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Crystallization of *Escherichia coli* RuvA complexed with a synthetic Holliday junction

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During homologous recombination in *Escherichia coli* the RuvA, B and C proteins interact specifically with the Holliday junction formed by the action of RecA to promote the strand-exchange reaction. RuvA, a homotetrameric protein of molecular weight 88 kDa, has been overexpressed in *E. coli*, purified and co-crystallized with a synthetic Holliday junction substrate made from four 18-base deoxyoligonucleotides. Crystals were grown using the hanging-drop vapour-diffusion method with sodium acetate as the precipitant. The crystals diffract to a resolution of 6 Å and belong to the monoclinic system, space group $C2$, with cell parameters a = 148, b = 148, c = 106 Å and $\beta = 123^\circ$. The X-ray analysis of these crystals should reveal the structure of the Holliday junction and its mode of binding to RuvA, providing new insights into the molecular mechanism of genetic recombination.

1. Introduction

Maintaining the structural and genetic integrity of an organism's DNA along with its accurate replication are fundamental to survival and proliferation, and there are many processes within the cell that are dedicated to these ends. *E. coli* RuvA is a homotetrameric DNA-binding protein of subunit M_r = 22 kDa (Tsaneva, Illing *et al.*, 1992) which, in concert with the RuvB and RuvC proteins, is involved in the recognition, migration and resolution of Holliday junction intermediates in recombination (West, 1996). Specifically, the function of RuvA is to bind the junction and hold the four duplex arms of the DNA in an appropriate orientation to permit branch migration (West, 1992). Once RuvA is bound to the junction, RuvB joins the complex and, in an ATP-dependent process, provides the driving force required for branch migration (Iwasaki *et al.*, 1992; Parsons & West, 1993; Hiom & West, 1995; Tsaneva, Muller *et al.*, 1992). The resolution of the Holliday junction intermediate is catalyzed by the action of a third protein, RuvC (Connolly *et al.*, 1991; Dunderdale *et al.*, 1991; Iwasaki *et al.*, 1991; Bennett *et al.*, 1993; Bennett & West, 1995) which is a dimeric sequence-specific endonuclease (Shah *et al.*, 1994) and cuts the junction DNA at the branch point.

The crystallographic structure of RuvA has been solved to a resolution of 1.9 Å and has led to the proposal of a model for the interaction of RuvA with a Holliday junction (Rafferty *et al.*, 1996). In this model, the Holliday junction DNA duplex arms lie along the four fourfold-symmetrical grooves on the positively charged face of RuvA, with the minor groove of the DNA adjacent to the protein at the point of exchange. Rising from the centre of the RuvA DNA-binding face is a negatively charged pin composed of four symmetry-related β turns which are arranged around the fourfold rotational axis of the tetramer. The pin is thought to be involved in the separation of the duplex strands during branch migration and also in preventing the binding of duplex DNA into opposing DNA-binding grooves across the face of the protein.

Structural comparisons of RuvA with other DNA-binding proteins have shown that each RuvA monomer contains two copies of a helix–hairpin–helix (HhH) DNA-binding motif, first described in the structure of endonuclease III and also observed in DNA polymerase β (Thayer *et al.*, 1995; Davies *et al.*, 1994; Pelletier *et al.*, 1994). Analysis of the structure of a complex of polymerase β with DNA has shown that this motif interacts directly with the DNA, suggesting that in RuvA these motifs interact with the phosphate backbone of the four duplex arms (Rafferty *et al.*, 1998). However, structural comparisons of the HhH motifs in RuvA and polymerase β suggest that the face of the DNA bound by RuvA might be opposite to that originally proposed, so that the minor groove rather than the major groove makes significant interactions with RuvA (Rafferty *et al.*, 1998). In order to resolve these ambiguities and to provide the first detailed structure for the Holliday junction as bound to RuvA, we have embarked on a structural study of RuvA complexed with its Holliday junction substrate.

2. Experimental

RuvA was overexpressed by IPTG induction from *E. coli* strain K12 BL21(DE3) (Tabor & Richardson, 1985; Studier *et al.*, 1991) carrying the plasmid pAM159 which harbours the *RuvA* gene. The protein was initially purified by ion-exchange chromatography as previously described (Sedelnikova *et al.*, 1997) and stored as an ammonium sulfate precipitate at 277 K. To remove any contaminant nucleases from the RuvA protein, a further purification step was added to the protocol. The RuvA precipitate was separated from the ammonium sulfate solution by centrifugation in a JA-20 rotor (Beckman) at 19000 rev min^{-1} and 277 K for 10 min and dissolved in a small volume of buffer (50 m*M* Tris–HCl pH 7.5, 100 m*M* NaCl). The solution of RuvA was then applied to a P-10 prepacked column containing Sephadex-6-25 (Pharmacia) in order to remove the remaining ammonium sulfate. The eluted protein solution was then applied to a 10 ml column packed with heparin-Sepharose CL-6B (Pharmacia) pre-equilibrated using the same buffer. The protein was then eluted with a gradient of 0.1–1.0 *M* NaCl in 50 m*M* Tris–HCl pH 7.5 at a flow rate of 1 ml min^{-1}, and the protein-containing fractions were detected spectroscopically at 280 nm. Purity of the fractions was examined using sodium dodecyl sulfate gel electrophoresis and those with a purity of >98% were pooled, precipitated with solid ammonium sulfate and stored at 277 K.

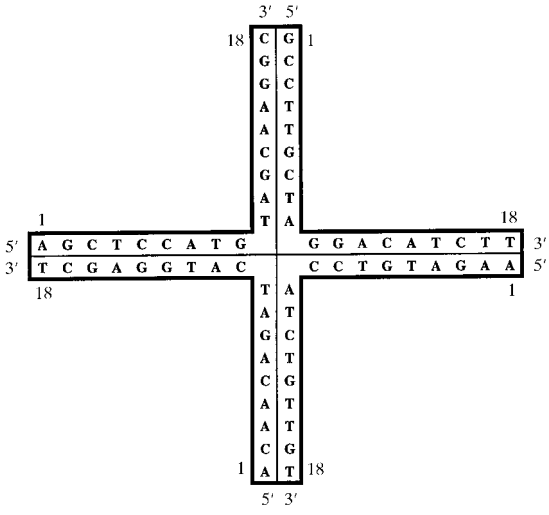

Figure 1
The sequence of the Holliday junction.

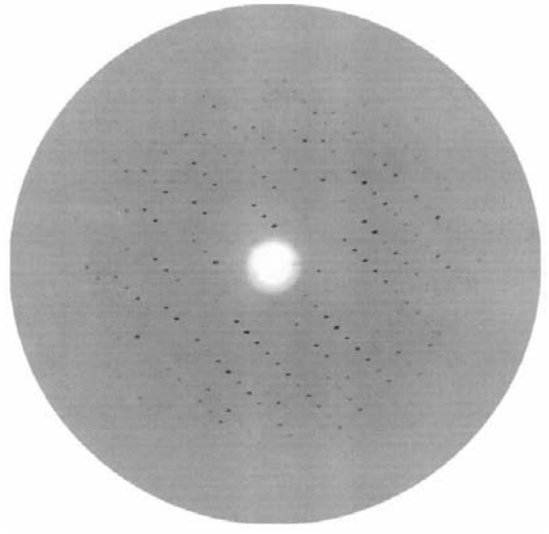

Figure 2
A 3° oscillation image taken from a crystal of *E. coli* RuvA–Holliday junction complex at 100 K. This image was taken on a MAR Research image plate on PX9.6 at the SRS Daresbury Laboratory. The resolution is 5.8 Å at the edge of the plate.

2.1. Preparation of the DNA

The design of the Holliday junction was based on that of an immobile junction (Bennett *et al.*, 1993) and the sequence is shown in Fig. 1. The four deoxyoligonucleotides required for the formation of the Holliday junction were synthesized on an ABI 394 DNA synthesiser using phosphoramidite chemistry and were supplied with the trityl group attached. The deoxyoligonucleotides were then purified by reverse-phase HPLC using a Rainin Dynamax 300 Pure DNA column (21.4 mm internal diameter) attached to an LKB 2152 dual controller running two 2150 pumps. Peak detection was achieved by coupling a Spectromonitor III to the column output and reading the optical density of the eluent at 254 nm. Separation of the required oligonucleotide was achieved by loading 0.2 µmol of DNA (buffered in 10 m*M* Tris–HCl, 1 m*M* EDTA) onto the column while running the initial mobile phase of 0.1 *M* triethylammonium acetate at pH 7.0 (mobile phase *A*) at 5 ml min^{-1}. After equilibration for 10 min, mobile phase *A* was adjusted to contain 10% methyl cyanide and held for 10 min to wash off DNA sequences that did not possess the hydrophobic trityl group, corresponding to the sequences which failed during synthesis. This was followed by a 10 min wash with mobile phase *A* to remove the MeCN. The oligonucleotides were then detritylated *in situ* by switching the mobile phase to 0.5% triflouroacetic acid in water and washing them for 10 min followed by another 10 min wash with mobile phase *A*. Finally, a gradient of 0–15% MeCN in mobile phase *A* was applied over 30 min and the eluted fractions collected by an LKB 2211 Superrac (fraction volumes were approximately 1 ml). The purity of each fraction was then ascertained by urea-denaturing polyacrylamide gel electrophoresis. The concentration of each fraction was estimated spectroscopically at a wavelength of 254 nm and volumes containing 1 µg of DNA were removed from each fraction and mixed with 25 µl of loading buffer (formamide containing 0.1 *M* TBE buffer pH 8.0 and bromophenol blue as a visual marker). 20 µl of these samples were then run out on a 20% polyacrylamide (2% bis-acrylamide), 6 *M* urea, 160 × 1.0 × 160 mm gel using 0.1 *M* TBE at pH 8.0 as the running buffer. Bands on the gel were visualized by staining with ethidium bromide and transilluminating with UV light at 340 nm. Fractions that were observed to be of highest purity were then pooled and freeze-dried overnight. When dry, the oligonucleotides were redissolved in sterile milliQ and the concentration adjusted to be equivalent to 0.1 m*M*. The oligonucleotide solutions were then stored at 255 K until required.

2.2. Preparation of the RuvA–Holliday complex

The four deoxyoligonucleotides, at a concentration of approximately 0.1 m*M*, were mixed together in equimolar amounts and 1 *M* Tris–HCl pH 8.0 was added to make a 40 m*M* buffered solution. Following heating to 363 K for 5 min the deoxyoligonucleotide solution was immediately plunged into liquid nitrogen and then stored at 255 K.

RuvA protein was prepared from the ammonium sulfate precipitate by dialysis against 600 m*M* sodium chloride buffered with 20 m*M* ammonium acetate pH 6.4 for 24 h at 277 K. The dialysed protein was then concentrated using a Centricon 10k centrifugal concentrator until an OD_{280} reading of 3.0 was achieved, which corresponded to an approximate protein concentration of 9 mg ml^{-1} (0.1 m*M*). Equal volumes of RuvA and the deoxyoligonucleotide solution were then mixed together and used immediately in crystallization trials.

An initial screen for crystallization conditions was carried out using the Hampton Crystal Screen kits I and II using the hanging-drop vapour-diffusion method. The conditions that gave the best crystals used 0.1 *M* imidazole–HCl pH 6.5–7.5 as the buffer and 0.70–0.95 *M* sodium acetate as the precipitant. Crystals grown using these conditions had a square plate-like morphology and grew to approximately 0.5 × 0.5 × 0.05 mm.

To verify that the crystals contained both DNA and protein, a number of them were harvested and washed thoroughly in stabilizing solution before being dissolved in water and run out on a 6% native polyacrylamide gel. The gel was stained with ethidium bromide to test for the presence of DNA and then with Coomassie blue to probe for protein (data not shown). A coincident band for both protein and DNA was observed in the lanes where the dissolved crystals had been loaded, and comparison of this band with the native protein and DNA controls showed the species to be of a higher molecular weight than either native RuvA or the DNA.

2.3. Data collection

The thin plate-like morphology of the crystals made them ideal candidates for cryocooling because of their high surface-area-to-volume ratio. The crystals also had a tendency to crack under their own weight when mounted in capillaries, so it was decided to screen for a suitable cryoprotectant and collect data at 100 K. Crystals were soaked in various cryoprotectant solutions (Garman, 1995) and mounted in Hampton Cryo loops before being flash frozen to 100 K with an Oxford Cryostream system. The ability of the various cryoprotectant solutions to stabilize the crystals were then assessed by analysing diffraction patterns collected on a two-detector San Diego multiwire system (Hamlin, 1985; Xuong *et al.*, 1985) with a Rigaku AFC6 goniostat system mounted on a Rigaku RU200 rotating-copper-anode X-ray generator. The conditions that provided the best cryoprotectant of those tested were 24% glycerol in 0.1 *M* imidazole pH 6.5–7.5 and 1.5 *M* sodium acetate.

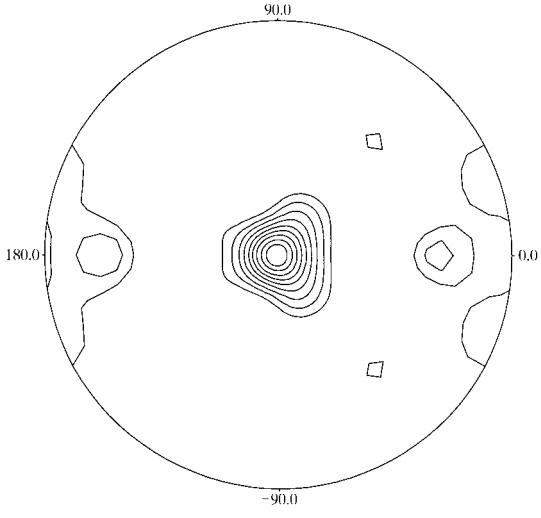

Figure 3
A stereographic projection of the $\kappa = 90^\circ$ section of the self-rotation function of the *E. coli* RuvA–Holliday junction complex. A non-crystallographic fourfold axis, collinear with c^*, can be seen at the centre of the plot (where $\omega = 0$ or 180°).

3. Results and discussion

A native data set was collected from a single crystal at 100 K on station 9.6 at the Synchrotron Radiation Source at Daresbury Laboratory. A total of 10623 measurements were recorded and these were merged with an *R* factor of 4.3% to give 5263 unique reflections with a resolution range 55–5.8 Å (Fig. 2). Analysis of the data using the autoindexing algorithm in *DENZO* (Otwinowski & Minor, 1997) showed that the RuvA–DNA complex crystals belonged to the monoclinic system, space group *C*2 with cell parameters $a = 148$, $b = 148$, $c = 106$ Å and $\beta = 123^\circ$. Calculation of the V_m (Matthews, 1968) showed the asymmetric unit contained at least a tetramer of RuvA. The self-rotation function of the native data set was calculated using the program *POLARRFN* (Kabsch, unpublished work; Collaborative Computational Project Number 4, 1994) for data in the resolution ranges of 30–7 Å and a radius of integration of 25 Å. Analysis of the $\kappa = 90^\circ$ section of the map showed the presence of a non-crystallographic fourfold axis parallel to c^* (Fig. 3). A search has now been undertaken to find suitable heavy-atom derivatives to use in an MIR solution of the structure.

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