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Crystallization and preliminary X-ray analysis of RecG, a replication-fork reversal helicase from *Thermotoga maritima* complexed with a three-way DNA junction

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The monomeric 3′-5′ helicase RecG from the thermophilic bacterium *Thermotoga maritima* has been crystallized in complex with a three-way DNA junction, the preferred physiological substrate. The crystals were obtained by hanging-drop vapour diffusion. The crystals belong to space group *C*2, with unit-cell parameters a = 133.7, b = 144.6, c = 84.0 Å, β = 113.8°. Native data to a resolution of 3.25 Å were collected from crystals flash-cooled to 100 K.

1. Introduction

For many years, it was envisaged that replication of the leading strand at each replication fork would be a continuous highly processive process that would continue uninterrupted around the chromosome between the origin and the termination site. Replication of the lagging strand would take place at regular intervals from newly synthesized RNA primers that initiate Okazaki fragments. However, more recently it has become apparent that this is a naïve view of the process (Cox *et al.*, 2000). A problem arises when the fork encounters DNA damage in the form of either a base lesion or a single-stranded nick in the DNA. In either of these situations, the replication fork stalls and the replisome disassembles. Fork progression can be recovered *via* any of a number of parallel pathways, probably dependent upon the nature of the DNA damage. At least two of these proposed pathways utilize the RecA protein to create a four-way (or Holliday) junction which is then migrated and resolved to create a new replication fork. A third pathway appears to involve direct fork reversal by the RecG helicase.

RecG shows significant activity in converting three-way (fork) junctions into Holliday junctions (McGlynn & Lloyd, 2000). This process is effectively the reversal of the fork-migration process catalysed by the replicative helicase DnaB. The function of this process appears to be to allow reversal of a replication fork that has stalled owing to a base lesion so that replication can continue by template switching (McGlynn & Lloyd, 2000). Fork reversal results in the formation of 'chicken-foot' intermediates. Once the chicken foot has been formed, DNA synthesis on the stalled strand can continue by switching template strands, thereby bypassing the lesion. The Holliday junction can then be migrated back past the lesion, presumably by RecG or RuvAB, to recover the fork, with repair of the lesion taking place at a later stage.

It seems that most (perhaps all) replication forks encounter some form of damage and have to restart during each cycle of chromosomal replication. It is not surprising, therefore, that there are a large number of proteins associated with this essential function. Recent estimates for *Escherichia coli* are that at least 26 proteins are implicated, in addition to the multisubunit DNA polymerase III holoenzyme (Cox *et al.*, 2000). At the present time, there is structural information for only a handful of these proteins.

2. Materials and methods

2.1. Protein preparation

The *T. maritima recG* gene was amplified from genomic DNA and inserted into the expression plasmid pET28a under the control of the inducible T7 promoter. The protein was overexpressed in *E. coli* B834 (DE3) pLysS with plasmid pSJS1240 (a gift from S. J. Sandler) expressing the rare codons AGA, AGG and AUA. Cells were induced at mid-log phase with 1 m*M* IPTG and subsequently grown for 3 h. The cell pellet was resuspended in buffer *A* (50 m*M* Tris–HCl pH 7.5, 200 m*M* NaCl, 1 m*M* EDTA, 1 m*M* DTT) and lysed by sonication. After clarification by centrifugation, the supernatant was taken to 65% saturation in $(NH_4)_2SO_4$ and the precipitated protein was pelleted by centrifugation and resuspended in buffer *A*. This extract was heated at 338 K for 5 min and the denatured protein was then removed by centrifugation. The supernatant was applied to a HiTrap heparin column (Pharmacia) equilibrated in buffer *A* and eluted with a gradient of 0.2–1.0 *M* NaCl. The RecG-containing fractions

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were pooled and $(NH_4)_2SO_4$ was added to a final concentration of 1.0 *M*. This was then applied to an isopropyl SOURCE column (Pharmacia) equilibrated with the same buffer and eluted with a 1.0–0 *M* $(NH_4)_2SO_4$ gradient. The RecG-containing fractions were diluted to the conductivity of buffer *A* and applied to a low-substitution Sepharose blue column (Cibacron Blue 3 GA) and eluted with a 0.2–1.5 *M* NaCl gradient. For crystallization, the protein was concentrated to 15 mg ml^{-1} using a Centricon 50 (Amicon) in buffer *B* (10 m*M* Tris–HCl pH 7.5, 200 m*M* NaCl, 1 m*M* DTT).

2.2. DNA preparation

The DNA substrate employed for crystallization was a three-way fork. The constituent oligonucleotides J1A (5′-CAGCTCCATGATCATTGGCA-3′), J1B (5′-GCAGTGCTCGCATGGAGCTG-3′) and J1C (5′-GAGCACTGC-3′) were supplied by Oswel Oligonucleotides and purified either by HPLC or FPLC depending on length. The oligonucleotides were concentrated to approximately 2 m*M* in dH_2O and annealed in a buffer of 50 m*M* KH_2PO_4/K_2HPO_4 pH 6.8, 200 m*M* KCl. The annealing program comprised an initial denaturing step of 1 min at 368 K, a ramp to 343 K over 10 min, a slow ramp to 323 K over 40 min, a ramp to 293 K over 10 min followed by a final ramp to 277 K over 10 min. The molar ratio of the oligonucleotides in the annealing reaction was 1:1.2:1.2 J1A:J1B:J1C and the final reaction volume approximately was 500 µl. The annealed substrate was applied to a 6 ml SOURCE Q column (Pharmacia) equilibrated in 50 m*M* KH_2PO_4/K_2HPO_4 pH 6.8 and eluted with a 0–0.6 *M* KCl gradient in the same buffer over 40 column volumes. The purified three-way junction was desalted on a Sephadex G25 column (Pharmacia) and vacuum concentrated to 1 m*M* in dH_2O.

3. Results

3.1. Crystallization

For the purposes of crystallization, the DNA and protein were mixed in a 1.2:1 DNA:protein molar ratio in buffer *B* with the addition of 5 m*M* $MgCl_2$ and 1 m*M* ADP. Initial screens were carried out using the 'clear strategy screen' (Brzozowski & Walton, 2001) using hanging-drop vapour diffusion at room temperature. Drops comprised 2 µl protein solution with 2 µl resevoir solution over 1 ml reservoirs. Crystals were obtained from conditions of 500 m*M* KH_2PO_4/K_2HPO_4 pH 5.0, 5 m*M* $MgCl_2$, 1 m*M* ADP in approximately two weeks. The crystal morphology was that of stacked rectangular or trapezoid plates with largest dimensions of 0.2 × 0.07 × 0.04 mm.

3.2. Data collection and processing

The crystals were transferred to a cryoprotectant solution of 300 m*M* KH_2PO_4/K_2HPO_4 pH 5.0, 5 m*M* $MgCl_2$, 1 m*M* ADP, 30%(*v*/*v*) ethylene glycol for approximately 10 s, then flash-cooled by plunging into liquid nitrogen. Initial characterization on a rotating-anode source showed the crystals to belong to space group *C*2, with unit-cell parameters a = 133.7, b = 144.6, c = 84.0 Å, β = 113.8°.

Subsequent data collection was carried out at 100 K. Diffraction data were collected on station ID14.1 of the European Synchrotron Radiation Facilty at 0.98 Å. Self-rotation functions calculated using the data showed no evidence of non-crystallographic symmetry. Assuming a single monomer of RecG bound to a single DNA fork in the asymmetric unit, with a total molecular mass of 105 kDa, the calculated Matthews coefficient is 3.6 Å^3 Da^{-1}, corresponding to a solvent content of 65%. A complete data set was collected on the native crystals, statistics for which are shown in Table 1. A search for heavy-atom derivatives is currently in progress.

Table 1
Statistics of data collection.

Values in parentheses refer to the highest resolution shell of the data, 3.37–3.25 Å.

Space group	*C*2
Unit-cell parameters (Å, °)	a = 133.7, b = 144.6, c = 84.0, β = 113.8
Resolution (Å)	20–3.25
Total reflections	49645
Unique reflections	22536
R_{merge}	0.039 (0.317)
Completeness (%)	98.3 (99.4)
$I/\sigma(I)$	12.3 (3.4)
Redundancy	2.2 (2.1)

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