The RuvABC resolvasome

Quantitative analysis of RuvA and RuvC assembly on junction DNA

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The RuvABC resolvasome of Escherichia coli catalyses the resolution of Holliday junctions that arise during genetic recombination and DNA repair. This process involves two key steps: branch migration, catalysed by the RuvB protein that is targeted to the Holliday junction by the structure specific RuvA protein, and resolution, which is catalysed by the RuvC endonuclease. We have quantified the interaction of the RuvA protein with synthetic Holliday junctions and have shown that the binding of the protein is highly structure-specific, and leads to the formation of a complex containing two tetramers of RuvA per Holliday junction. Our data are consistent with two tetramers of RuvA binding to the DNA recombination intermediate in a co-operative manner. Once formed this complex prevents the binding of RuvC to the Holliday junction. However, the formation of a RuvAC complex can be observed following sequential addition of the RuvC and RuvA proteins. Moreover, by examining the DNA recognition properties of a mutant RuvA protein (E55R, D56K) we show that the charge on the central pin is critical for directing the structure-specific binding by RuvA.

Keywords: RuvABC resolvasome; Holliday junction; surface plasmon resonance.

Genetic recombination is a fundamental cellular process that serves both to protect and expand the coding potential of all organisms. Through recombination events the genes within and between chromosomes may be rearranged, segregation at cell division may be modulated and DNA repair facilitated. All organisms have evolved many different ways to repair the damage to DNA and failure of these mechanisms leads to chromosomal disorders that can result in mutation, malignant transformation or death. Studies in Escherichia coli and other bacteria have provided considerable insights into the molecular pathways of DNA repair. Prominent amongst these pathways is recombination. Genetic recombination occurs via breakage and reunion of DNA chains and generally conserves sequence [1]. DNA recombination has also been shown to play a role in re-establishing stalled replication forks [2].

During the late stages of recombination in E. coli, Holliday junction intermediates made by RecA-mediated homologous pairing and strand exchange are processed into mature recombinants by the RuvA, RuvB and RuvC proteins [3,4]. RuvA has been shown to be a highly structure specific DNA binding protein whose function is to target RuvB to the Holliday junction [5,6]. RuvB assembles around the DNA as hexameric rings, which are thought to move along the DNA using energy derived from the hydrolysis of ATP. RuvC is an endonuclease that cleaves the junction via a process involving a dual incision mechanism at base specific contacts in which nicks are introduced into two strands having the same polarity, around a defined target sequence with the consensus 5’ (A/T)TT(G/C) [7,8]. Genetic studies have indicated that RuvAB mediated branch migration is intrinsically linked to RuvC mediated Holliday junction resolution [9–11]. Taking into account the sequence specificity of RuvC, different models have been proposed to reconcile the genetic and biochemical data on the resolution of Holliday junctions. In one scenario RuvAB promotes branch migration until suitable sequences are encountered, at which point the complex dissociates to allow RuvC binding. Alternatively RuvC may act as part of a RuvABC complex in which migration and resolution are coupled.

RuvA is a 22-kDa protein which exists as a tetramer in solution [12] and binds to both ssDNA and dsDNA [13], but binds with the greatest affinity to Holliday junctions. Binding is structure specific and completely independent of DNA sequence. When the RuvA protein is bound to the Holliday junction in solution and is subjected to non-denaturing gel electrophoresis, two different complexes are observed. Their electrophoretic mobilities are consistent with the formation of protein–DNA complexes containing one and two tetramers of RuvA [14,15]. Under conditions in which RuvB exhibits a low affinity for DNA, the presence of RuvA results in the formation of a RuvAB–Holliday junction complex, indicating that RuvA targets RuvB to the junction [16]. The determination of the molecular structure of RuvA [17] has shown that the four monomers of RuvA are related by fourfold symmetry (see Fig. 1), similar to a four petal flower with concave and convex surfaces normal to the fourfold axis.

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The two faces of the RuvA tetramer are different; one face is convex and is mainly negatively charged whereas the opposite face is concave and positively charged. The negatively charged central pin containing the conserved Glu55 and Asp56 residues is shown in Fig. 2.

Three crystal structures have been determined for a RuvA–synthetic Holliday junction complex [18–20]. The crystal structures of the *E. coli* RuvA–DNA complex consist of a single RuvA tetramer with a nearly square planar DNA molecule bound to its concave surface [18–20]. This is in agreement with a predicted model derived from the RuvA crystal structure [17]. The DNA duplex arms in the junction are located in the grooves on the surface of the RuvA as predicted, and a central pin region of the concave surface, which includes the conserved Glu55 and Asp56 residues, perfectly matches a hole of approximately 20 Å diameter at the centre of the junction. The crystal structure of the *Mycobacterium leprae* RuvA–junction complex contains a RuvA tetramer on both faces of the junction, such that the DNA is sandwiched between two tetramers [19]. In the latter structure RuvA forms an octameric shell through which the DNA must pass during branch migration. Recently it has been shown that the acidic pin of *E. coli* RuvA modulates Holliday junction resolution by preventing binding to duplex DNA and constraining the role of branch migration in the RuvAB complex [21].

**MATERIALS AND METHODS**

Synthesis and purification of oligodeoxynucleotides used in the binding studies

Oligonucleotide synthesis was performed on an Applied Biosystems 394 DNA synthesiser using cyanoethyl phosphoramidite chemistry. The biotin phosphoramidite was obtained from Glen Research. Oligodeoxynucleotides were provided in solution after deprotection in 30% ammonia. The oligonucleotides were purified using denaturing PAGE and subsequently evaporated to dryness and desalted using a Pharmacia NAP 10 column according to the manufacturer’s instructions. Synthetic Holliday junctions, HJ24 and HJ30, each comprised four 24-mer and four 50-mer oligonucleotides. DNA annealing and purification were essentially as described previously [33]. HJ50 contained HJ5, 6, 7 and 8. HJ 25 contained HJ1, 2, 3 and 4. Three way junctions were prepared using HJ5, 6 and 7 and duplex DNA annealed using D1 and D2, ASP1 and ASP2. All oligonucleotides used in the binding studies are shown in Table 3.
Synthesis and purification of proteins used in the binding studies

RuvA was purified as described [31]. RuvC was overexpressed to about 10% of total cell protein in BL21 plysS (Cm') harbouring pGST75 (RuvC + cloned in pT7-7) (Ap'). RuvC was then purified to 90% using two stages of chromatography. The first step involved pseudo-affinity chromatography using heparin-Sepharose. The second step was gel filtration on a Hi-Load Superdex-200 column (Pharmacia) in buffer A (0.5M NaCl, 50mM Tris/HCl, pH 7.5). Protein was then concentrated by precipitation with ammonium sulphate (0.55g binding, ammonium sulphate (0.55 g)) and dialysed against buffer A to remove the ammonium sulphate. The RuvA and RuvC proteins were diluted in the appropriate running buffer to give final concentrations between 2000 and 1 nm.

Binding assays using surface plasmon resonance

The SPR analysis was performed using a BIAcore 2000™ (BIAcore, Uppsala, Sweden). The oligonucleotides were diluted in HBS [0.01 m Heps (pH 7.4), 0.15 m NaCl, 3 mM EDTA, 0.05% (v/v) surfactant P20] buffer to final concentration of 1 ng/mL and passed over a streptavidin sensor chip (SA) at a flow rate of 10 µL min⁻¹ until approximately 100–200 response units of the oligonucleotide was bound to the sensor chip surface. The protein was diluted in HBS. A range of protein concentrations (1–2000 nm) were injected over the DNA attached to the sensor chip at a flow rate of 20 µL minute⁻¹ for 3 min and were allowed to dissociate for 5 min. The bound protein was then removed by injecting 10 µL of 1 mM NaCl. This regeneration procedure did not alter to any measurable extent the ability of the Holliday junction to bind to RuvA. Analysis of the data was performed using the BIAevaluation software supplied with the BIAcore. To remove the effects of the bulk refractive index change at the beginning and end of the injections (which occur as a result of a difference in the composition of the running buffer and the injected protein), a control sensorgram obtained over the streptavidin surface was subtracted from each protein injection.

Stoichiometry analysis

The biotinylated Holliday junctions were injected over the surface of the streptavidin coated sensor chip and the changes in response recorded. RuvA (1 µm) diluted in HBS buffer was then injected over the Holliday junction attached to the sensor chip surface. The change in response of RuvA binding to the junction was recorded, the stoichiometry was calculated using Eqn (1):

\[
S = \frac{R_{\text{max}} \text{RuvA}}{R_{\text{max}, \text{RuvA}}}
\]

where \( R_{\text{max}} \text{RuvA} \) is the maximum response of RuvA binding, \( R_{\text{max}, \text{RuvA}} \) is the response of the binding of the biotinylated Holliday junction, \( M_r \text{RuvA} \) is the molecular mass of RuvA and \( M_r, \text{junc} \) is the molecular mass of the Holliday junction. The following values were used: \( M_r \text{RuvA} \) 88 000, \( M_r, \text{junc} \) (HJ50) 63 200, \( M_r, \text{junc} \) (HJ24) 32 000, \( M_r \text{RuvC} \) 38 000.

Kinetic analysis

The dissociation rate constants were calculated using linear regression analysis assuming a zero order dissociation using Eqn 2:

\[
dR/dt = -k_d R_0 e^{-k_d (t - t_0)}
\]

where \( dR/dt \) is the rate of change of the SPR signal, \( R \) and \( R_0 \) is the response at time \( t \) and \( t_0 \). \( k_d \) is the dissociation rate constant.

Nonlinear regression analysis was used to determine the equilibrium dissociation constant from the sensorgrams and allows the calculation of both association and dissociation rate constants from a single sensorgram using the following equation. Using a 1:1 homogenous single site binding model:

\[
R = \frac{(k_a C R_{\text{max}}) / (k_a C + k_d)}{(1 - e^{-(k_a C + k_d) t})}
\]

where \( C \) is the concentration of analyte, \( R_{\text{max}} \) is the maximum analyte binding capacity in RU and \( R \) is the SPR signal in RU at time \( t \), \( k_a \) the association rate constant and \( k_d \) the dissociation rate constant.

Using a heterogenous model (where the component interactions are independent of each other, the response curve is the sum of the separate binding events), the following equation can be used:

\[
R = \sum_{n=1}^{n} \frac{(k_{a,n} C_n R_{\text{max},n}) / (k_{a,n} C_n + k_{d,n})}{(1 - e^{-(k_{a,n} C_n + k_{d,n}) t})}
\]

This equation assumes the analyte species bind independently to separate ligand sites (BIA evaluation software).

Equilibrium binding analysis

BIACore equilibrium binding experiments were performed as described by Myszka et al. [28] with minor modifications. The instrument was equilibrated at 25 °C with HBS buffer (0.01 m Heps (pH 7.4), 0.15 m NaCl, 3 mM EDTA, 0.05% (v/v) surfactant P20) at a flow rate of 100 µL min⁻¹. Baseline data were collected for 45 min at the start of the experiment, before the incorporation of the protein into the running buffer. After equilibrium binding profiles had been generated, the responses from the four flow cells were baseline corrected during the initial washing phase. The response from the reference flow cell was subtracted from the other three flow cells to correct for refractive index changes, nonspecific binding and instrument drift.

RESULTS

Stoichiometry and kinetics of the RuvA–Holliday junction interaction

The stoichiometry and kinetics of the RuvA–Holliday junction interaction was analysed using surface plasmon resonance (SPR) on a BIACore 2000. The biotinylated synthetic Holliday junctions (HJ50/HJ24, see Materials and methods) were immobilized on a streptavidin coated sensor chip (SA) and the protein injected over the surface of the immobilized Holliday junction. The sensorgram can be used to derive kinetic and equilibrium constants and also allows
the calculation of the stoichiometry of the interaction. This method has been used previously to study protein–DNA interactions [22,23].

To calculate the stoichiometry of the interaction of RuvA with the Holliday junction, the biotinylated Holliday junctions were injected over the surface of the streptavidin coated sensor chip and the change in response recorded. 1 RU corresponds to 1 pg mm$^{-2}$ protein [24]. For DNA, a value of 1 RU corresponding to 0.73 pg mm$^{-2}$ was used, as determined by Speck et al. [23] The change in response for the binding of the RuvA to the junction was recorded and using Eqn (1) (see Materials and methods), the stoichiometry at a given RuvA concentration was calculated and the results are summarized in Table 1. From these results it can be concluded that two RuvA tetramers bind to the Holliday junction, this occurs in both the 24-mer strand and 50-mer strand junctions. This experiment was repeated using different amounts of junction bound to the sensor chip surface. The results were consistent with two RuvA tetramers bound per Holliday junction. These results support the evidence from both gel shift assays [15], electron microscopy studies [25] and neutron scattering data [26], which demonstrate that two RuvA tetramers can bind to synthetic Holliday junctions. Crystallographic studies of the RuvA–synthetic Holliday junction show either one [18,20] or two [19] RuvA tetramers bound to the Holliday junction. The results from the SPR experiments described here support the latter model in solution. These experiments also show that complete removal of RuvA from DNA can be affected by addition of 1 M salt, highlighting the role of electrostatic interactions in the binding of RuvA to the junction (data not shown).

The binding of RuvA was studied using the BIAcore to obtain kinetic parameters for the interaction of RuvA with the synthetic Holliday junctions attached to a streptavidin sensor chip. The interaction of RuvA with both the 24-mer and the 50-mer Holliday junctions was then investigated. From the sensograms obtained (see Fig. 3) the data were fitted to a mathematical model which describes the interaction of two analyte molecules (two RuvA tetramers) binding to a single ligand (Holliday junction) at different sites. The model used was a heterogeneous parallel model (BIAevaluation software) which describes the interaction:

$$A + B \rightleftharpoons AB + B \rightleftharpoons AB_2$$

This mathematical model was used to obtain association and dissociation rate constants for the above reaction (see Eqn 4). However, the results clearly show that this model produces an unsatisfactory fit to the data. The residuals shown in Fig. 3B indicate how well the mathematical model fits the data. High residual values, indicating a poor fit to the data, are obtained for the association phase. However, by contrast, low residuals were obtained for the dissociation phase indicating a very acceptable fit to the data. Several other mathematical models were used to fit the data, including a 1 : 1 Langmuir binding model. All yielded poor correlation with the association phase of the RuvA–Holliday junction interaction. The mathematical models employed, appeared to be unable to support the very fast association kinetics observed experimentally. Such a kinetic mechanism obtained for the interaction of two RuvA tetramers with the Holliday junction could be explained by a co-operative effect during the association phase. Two analyte molecules interacting with a single ligand at different sites may introduce a co-operative function not described by the mathematical models. This co-operativity may lead to the fast association kinetics observed in the binding of RuvA to the Holliday junction. Whilst there are other possible explanations for the binding data, the experiments carried out below seem to be consistent with a co-operative component to the RuvA–DNA interaction.

Table 1. Stoichiometry of RuvA and RuvC bound to Holliday junctions as determined by SPR analysis using Eqn (1) (see Materials and methods).

<table>
<thead>
<tr>
<th>Holliday Junction</th>
<th>RuvA</th>
<th>RuvC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$1 , \mu$m</td>
<td>$2 , \mu$m</td>
</tr>
<tr>
<td>HJ 24</td>
<td>2.3</td>
<td>2.5</td>
</tr>
<tr>
<td>HJ 50</td>
<td>2.6</td>
<td>2.7</td>
</tr>
</tbody>
</table>

$^a$ Tetramers bound per Holliday junction. $^b$ Dimers bound per Holliday junction.

Equilibrium binding profile of the RuvA–Holliday junction interaction

To further evaluate the interaction of RuvA with the Holliday junction, equilibrium binding analysis was
RuvA–Holliday junction complex [19]. Whilst complex tetramer–tetramer interactions observed in the junction interaction; an idea supported by the RuvA co-operative mode of binding in the RuvA–Holliday occupied. These results provide further evidence to support RuvA concentration until 100% of the binding sites are occupied. However, the RuvA–Holliday magnitude of protein concentration until 100% of the binding sites are occupied. The results demonstrate that the E. coli RuvA has the ability to target Holliday junctions over duplex DNA with approximately 1000-fold greater efficiency. Also, E. coli RuvA has the ability to bind to the duplex arms of the Holliday junctions after specifically binding to the Holliday junction at the crossover point.

From the analysis of the equilibrium binding profile it can also be shown that at low concentrations of RuvA (0.0026 and 0.026 nm) only very small amounts of protein bind to the Holliday junction even after 2.5 h of incubation. Only after the concentration of RuvA is increased to 0.22 nm, is significant binding to the Holliday junction observed. However, at this concentration, equilibrium is soon reached with only small amounts of additional binding to the junction at concentrations above this value. Therefore over only a 10-fold increase in protein concentration, nearly 100% of the binding sites are occupied by RuvA. The concentration at which approximately 50% of the DNA binding sites are bound by RuvA is 0.2 nm. Previous analysis of the high affinity Ets1 protein–DNA interaction [27] showed that binding occurs over three to four orders of magnitude of protein concentration until 100% of the binding sites are occupied. However, the RuvA–Holliday junction binding occurs over two orders of magnitude of RuvA concentration until 100% of the binding sites are occupied. These results provide further evidence to support a co-operative mode of binding in the RuvA–Holliday junction interaction; an idea supported by the RuvA tetramer–tetramer interactions observed in the M. leprae RuvA–Holliday junction complex [19]. Whilst complex modes of interaction could account for the data, a co-operative mode of binding seems to be the most plausible explanation.

**SPR analysis of the RuvAC–Holliday junction ternary complex**

To further examine the hypothesis that a tetramer of RuvA can bind to each face of a Holliday junction, the effect of the addition of RuvC to the RuvA–Holliday junction complex was investigated. Analysis of the interaction of RuvA and RuvC with the Holliday junction was performed by sequential addition of the RuvA and RuvC proteins to the Holliday junction. The resulting sensorgram is shown in Fig. 5A and shows that after the addition of RuvA (1 μM) to the Holliday junction, RuvC (1 μM) does not bind to the junction. This would be expected, if RuvA occupies the site for this interaction.

A series of concentrations of RuvC (10–2000 nM) were injected over the immobilized Holliday junctions attached to the sensor chip surface and the stoichiometry calculated as previously. A summary of the values obtained are shown in Table 1. From the results it can be seen that at high concentrations (2 μM), RuvC forms a complex in which more than one dimer interacts with the junction, suggesting that RuvC possibly binds to both faces of the junction in a similar manner to RuvA. It had been thought previously that only one RuvC dimer binds to the junction, which is the active form of the complex [7]. However, at lower concentrations of RuvC, the complex only contains on average one RuvC dimer per DNA junction as expected. These experiments also show that complete removal of RuvC from DNA can be effected by addition of 1 M salt (data not shown), highlighting the role of electrostatic interactions in the binding of RuvC to the junction.

A further experiment was performed by first binding RuvC (2 μM) to the junction followed by the addition of RuvA (1 μM). The resulting sensorgram is shown in Fig. 5B. No significant RuvA binding was observed, which indicates that two RuvC dimers may bind to the junction in a similar manner to the two RuvA tetramers, and that the two bound RuvC dimers prevent the binding of RuvA under the conditions used in the experiment. A small amount of RuvA binding is observed, possibly due to RuvC dissociation from the Holliday junction before the injection of RuvA. A final experiment was carried out in which RuvC was added to the Holliday junction under conditions where the mean stoichiometric calculation shows one RuvC dimer was bound per junction. After adding RuvC (0.75 μM) to the junction, RuvA (1 μM) was then added and the resulting sensorgram is shown in Fig. 5C. These results show that under these conditions RuvA can bind to a RuvC–Holliday junction complex, allowing the formation of a RuvAC complex on the Holliday junction. The effect of addition of antibodies raised against RuvA (anti-RuvA) to the proposed RuvAC complex is shown in Fig. 5D. The sensorgram shows the binding of RuvA after the addition of RuvC, followed by the binding of the anti-RuvA. A large response is seen due to the large molecular mass of the anti-RuvA complex. These results demonstrate that after the addition of RuvC, RuvA can bind to the complex as confirmed by the binding of the anti-RuvA. No binding of the anti-RuvA is seen on the RuvC complex (data not shown).
Comparison of DNA recognition by RuvA and a mutant RuvA (E55R D56K)

SPR analysis demonstrated that two tetramers of E. coli RuvA bind to Holliday junctions in a structure specific manner, with a significantly greater affinity than for duplex DNA. A further experiment was performed to compare the binding of RuvA and a mutant RuvA. The mutant E. coli RuvA has the negatively charged central pin residues Glu55 and Asp56 mutated to Arg55 and Lys56, which results in a positively charged central pin.

A biotinylated Holliday junction (HJ50), a three-way junction and a duplex DNA (D1/2, see Materials and methods) were immobilized on different flow cells on a streptavidin sensor chip. The binding of 2 μM E. coli RuvA with the different complexes is shown in Fig. 6A. The difference in stability of the duplex and three-strand-RuvA complex compared to the four-strand-RuvA–protein complex can clearly be seen in the sensorgram (note the gradient of the dissociation phase). The dissociation rate constants were calculated using Eqn (2) (see Materials and methods) and shows a three- to fourfold difference between the duplex/three-strand junctions, compared to the four-strand-RuvA complexes (see Table 2), indicating that the formation of the Holliday junction–protein complex is more stable than the duplex/three-way junction complex. Using small duplex DNA (< 25 bp) no significant binding of the E. coli RuvA was seen using SPR (data not shown). These experiments present further evidence that the E. coli RuvA is highly structure specific in its binding to Holliday junctions.

The effect of the charge on the central pin with respect to the specificity of the interaction was further investigated by SPR analysis of the mutant E. coli RuvA (RuvA E55R D56K). The protein was passed over the streptavidin sensor chip containing the duplex and the three/four-strand junctions. The resulting sensorgram is shown in Fig. 6B. The dissociation rate constants were calculated for the dissociation of the protein from the different complexes and are shown in Table 2. These results demonstrate that all the protein-DNA complexes have very slow dissociation rates, indicating the formation of very stable protein–DNA complexes. The calculated rate constants are lower than

Fig. 5. Binding of RuvA and RuvC to Holliday junctions. (A) SPR sensogram showing the binding of RuvA to the Holliday junction followed by the addition of RuvC. 1 shows the binding of RuvA (1 μM) to the Holliday junction to form a proposed complex containing two RuvA tetramers bound to the Holliday junction. 2 shows the subsequent addition of RuvC (1 μM) to the RuvA–Holliday junction complex, the sensogram indicates no binding of RuvC. (B) Sensogram showing the binding of RuvC to the Holliday junction followed by the addition of RuvA. 1 shows the binding of RuvC (2 μM) to the Holliday junction to form a proposed complex containing two RuvC dimers bound to the junction. 2 shows the subsequent addition of RuvA (1 μM) and indicates no significant binding to the RuvC–junction complex. (C) Sensogram showing the formation of a RuvAC-junction complex. 1 shows the binding of RuvC (0.75 μM) to the Holliday junction to form a proposed complex which contains one RuvC dimer bound to the junction. 2 shows the subsequent addition of RuvA (1 μM) to the RuvC–junction complex. The sensogram indicates after RuvC has bound to the junction RuvA can bind to the RuvC–junction complex to form a RuvAC complex. (D) Sensogram showing the binding of anti-RuvA to the RuvAC-junction complex. 1 shows the binding of RuvA to form the RuvAC complex. 2 shows the binding of the RuvA antibody to the RuvAC complex. The sensogram demonstrates that the antibody can bind to the complex, indicating further evidence of the formation of the RuvAC-complex.

Fig. 6. SPR sensogram showing the DNA binding specificity of RuvA. (A) Binding of wild-type RuvA (2 μM) and (B) binding of the mutant RuvA (E55R D56K) (1.2 μM) to Holliday junction, linear duplex and 3-strand junction substrates.
previous values obtained for the binding of the wild-type *E. coli* RuvA protein, and show similar values for the binding to the duplex, three-strand and four-strand junctions. Demonstrating that the mutant RuvA protein forms a complex with duplex DNA which is of equal stability as the Holliday junction–protein complex. The sensorgram in Fig. 6B also illustrates that increased amounts of the protein interact with the DNA, as shown by the larger response observed on the sensorgram compared to the wild-type *E. coli* RuvA. These results give stoichiometry values of five RuvA tetramers bound per DNA.

From the SPR analysis it shows that the effective charge of the central pin region dramatically influences the binding of duplex DNA to the protein: changing the charge on the central pin from negative to positive, increases the stability of the duplex DNA–protein complexes. These results are consistent with those obtained by Ingleston et al. [21] using gel retardation assays and indicate that the charge on the central pin of the RuvA has a substantial effect on the ability of the protein to bind to duplex DNA, and therefore to direct the structure specificity involved in binding a Holliday junction. The mutant *E. coli* RuvA forms a stable complex with duplex DNA, there is no additional stability of the Holliday junction–protein interaction over the duplex DNA–protein interaction. These data suggest that the protein may now be binding to the duplex arms of the junction, as opposed to only the crossover points of the junction, suggesting that the protein may no longer be binding in a structure specific manner to the junction, but in a nonspecific fashion to duplex DNA.

**Equilibrium binding profile of the mutant *E. coli* RuvA (E55R D56K) protein**

To further analyse the interaction of the mutant *E. coli* RuvA protein, equilibrium binding analysis was performed similar to that performed with wild-type *E. coli* RuvA. The equilibrium binding profiles were generated to obtain further information on the specificity of binding. Figure 7A shows the binding profile of the mutant *E. coli* RuvA protein to the DNA complexes. From this profile it can clearly be seen that the protein binds to both the Holliday junction and the duplex DNA at the same concentration (2.6 nM), indicating that the protein has a similar affinity for the duplex DNA and the Holliday junction. These results demonstrate that the mutant *E. coli* protein is binding to the duplex arms of the Holliday junction and is no longer binding in a structure specific manner to the Holliday junction.

![Equilibrium binding of the mutant RuvA (E55R D56K) protein to linear duplex and Holliday junction substrates.](image)

**Table 2.** Dissociation rate constants ($k_d$) for RuvA–DNA complexes as determined by SPR analysis.

<table>
<thead>
<tr>
<th>DNA</th>
<th>RuvA wild-type (1/s) ± SD</th>
<th>RuvA E55R,D56K (1/s) ± SD</th>
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</thead>
<tbody>
<tr>
<td>Duplex</td>
<td>$19 \times 10^{-4} \pm 2.2 \times 10^{-4}$</td>
<td>$8 \times 10^{-5} \pm 4.2 \times 10^{-6}$</td>
</tr>
<tr>
<td>3-strand junction</td>
<td>$17 \times 10^{-4} \pm 1.9 \times 10^{-4}$</td>
<td>$2.7 \times 10^{-5} \pm 6.2 \times 10^{-6}$</td>
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<tr>
<td>4-strand junction</td>
<td>$5.5 \times 10^{-5} \pm 4.2 \times 10^{-5}$</td>
<td>$4.7 \times 10^{-5} \pm 6.4 \times 10^{-6}$</td>
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**Table 3.** Oligodeoxynucleotides.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5'-3')</th>
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<tbody>
<tr>
<td>ASP1</td>
<td>Bio-AATGCTACAGTATCGTCCGGTCACGTACAACATCCAG</td>
</tr>
<tr>
<td>ASP2</td>
<td>CTGGATGTGTACGTGACGGGAGCTGACATACGGTAC</td>
</tr>
<tr>
<td>DU1</td>
<td>Bio-GTACGAGACCTCCCGGGTCAGTCTGCCTA</td>
</tr>
<tr>
<td>DU2</td>
<td>TAGGCAAGACTGACCGGAGCTGTCGCTAC</td>
</tr>
<tr>
<td>HJ5</td>
<td>Bio-AAAATGGGTCAGCTGGGCAAGATGTCTGACAGTCATGACGTT</td>
</tr>
<tr>
<td>HJ6</td>
<td>GTCCGGATCTCTAGACGTCCTCAGCAGTGGCACTGGCATGAGTGAC</td>
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<td>HJ7</td>
<td>TGCCGAATCTCACTGATGCGAGCTTCTATGACGTT</td>
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<td>HJ8</td>
<td>CAACGCTATAAGCGATTACATCTGCTACATGGAGCTGTCAGAGGATCAG</td>
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<td>HJ1</td>
<td>AGAAGCTCCATGTAGCAAGGCTAG</td>
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Fig. 7. Equilibrium binding of the mutant RuvA (E55R D56K) protein to linear duplex and Holliday junction substrates. The profiles shown were obtained by incorporating the mutant RuvA in the running buffer at concentrations of 0.064 nM (A), 0.64 nM (B), 6.4 nM (C) and 37 nM (D). The arrows indicate the time at which the concentration of the protein was altered.
DISCUSSION

Co-operative binding of RuvA tetramers to Holliday junctions

The stoichiometry analysis presented here (see Table 1), clearly shows that two RuvA tetramers bind to synthetic Holliday junctions, which is in agreement with the crystal structure obtained for the *M. leprae* RuvA–Holliday junction complex [19]. In this structure the two RuvA tetramers make direct protein–protein contacts at four equivalent points. The protein–protein contacts involve side chain interactions between the helix from residues 117–129 of an A chain in one tetramer, with the same helix in a B chain on the other tetramer. A total of six ion-pair interactions are formed at the helix–helix interface (see Fig. 8). The residues involved in protein–protein interactions are also conserved in the *E. coli* RuvA protein. These protein–protein interactions may be the source of the co-operativity proposed from the binding profiles observed for *E. coli* RuvA and synthetic Holliday junctions presented here. The two binding surfaces of the Holliday junction are expected to bind to the RuvA tetramers with different affinities: the binding surface of the Holliday junction in the crystal structure obtained by Hargreaves *et al.* [18] is predicted to be the optimal binding site. The second RuvA tetramer that binds to the opposite surface of the Holliday junction may bind with lower affinity. The results obtained from the equilibrium binding profile of the interaction of RuvA with the Holliday junction demonstrate that only a 10-fold increase in protein concentration is required for the formation of a complex with two RuvA tetramers bound to the Holliday junction. No intermediate is seen where equilibration is reached with one RuvA tetramer bound to the Holliday junction. This suggests co-operativity in the binding of the tetramers, which may involve protein-protein contacts between the two tetramers, leading to a possible stabilization of the weaker binding RuvA tetramer.

A model for the active RuvAB branch migration complex bound to the Holliday junction has been proposed [5,24]. The complex comprises a central RuvA oligomer with RuvB hexameric rings bound to the duplex arms on opposite sides of the Holliday junction. Roe *et al.* [19] propose that the RuvB ATPase is anchored to the complex to achieve maximum efficiency of branch migration, and this can only be achieved by the presence of two RuvA tetramers. Our data suggest that two RuvA tetramers bind to the Holliday junction in the absence of RuvB in a co-operative manner, with no observable intermediate containing one RuvA tetramer. These data provide further evidence to support the formation of a RuvAB complex containing two RuvA tetramers which subsequently undergoes branch migration.

Observation of a RuvAC–Holliday junction complex

The formation of the RuvAC–Holliday junction complex, in which one RuvA tetramer and one RuvC dimer are bound on opposite faces of the junction, is significant. Its formation may represent an important stage in the transition between RuvAB mediated branch migration and RuvC mediated cleavage. Alternatively this structure could be part of a larger RuvABC–junction complex. The assembly of a RuvABC complex has been supported through various experiments [15,28,29], RuvBC promoted branch migration has been observed [29] providing additional support for a RuvABC active complex. However, formation of a RuvABC complex by displacement of a RuvA tetramer from the octameric RuvAB complex is not supported by our results. Figure 5A demonstrates that there was no displacement of RuvA when RuvC was added to the octameric RuvA complex. However the formation of a RuvABC complex after the formation of a RuvBC complex containing a single RuvC dimer is supported by the binding of RuvA after the addition of RuvC to form a RuvAC complex (see Fig. 5C). Eggleston *et al.* [28] proposed that an equilibrium may exist between two types of complex: a RuvAB branch migration complex and a RuvABC branch migration/resolution complex.

The charge on the central pin modulates DNA recognition

The SPR profiles reveal that *E. coli* RuvA is a structure specific protein that binds with a much greater affinity to Holliday junctions compared to duplex DNA. The *E. coli* RuvA was demonstrated to target Holliday junctions 1000 times more efficiently than duplex DNA. The mutant *E. coli* RuvA (E55R D56K), which contains a positively charged pin region, binds to duplex DNA with high affinity. These results are consistent with those obtained using gel retardation assays, which demonstrated that the protein binds Holliday junctions with approximately the same affinity as it binds duplex DNA [21]. The SPR analysis shows that the mutant protein no longer binds to Holliday junctions in a structure specific manner but binds to the duplex arms of the junction (see Fig. 7). The data also show that the protein binds with a greater stoichiometry (five tetramers) compared to the binding of the wild-type *E. coli* RuvA.
(two tetramers) to Holliday junctions, indicating that more than one tetramer or octamer binds to the DNA molecule. This suggests that the protein does not simply bind at the ends of the duplex DNA arms but binds along the linear duplex DNA across the central pin. The formation of a positive charge on the pin region also leads to a further increase in affinity for duplex DNA. There remains the caveat that alteration of these amino acids may have affected the conformation of this pin. However, the mutant protein was purified using the same procedure designed for wild-type RuvA, indicating that the mutation caused little effect on the overall structure of the protein [21]. The overall structure of the pin region, which exquisitely matches a cavity of approximately 20 Å diameter at the center of the junction, was expected to direct the structure specific binding of the protein to the Holliday junction. The observed structural specificity seems to originate both from the charge and the structure of the pin region. However, the charge carried by the pin region appears to have an equally important role.

Ingleston et al. [21] demonstrated that the E55R D56K mutant E. coli protein was unable to block the activity of RuvA resolvasome in vivo and inhibited junction resolution and branch migration with respect to the wild-type RuvA. These data are consistent with the SPR analysis, which indicates that the protein does not target Holliday junctions, and multiple tetramers or octamers bind along linear duplex DNA. However, Ingleston et al. [21] also demonstrated that the E. coli RuvA mutants with a more positively charged pin were unable to promote repair more efficiently than those with a more negative pin. Excluding the E55R D56K protein their results demonstrated that the mutant proteins increased the rate of branch migration in the RuvAB complex compared to the wild-type RuvA. The changes also reduced the ability to stimulate RuvC in the RuvABC resolvasome. It still remains to be seen if the increased rate of branch migration directly inhibits the ability of RuvC to perform junction cleavage in the resolvasome complex. Ingleston et al. had previously shown the RuvA mutants with an increased rate of branch migration of the RuvAB complex and in conjunction with the reduced ability to target Holliday junctions this may explain the inability of the mutant proteins to promote DNA repair.

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