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Crystallization of *E. coli* RuvA gives insights into the symmetry of a Holliday junction/protein complex

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Abstract

The *E. coli* protein RuvA (resistance to ultraviolet light) has been overexpressed in *E. coli*, purified and crystallized using the hanging-drop vapour-diffusion method with sodium chloride as the precipitant. The crystals, which diffract to beyond 1.9 Å, belong to the tetragonal system, space group *P*4 with unit-cell dimensions of $a = 83.7$, $c = 33.1$ Å with a monomer in the asymmetric unit. RuvA is known to be a tetramer and thus the crystal symmetry implies that its quaternary structure will be based on fourfold rotation symmetry rather than 222 symmetry. This is consistent with electron microscopy data on Holliday junction DNA complexes and implies that the arms of the four DNA duplexes involved in recombination adopt fourfold rotation symmetry.

1. Introduction

Accurate replication of DNA and the maintenance of its structural and genetic integrity are fundamental to an organism's survival and proliferation and as such there are many processes within the cell that are dedicated to these ends. Underpinning these processes are a host of proteins such as polymerases, nucleases and helicases. RuvA is a homotetrameric DNA binding protein of subunit M_r 22 kDa (Tsaneva, Illing, Lloyd & West, 1992). In concert with the products of the *ruvB* and *ruvC* genes, RuvA is involved in the recognition, migration and resolution of Holliday junction intermediates in recombination (West, 1996). Both RuvA and RuvB are under the control of the DNA-damage inducible SOS regulon (Shurvinton & Lloyd, 1982) with their loci at 41′ on the *E. coli* chromosome (Otsuji, Iyehara & Hideshima, 1974; Benson, Illing, Sharples & Lloyd, 1988; Shinagawa *et al.*, 1988; Sharples, Benson, Illing & Lloyd, 1990). RuvA specifically binds the four-stranded DNA junction that is produced by the action of RecA during homologous recombination (West, 1992). Once bound, RuvB joins the complex and, in an ATP-dependent process, provides the driving force required for branch migration (Iwasaki, Takahagi, Nakata & Shinagawa, 1992; Parsons & West, 1993; Hiom & West, 1995; Tsaneva, Muller & West, 1992). The resolution of the Holliday junction intermediate is achieved by the action of a third protein, RuvC (Connolly *et al.*, 1991; Dunderdale *et al.*, 1991; Iwasaki, Takahagi, Shiba, Nakata & Shinagawa, 1991; Bennet, Dunderdale & West, 1993; Bennet & West, 1995*a*,*b*) which is a sequence specific nuclease (Shah, Bennet & West, 1994) which functions as a dimer to cut junction DNA on the two non-crossover strands. In order to contribute to the understanding of the molecular basis of recombination we have initiated a structural study of RuvA.

2. Methods

The *ruvA* gene was amplified by the polymerase chain reaction (PCR) from pPVA101, a *ruvABC*$^+$ construct (Attfield, Benson & Lloyd, 1985), using the oligonucleotide primers 5′-TCTT*GAATTC*ATTACGCAGGAG**G**GT**G**ATATGATA-3′ and 5′-TCAGACGGT*AAGCTT*CAATCATCC-3′ to engineer *Eco*R1 and *Hind*III restriction sites (in italic) at the 5′ and 3′ ends of the coding sequence, respectively. The 5′ primer also included nucleotide changes (shown in bold) to improve ribosome binding and to substitute an ATG initiation codon for the wild-type GTG (Benson, Illing, Sharples & Lloyd, 1988). After digestion with *Eco*R1 and *Hind*III the PCR product was ligated into pT7-7 (Tabor & Richardson, 1985), which places the *ruvA* gene under control of the phage T7φ10 promoter. The construct, pAM159, was verified by sequencing the cloned gene and testing its ability to restore UV resistance to a *ruvA* mutant strain.

RuvA protein was overexpressed by IPTG induction of *E. coli* strain 12 BL21(DE3) (Tabor & Richardson, 1985; Studier, Rosenberg & Dunn, 1991) carrying the plasmid pAM159. Induced cells obtained from four 500 ml cultures were harvested by centrifugation at 277 K and the cell pellet was resuspended in 3 ml of the buffer containing 0.1 *M* Tris–HCl, 2 m*M* EDTA, 5% glycerol and stored frozen at 253 K. For purification of RuvA, 2 ml of 5 *M* sodium chloride was added to the defrosted cells from the 2 l culture which were then disrupted by two passes through a French pressure cell at 20000 p.s.i. The cell debris was removed by centrifugation and the supernatant was diluted with buffer *A* (40 m*M* Tris–HCl pH 8.5, 2 m*M* EDTA) to bring its conductivity to below 9 mS. The diluted supernatant (typically containing 300 mg of total protein) was applied at a flow rate of 300 ml h^{-1} to a DEAE–Sepharose Fast Flow column (20 ml bed volume) equilibrated with the buffer *A*. The protein was eluted with a 300 ml linear gradient of 0.0–0.25 *M* NaCl in the same buffer at a flow rate of 150 ml h^{-1}. Fractions containing RuvA protein eluted at a sodium chloride concentration of 0.15–0.16 *M*. These fractions were pooled and 4 *M* ammonium sulfate solution was added to a final concentration of between 1.0 and 1.2 *M*. The resulting solution was loaded on a Butyl-Toyopearl 650S column (20 ml bed volume) equilibrated with 1.0 *M* ammonium sulfate in buffer *A* and eluted with a 300 ml linear gradient of 1.0–0.0 *M* ammonium sulfate in buffer *A* at flow rate 150 ml h^{-1}. RuvA was eluted in a broad peak from 0.6–0.3 *M* ammonium sulfate. Fractions containing RuvA were pooled and the protein was precipitated by the addition of ammonium sulfate to give a final concentration of 80% saturation. The typical yield of RuvA was between 35 and 40 mg. Rather than the use of a hydrophobic column as described above, previous work had reported a purification step which involved precipitation of RuvA by

dialysis against a neutral, low-salt buffer following ion-exchange chromatography (Shiba, Iwasaki, Nakata & Shinagawa, 1991). When we prepared protein using this method, examination of the precipitate showed that it appeared to be composed of a mass of crystals in the form of long thin needles. Furthermore, we noted that the purity appeared to be better but there was a significant reduction in yield. However, whilst developing the purification protocol for RuvA, we also found that the peptide bond between Ser150 and Thr151 was sensitive to cleavage particularly under dialysis at a pH above 7 and under conditions of low ionic strength. Sequencing studies have shown that the use of the combination of ion-exchange/hydrophobic chromatography gives rise to material in which the level of cleavage is very low and hence we chose to use this method in further work.

The crystals obtained from the low salt conditions, described above, were very thin needles and were unsuitable for X-ray analysis. New crystallization conditions from trials by the hanging-drop method were found with the Hampton Crystal Screen II using sodium chloride as the precipitant. The refined crystallization protocol is as follows; the RuvA protein from the ammonium sulfate precipitate stock was pelleted by brief centrifugation and resuspended in buffer containing 20 m*M* ammonium acetate, 600 m*M* sodium chloride made up in Milli-Q water at pH 6.4. The sample was then dialysed against this buffer for 24 h at 277 K and then concentrated using a Centricon 10k until the OD_{280} was equal to 2 (approximately equal to a concentration of 10–15 mg ml^{-1}). The ammonium acetate buffer was then swamped by adding 1 *M* potassium citrate buffer at pH 4.9 to give a final concentration of 50 m*M*. The optimum conditions for the growth of large crystals are based on suspending 5 µl drops of protein solution over a well containing 1.5–2.0 *M* sodium chloride, 20 m*M* ammonium acetate, 50 m*M* potassium citrate (pH 4.9) without addition of this mixture to the drop. Crystals with maximum dimensions of $2.5 \times 0.125 \times 0.125$ mm grew within two weeks and the crystals were confirmed as being RuvA by sequencing the first ten N-terminal residues.

3. Results and discussion

Preliminary data were collected from crystals mounted in glass capillaries to a resolution of 2.3 Å on a two-detector San Diego multiwire area detector (Hamlin, 1985; Xuong, Nielsen, Hamlin & Anderson, 1985) with a Rigaku AFC6 goniostat system mounted on a Rigaku RU200 rotating copper anode X-ray generator. A total of 29 152 measurements were made of 10 786 independent relections and the data were merged to an R factor of 7.3% with 91% completeness of the data from 20 to 2.3 Å resolution and for the highest resolution shell (2.3 to 3 Å); $R_{\rm merge} = 20\%$, completeness = 87%, and $I/\sigma I = 1.8$. Analysis of the data using the autoindexing algorithm supplied with the SDMS detector system (Howard, Nielsen & Xuong, 1985) showed that the RuvA crystals belong to the tetragonal system, space group $P4$, with unit-cell dimensions of $a = 83.7$, $c = 33.1$ Å. The unit-cell volume is 2.3×10^5 Å^3 and, given the subunit M_r of 22 kDa, the V_m value for a monomer in the asymmetric unit is 2.6 Å^3 Da^{-1} which lies within the range observed for globular proteins (Matthews, 1977), whereas values of V_m for multiple subunits in the asymmetric unit fall outside this range. Subsequent test exposures using the synchrotron radiation source at CCLRC Daresbury Laboratory have indicated that the RuvA crystals diffract to beyond 1.9 Å (Fig. 1).

Previous gel electrophoretic studies on free Holliday junctions which had been engineered with a unique restriction site on each of the four arms and then digested with pairs of restriction enzymes showed that the six possible cleavage products (cleavage of either the opposite or adjacent arms) migrated as one of three species; fast, medium or slow. This pattern of relative migration has been taken to indicate that the free DNA adopts twofold symmetric so-called stacked-X structure (Duckett *et al.*, 1988). However, biochemical studies coupled with modelling on Holliday junction/RuvC complexes, using the known structure of RuvC as a guide (Ariyoshi *et al.*, 1994), suggest that the junction shows twofold symmetry but with a considerable modification of the crossover point compared to the stacked-X structure, which involves the insertion of extended linkers of at least two nucleotides (Ariyoshi *et al.*, 1994; Bennett & West, 1995*b*).

However, since RuvA is known to be a tetramer, the observed crystal symmetry implies that its quaternary structure will be based on fourfold rotation symmetry rather than 222 symmetry. This implies that the four DNA duplexes that form the Holliday junction substrate would be held in a fourfold arrangement on the surface of RuvA. This is consistent with electron-microscopy images of the RuvA/Holliday junction complex which also indicate that the complex has fourfold symmetry (Parsons, Stasiak, Bennet & West, 1995).

The direct evidence for the symmetry of the Holliday junction/RuvA complex provided by the analysis of the crystal symmetry is also supported by electrophoretic mobility studies on complexes of RuvA with Holliday junctions. Double restriction digests of these complexes have showed the presence of two migrating species; fast or slow. This pattern of relative migration has been taken to indicate that the Holliday junction

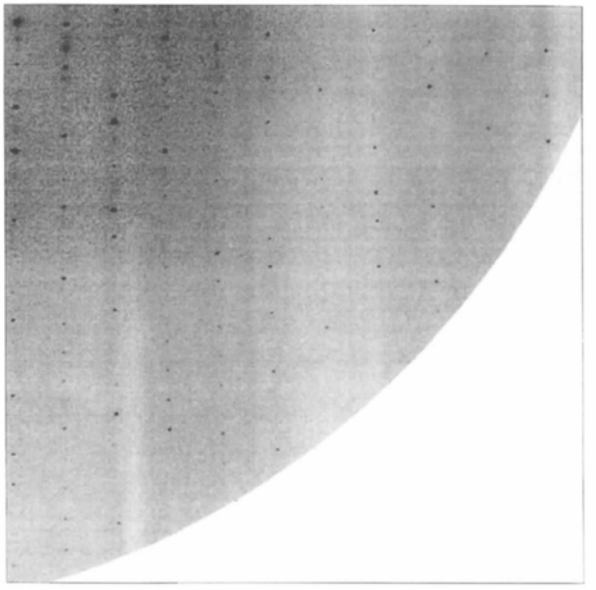

Fig. 1. A 1.2° rotation diffraction pattern from a crystal of *E. coli* RuvA. The image was taken on a MAR Research image plate on station 9.5 at the CCLRC Daresbury Laboratory Synchrotron and shows data to 1.9 Å resolution at the edge of the plate.

adopts a structure with fourfold symmetry when bound to RuvA (Parsons, Stasiak, Bennet & West, 1995).

The solution of the atomic structure of RuvA will contribute to the growing body of research into the processes of genetic recombination and DNA repair and play a key role in the evolution of a complete molecular model for the mechanism of genetic recombination.

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