3-fold faster when the cleavage site is present on a strand constrained to be continuous than when the strand is forced to be exchanging. However, the unconstrained junction is cut 3-fold faster than the preferred tethered junction J1T1, suggesting that the tether may interfere with efficient junction cleavage, perhaps by reducing conformational freedom.

**Creation of an inactive mutant of YDC2**

In order to characterise the nature of the interaction of YDC2 with the four-way junction, it was desirable to create a mutant enzyme that could bind, but not cleave, the DNA substrate. Such mutants have been produced for RuvC (22), T4 endonuclease VII (17,23) and T7 endonuclease I (24), in each case by mutation of aspartate or glutamate residues. These acidic side chains may act as ligands for catalytic metal ions required by all the junction-resolving enzymes, in common with most other endonucleases. Alignment of the sequences of YDC2 and CCE1 shows that out of 71
identical amino acids, five are glutamate and five aspartate. Using site-directed mutagenesis, we converted conserved Asp26 in YDC2 to an asparagine, thus converting the carboxylate moiety to an amide. The D226N mutant protein was expressed and purified using the same procedure as that used for the wild-type enzyme and its ability to bind to and cleave four-way junctions was tested. Gel electrophoretic retardation experiments showed that the D226N mutant retained the ability to bind to a four-way junction (Fig. 2), with a calculated dissociation constant of 1.4 nM for complex I, comparable with the value for the wild-type enzyme of 6.3 nM. The slightly tighter binding of the mutant enzyme may reflect the increased basicity of this protein compared with the wild-type. In contrast, under single turnover conditions, wild-type and D226N YDC2 cleaved the r strand of complex I, comparable with the value for the wild-type enzyme of 6.3 nM. The slightly tighter binding of the mutant enzyme may reflect the increased basicity of this protein compared with the wild-type. In contrast, under single turnover conditions, wild-type and D226N YDC2 cleaved the r strand of junction 1 with rates of $2.9 \times 10^{-9}/s$ and $6.0 \times 10^{-9}/s$, respectively. The D226N mutation therefore results in an enzyme with almost unaltered binding properties with respect to four-way DNA junctions, but with an extremely low catalytic rate: reduced 480-fold compared with wild-type. These properties are suitable for the characterisation of enzyme–junction interactions both in the presence and absence of magnesium ions.

**Global conformation of the four-way junction when bound by YDC2 D226N**

The stacked X structure of the four-way junction in the presence of magnesium ions and the extended, square configuration of junctions in the presence of EDTA were first demonstrated by comparative gel electrophoresis and subsequently confirmed by a number of other techniques, including fluorescence resonance energy transfer (reviewed in 25-27). The electrophoretic technique has been extended by carrying out gel electrophoretic retardation analysis with junction-binding proteins to examine the global conformation of the bound junction. All the proteins studied to date alter the stacked X structure of the junction by unfolding it to a greater or lesser extent (reviewed in 1). Perhaps the most extreme distortion is observed with CCE1 (9) and RuvA (28), both of which impose an extended, four-fold symmetric structure on the junction characteristic of the free junction structure in the presence of EDTA. To examine the effect of YDC2 D226N binding on the junction structure, we carried out comparative gel electrophoretic retardation analysis using the six two-long, two-short arm species derived from junction 3. DNA binding and gel electrophoreses were carried out in the presence of either 1 mM EDTA or 200 µM magnesium ions and the results are shown in Figure 3. In EDTA, the free junction species display the expected slow-fast-slow-slow-fast migration pattern characteristic of the four-fold symmetric extended form of the junction. The retarded species display a similar pattern, suggesting that YDC2 preserves the open structure of the junction on binding in EDTA. When magnesium ions are present during binding and electrophoresis, the free junction exhibits the slow-intermediate-fast-fast-intermediate-slow pattern indicative of the stacked X structure with B upon X coaxial stacking. In contrast, the bound species display the same pattern observed in the presence of EDTA. YDC2 D226N binding therefore imposes an open, extended conformation on the four-way junction, as observed previously for CCE1 and RuvA.

**Footprinting of YDC2 D226N–junction complexes**

YDC2, in common with CCE1, binds to four-way junctions to form two distinct complexes, complex I and complex II, which probably correspond to binding of one and two dimers of protein bound per junction, respectively (8,12). We used both DNase I and DMS to probe the extent of protection afforded to the four-way junction present in complexes I and II. In these experiments, 80 nM junction 1, radioactively $^{5'}$-32P-labelled on each arm individually, was incubated without protein and with

![Figure 2](https://academic.oup.com/nar/article-abstract/26/24/5609/1460042/Interaction-of-the-resolving-enzyme-YDC2-with-the)
200 nM YDC2 D226N in the presence or absence of duplex competitor DNA. This was carried out in binding buffer supplemented with 5 mM MgCl2 and 2.5 mM CaCl2, in a total volume of 22 µl. After incubation at 20°C for 5 min, 2 µl were removed, loaded on a 5% non-denaturing polyacrylamide gel and subjected to electrophoresis in order to examine the proportion of the junction present in complex I, complex II and in free solution. The remaining 20 µl was split into two equal aliquots and incubated with either DNase I or DMS prior to denaturing gel electrophoresis as described in Materials and Methods. Gel electrophoretic retardation analysis indicated that junction incubated with 200 nM YDC2 D226N in the absence of competitor DNA is almost exclusively in complex II, whilst in the presence of duplex competitor complex I and free junction exist in approximately equal amounts, with a very small proportion of complex II present (Fig. 4).

Using DNase I as a probe (Fig. 5), regions of protection are observed on all four strands of the unbound four-way junction. This observation is in agreement with previous work (29) which demonstrated that the central region of the junction is refractory to DNase I cleavage and is consistent with the structure of the folded, stacked X-structure of the junction that exists in the presence of magnesium ions. The area of protection is more extensive for the exchanging strands (strands h and x for junction 1) than for the continuous strands (b and r). When 50% of the junction is present in complex I with YDC2, the area of protection is extended by a modest amount in the continuous strands b and r, but is unchanged for the exchanging strand x; strand h displays some further protection 3′ of the junction centre, whilst on the 5′-side there is increased reactivity immediately adjacent to the protected area of the free junction and partial protection of 5 nt more distant from the junction centre. For each strand, 2–3 nt are protected 5′ of the junction centre and 4–5 nt 3′ of the junction centre. These observations may reflect the rearrangement of the structure of the junction on YDC2 D226N binding, from the stacked X-structure to an open, square structure, as suggested by comparative gel electrophoresis. When essentially all of the junction is present in complex II a much more extensive area of protection from DNase I is observed, extending to 9–11 nt on the 5′-side and 12–13 nt on the 3′-side of the point of strand exchange. The areas protected from DNase I digestion have been projected onto the open structure of junction 1 to show the footprints due to complexes I and II (Fig. 6).
4.

Figure 4. Gel electrophoretic retardation analysis of junction–protein complexes used in footprinting experiments. An aliquot of each sample from the DNase I/DMS footprinting experiments was removed prior to addition of the probe, subjected to polyacrylamide gel electrophoresis in non-denaturing conditions in order to characterise the protein–DNA complexes present and quantified by phosphorimaging. The migration positions of free junction 1 (J), complex I (cI) and complex II (cII) are indicated by arrows. Lane 1, b strand labelled, no YDC2; lane 2, b strand labelled, 200 nM YDC2 and competitor DNA; lane 3, b strand labelled, 200 nM YDC2, no competitor DNA; lane 4, junction 1 h strand labelled, no YDC2; lane 5, h strand labelled, 200 nM YDC2 and competitor DNA; lane 6, h strand labelled, 200 nM YDC2, no competitor DNA; lane 7, r strand labelled, no YDC2; lane 8, r strand labelled, 200 nM YDC2 and competitor DNA; lane 9, r strand labelled, 200 nM YDC2, no competitor DNA; lane 10, x strand labelled, no YDC2; lane 11, x strand labelled, 200 nM YDC2 and competitor DNA; lane 12, x strand labelled, 200 nM YDC2, no competitor DNA.

The large footprint observed for the free four-way junction in DNase I probing experiments is an impediment to the definitive interpretation of the footprint of complex I. We therefore repeated the footprinting experiment on the same complexes, using the much smaller probe DMS. DMS specifically modifies guanine bases, rendering the sugar–phosphate backbone susceptible to base degradation by piperidine, and free four-way junction has been shown to provide no protection against DMS modification (29). We found that DMS footprinting for complex I and complex II, suggesting that the major groove of the junction remains accessible to this small hydrophobic probe when bound by YDC2 D226N (data not shown).

DISCUSSION

In order to investigate the nature of the interaction of YDC2 with the four-way junction, we required a mutant enzyme that retained the ability to bind junction as normal, but had lost all or most of its catalytic activity. Such mutants have been identified for RuvC by altering one of the aspartate residues conserved (though not binding) are presumed to constitute the metal binding site (31). By altering one of the aspartate residues conserved acidic residues that are essential for enzyme activity to disruption of binding of the catalytic metal(s). For RuvC, four conserved acidic residues which have been identified for RuvC (22), T4 endonuclease VII (17,30), T7 endonuclease I (24) and RusA (21). In most cases an acidic side chain has been replaced by a neutral or basic one and their lack of activity is probably due to disruption of binding of the catalytic metal(s). For RuvC, four conserved acidic residues which are essential for enzyme activity (though not binding) are presumed to constitute the metal binding site (31). By altering one of the aspartate residues conserved between YDC2 and CCE1 to an asparagine (D226N), we have created a mutant enzyme that retains the binding specificity of wild-type YDC2, but is catalytically compromised.

The two major structural factors that can influence the efficiency of cleavage of a four-way junction by a junction-resolving enzyme are the local nucleotide sequence at the cleavage site and the strand type, i.e. whether a strand is continuous or exchanging in the stacked X structure of the junction. Resolving enzymes are affected to differing extents by these two factors; the two extremes represented by CCE1, which displays an exquisite sequence specificity for cleavage, but little distinction for strand type, and T4 endonuclease VII, which has little sequence discrimination but strongly favours cleavage of exchanging over continuous strands. The use of tethered junctions allows the effects of sequence to be disregarded by forcing strands of identical sequence to adopt either continuous or exchanging character. Using tethered forms of junction 1 in a single turnover kinetic assay, we have shown that YDC2 cleaves a target sequence in a continuous strand ~3-fold faster than the same sequence present in an exchanging strand. This preference for continuous over exchanging strands appears to be a general feature of the cellular junction endonucleases. However, interpretation of these findings must take into account the degree to which the protein alters the conformation of the four-way junction on binding, and comparative gel electrophoretic experiments.
suggest that large-scale manipulation of the global conformation of the junction is the rule. In the case of CCE1 for example, the distinction between exchanging and continuous strands is unclear, as the enzyme appears to open the junction into a square, four-fold symmetric structure where all four strands are equivalent. A second problem encountered is the observation that the effect of constraining a four-way junction with a tether may have a larger deleterious effect on the cleavage rate than any effect resulting from the position of the tether. For YDC2, the unconstrained junction is cut 3-fold faster than the preferred tethered species, while for RuvA, the effect is larger, with untethered junction being cleaved 10-fold more quickly (21).

Using the inactive YDC2 mutant, we have shown by comparative gel electrophoresis that the enzyme manipulates the structure of the four-way DNA junction in complex I such that the arms exist in the extended, square configuration. This effect is independent of the presence or absence of metal ions, i.e. the stacked X structure adopted by the junction in magnesium is unfolded by the protein. Thus the arms of a junction bound by YDC2 appear to have approximately four-fold symmetry, as is seen for four-way junctions complexed with CCE1 (9) and RuvA (28). The lack of distinction between continuous and exchanging strands in the complex explains the rather low degree of discrimination observed for cleavage of the two tethered junction species. Junctions bound in complex II with YDC2 still adopt the four-fold symmetry seen for complex I (data not shown), suggesting that binding of a second dimer of YDC2 does not further alter the global conformation of the junction. Comparative gel electrophoretic retardation analysis of junction 3 bound by wild-type YDC2 in the presence of EDTA shows the same four-fold symmetry observed with the D226N mutant (data not shown).

DNase I probing of junction–protein complexes is complicated by the extensive protection observed for the free junction, which is consistent with the stacked X structure of the junction (29). When junction 1 is bound by YDC2 D226N in complex I, we see a slight increase in the area protected at the centre of the junction. Unfortunately, it is not possible to distinguish protection due to protein binding from intrinsic protection due to the junction structure. In addition, the structure of the junction itself has probably changed from the stacked X structure of the free junction in magnesium to the square, extended structure seen in complex I by comparative gel electrophoresis. The footprint of the open form of the free four-way junction is probably much smaller than that of the stacked X structure, but cannot be quantified as the levels of magnesium ions required for DNase I activity result in junction stacking. The YDC2 D226N complex I footprint extends fairly equally on all four strands, consistent with the four-fold symmetric structure of the complex predicted by comparative gel electrophoresis, and appears to be surprisingly small compared with that observed for the significantly smaller protein RuvC; YDC2 in complex I affords protection for 7–8 nt in each strand, compared with up to 14 nt protected by RuvC (32).

For complex II, a much more significant footprint is observed on all four arms, extending to 13 nt from the junction centre, with a slight bias for greater protection on the 3′-side of the point of strand exchange. Formation of complex II probably involves a second dimer of YDC2 binding to a YDC2–junction complex and the DNase I footprinting clearly indicates that this interaction occurs at the junction centre. However, we cannot rule out the possibility that complex II formation requires binding of two further dimers of YDC2, each with equal affinity. Complex II formation is observed only at relatively high levels of YDC2: the $K_d$ can be estimated from Figure 2 (by quantifying complex I as ‘unbound’ and complex II as ‘bound’) as $\sim 50$ nM for wild-type YDC2 and 15 nM for D226N, values that are $\sim 10$-fold higher than those for complex I in each case.

The observation that both complexes I and II are formed at lower protein concentrations of the mutant D226N than the wild-type YDC2 protein suggests that the mutation has affected both binding constants. This may indicate that the second dimer of YDC2 binds in a similar fashion to the first. One possible model is that the second YDC2 dimer binds the junction on the opposite face to the first dimer to form a protein–DNA ‘sandwich’, comparable with the binding of one and two tetramers of RuvA to four-way DNA junctions (33). The second dimer could be symmetrical with the first or rotated by 90°. Both the mode of binding and the functional significance of complex II for YDC2 and CCE1 remain unclear and will be a target for future study.

**ACKNOWLEDGEMENTS**

We thank our colleagues Richard Pöhler and Mark Schofield for helpful discussions and David Norman for assistance with the molecular graphics. We thank the Cancer Research Campaign for financial support. M.F.W. is a Royal Society University Research Fellow.
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