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Crystallization and preliminary X-ray diffraction analysis of the *Bacillus subtilis* replication termination protein in complex with the 37-base-pair TerI-binding site

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The replication terminator protein (RTP) of *Bacillus subtilis* binds to specific DNA sequences that halt the progression of the replisome in a polar manner. These terminator complexes flank a defined region of the chromosome into which they allow replication forks to enter but not exit. Forcing the fusion of replication forks in a specific zone is thought to allow the coordination of post-replicative processes. The functional terminator complex comprises two homodimers each of 29 kDa bound to overlapping binding sites. A preparation of RTP and a 37-base-pair TerI sequence (comprising two binding sites for RTP) has been purified and crystallized. A data set to 3.9 Å resolution with 97.0% completeness and an $R_{\rm sym}$ of 12% was collected from a single flash-cooled crystal using synchrotron radiation. The diffraction data are consistent with space group *P*622, with unit-cell parameters $a = b = 118.8$, $c = 142.6$ Å.

1. Introduction

Much of our understanding of DNA replication comes from biochemical investigations into two systems: those of *Escherichia coli* and *Bacillus subtilis* (Kornberg & Baker, 1992). In these two systems, many of the components of the molecular assemblies that initiate and coordinate DNA replication are homologous in function without necessarily sharing significant sequence homology (Wijffels *et al.*, 2005; Schaeffer *et al.*, 2005; Johnson & O'Donnell, 2005). Interestingly, the proteins that halt the replication forks at the initial stage of DNA replication termination have evolved independently. The replication termination protein of *E. coli* (Tus) has been solved in complex with its associated DNA-binding site (Ter; Kamada *et al.*, 1996). Tus is a monomeric protein of unique fold that binds Ter very tightly ($K_{\rm d} = 3.4 \times 10^{-13}$ *M*) to halt the progress of approaching forks in a polar manner (Gottlieb *et al.*, 1992). The terminator complex of *B. subtilis*, components of which have also had their crystal structures determined (Bussiere *et al.*, 1995; Wilce *et al.*, 2001), performs the same function yet bears neither structural nor sequence similarity with either Tus or Ter of *E. coli*. Here, we report the crystallization of the full-length *B. subtilis* TerI sequence in complex with the replication terminator protein and preliminary analysis of these crystals.

Replication of the circular chromosome of *B. subtilis* occurs bidirectionally from *oriC*, with replication forks migrating until they fuse in a region approximately 180° from the origin. The arrest of replication forks and the coordinated termination of DNA replication is dependent upon the binding of the replication terminator protein (RTP) to its cognate DNA-binding sites known as terminator sites (Ter sites; Smith & Wake, 1988, 1989). Each Ter site comprises two pseudosymmetric binding sites for RTP: a low-affinity A site and a high-affinity B site. Two RTP molecules bind the complete TerI sequence with an apparent $K_{\rm d}$ of 1×10^{-13} *M* (Lewis *et al.*, 1990). Binding of RTP to the Ter site is cooperative in nature, with binding at the A site not occurring until prior filling of the B site (Langley *et al.*, 1993). Binding at the B site alone is tight ($K_{\rm d,app} = 4 \times 10^{-11}$ *M*), yet is not sufficient to arrest the progression of the replication fork (Smith & Wake, 1992).

The functional terminator complex arrests replication-fork progression in a polar manner. Replication forks that approach the

Table 1
Data-collection and processing statistics.

Values in parentheses refer to the highest resolution shell.

Space group	*P*622
Unit-cell parameters (Å, °)	a = 118.8, b = 118.8, c = 142.6, α = 90, β = 90, γ = 120
Resolution range (Å)	50.0–3.90 (4.09–3.90)
Total reflections	55734
Unique reflections	6098
Redundancy	9.1
Completeness (%)	98.2 (93.7)
$R_{\rm sym}$†	0.12 (0.46)
Mean $I/\sigma(I)$	15.3 (3.0)

† $R_{\rm sym} = \sum_{\mathbf{h}} \sum_{l} |I_{\mathbf{h}l} - \langle I(h)\rangle| / \sum_{\mathbf{h}} \sum_{l} \langle I_{\mathbf{h}}\rangle$, where $I_{\mathbf{h}l}$ is the lth observation of reflection **h** and $\langle I_{\mathbf{h}}\rangle$ is the weighted average intensity for all observations l of reflection **h**.

terminator complex from the A site are able to progress through the complex. Replication-fork arrest only occurs when the B site is proximal to the approaching replication fork (Smith & Wake, 1992). Nine Ter sites flank the terminus region of the chromosome in two opposed groups that allow the replication forks to enter the terminus region but do not allow them to exit. TerI, TerIII, TerV and TerIX are arranged so that the B site is proximal to the clockwise replication fork, while TerII, TerIV, TerVI, TerVII and TerVIII are arranged so that the B site is proximal to the anticlockwise replication fork (Griffiths *et al.*, 1998). This arrangement establishes a 'replication-fork trap' that ensures the replication forks always fuse in the region spanned by the terminators.

RTP (gene code P68732) is a protein comprising 122 amino acids that previous crystal structures have identified as a homodimeric member of the 'winged-helix' fold (Bussiere *et al.*, 1995). This structural motif comprises a three-helical bundle flanked by a two-stranded β-sheet extension or 'wing' (Gajiwala & Burley, 2000). RTP has an extra helix (α4). The α4 helices form an antiparallel coiled coil that contributes to the dimerization of the molecule. To date, the DNA used in crystallographic studies has comprised only one B site (Wilce *et al.*, 2001). This structure revealed that the majority of contacts are made to the major groove by the third helix or recognition helix. The structure also suggested a model of cooperative binding across the A and B sites. However, as yet no crystallographic structure of the complete RTP–TerI structure exists.

This paper describes the preparation and crystallization of RTP C110S in complex with 37 base pairs of the TerI sequence. This sequence contains both A and B binding sites for RTP. Elucidation of the three-dimensional structure of RTP C110S–TerI is expected to yield insights into the molecular basis for the binding affinity and cooperative binding of RTP across the A and B sites. Knowledge of the formation of the functional complex will help to elucidate the establishment of polarity and the mechanism of replication-fork arrest.

2. Materials and methods

2.1. Protein expression and purification

The wild-type RTP gene was previously isolated from *B. subtilis* strain 168 (Ahn & Wake, 1991). The protein used in this study was a mutant in which the only cysteine was converted to a serine (RTP C110S). RTP C110S has previously been characterized (Vivian *et al.*, 2003) and found to be structurally and functionally similar to the wild-type protein, yet it had much improved solubility properties. ^{15}N-RTP C110S was overexpressed from a pET-9 derived vector in *E. coli* BL21(DE3) *pLysS* grown in minimal M9ZB media supplemented with trace metals, vitamins and $^{15}NH_4Cl$. The protein was purified by the method of Kralicek *et al.* (1993), with the exception that dithiothreitol was excluded from all buffers. In brief, the cell lysate was cleared of nucleic acid fragments by the addition of one-fifth volume of 10 mg ml^{-1} protamine sulfate. The precipitate was cleared by centrifugation and the resultant supernatant was dialysed into 50 m*M* phosphate pH 6.7. Preliminary purification of RTP C110S was achieved by batch cation exchange using CM-Sephadex C25. RTP C110S was eluted from the beads with 0.7 *M* NaCl in 50 m*M* phosphate pH 6.7. The protein was purified to homogeniety by cation exchange on a BioRad UNO-S6 column equilibrated in 50 m*M* phosphate pH 6.7 and eluted with a linear gradient from 0 to 1 *M* NaCl at approximately 0.55 *M* NaCl.

2.2. DNA preparation

The TerI oligonucleotides (TerI-1, 5′-AAT AGA ACT AAG AAA ACT ATG TAC CAA ATG TTC AGT C-3′; TerI-2, 5′-GAC TGA ACA TTT GGT ACA TAG TTT TCT TAG TTC TAT T-3′) were purchased reverse-phase purified and deprotected from Alpha DNA (Montreal Canada). 100 µ*M* of the single-stranded oligonucleotides were combined in annealing buffer (10 m*M* Tris–HCl pH 8.0, 1 m*M* EDTA pH 8.0, 50 m*M* NaCl). The annealing mixture was heated to 368 K for 5 min and then cooled slowly to 295 K. After 2 h at 295 K, the annealing mixture was flash-frozen in liquid nitrogen and stored at 193 K.

2.3. Complex formation

Samples of RTP C110S and TerI were dialysed separately into 10 m*M* phosphate buffer pH 6.0, 700 m*M* NaCl, 100 µ*M* EDTA, 25 µ*M* chloramphenicol (the chloramphenicol was added to prevent bacterial growth). Mixtures of RTP C110S and duplex DNA were combined in 7 ml such that upon concentration (in a Vivascience Vivaspin 5 kDa molecular-weight cutoff centrifugal concentrator) to 500 µl the concentration of protein monomer was 600 µ*M*. D_2O was added to 5% and 2D heteronuclear single-quantum coherence (HSQC) NMR spectra were acquired on a Bruker 500 MHz at 298 K. Owing to the inability to determine the ratio of protein to DNA at which the complex was fully formed from the NMR experiments, a ratio of 2:1 was chosen for crystallization trials. This 2:1 ratio of dimeric RTP C110S to duplex DNA was prepared as described above. Following concentration, a white precipitate formed that was dissolved with gentle agitation. This sample was then used for crystallization trials.

2.4. Crystallization

Initial crystallization screens were performed using sparse-matrix screening methods utilizing Crystal Screen I (Hampton Research, USA; Jancarik & Kim, 1991). 1 µl of protein–DNA solution (570 µ*M* monomeric RTP, 142.5 µ*M* TerI DNA, 8 m*M* phosphate pH 6.0, 665 m*M* NaCl, 95 µ*M* EDTA and 23.75 µ*M* chloramphenicol) was added to 1 µl reservoir solution and the drop was equilibrated by the hanging-drop vapour-diffusion method over 300 µl reservoir solution at 294 K. Small hexagonal rods (less than 0.05 mm in each dimension) formed overnight in Crystal Screen I condition No. 50 [15%(*w*/*v*) PEG 8000, 0.5 *M* lithium sulfate]. Attempts to reproduce or optimize this crystallization condition using in-house solutions proved unsuccessful. All screening was conducted with solutions produced by Hampton Research. Diffraction-quality crystals were formed from drops with a 2:1 ratio of protein-complex solution to reservoir comprising 13.5%(*w*/*v*) PEG 8000, 0.45 *M* lithium sulfate and 10%

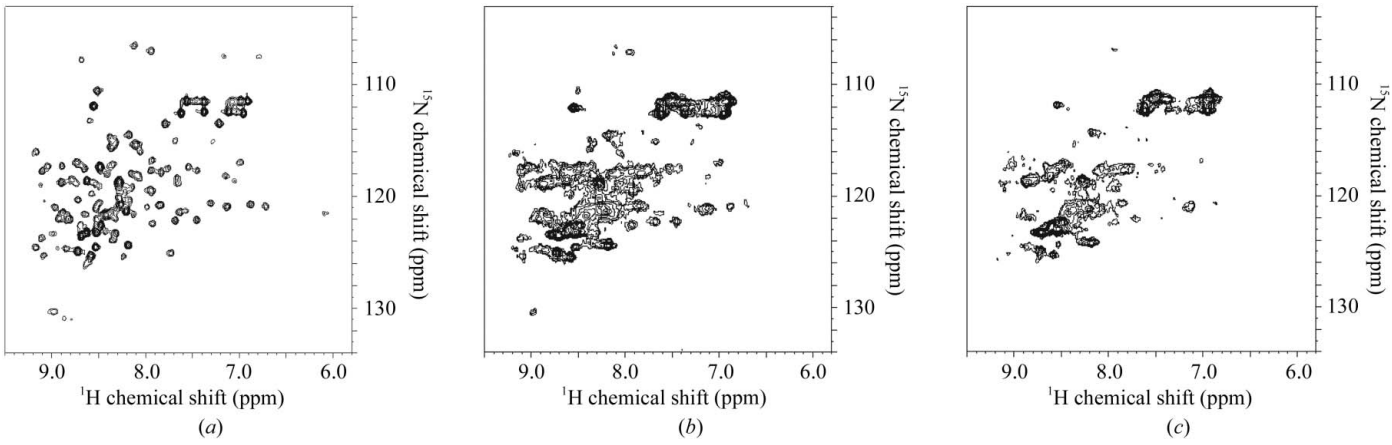

Figure 1
RTP C110S–TerI titration series. The backbone amide region of the ^{15}N–^{1}H HSQC spectra were recorded of ^{15}N-labelled RTP C110S to which TerI had been added. (*a*) RTP C110S only. (*b*) 6:5 RTP C110S:TerI. (*c*) 7:10 RTP C110S:TerI. All samples contained 10 m*M* phosphate buffer pH 6.0, 700 m*M* NaCl, 25 µ*M* chloramphenicol, 100 µ*M* EDTA, 5% D_2O and 300 µ*M* dimeric RTP C110S.

glycerol. Crystals grew within 3 d to maximal dimensions of approximately 0.25 × 0.07 × 0.07 mm.

2.5. Data collection and processing

Crystals were mounted in a nylon loop and flash-cooled in a stream of liquid nitrogen at 100 K without pre-soaking in further cryoprotectant. A complete data set was collected at beamline 14-BMC at the Advanced Photon Source (Argonne National Laboratories, Argonne Il, USA) at a wavelength of 0.90 Å and was recorded using an ADSC Quantum 315 CCD detector. The crystal was exposed for 20 s and rotated through a 0.25° oscillation per frame at a crystal-to-detector distance of 250 mm. Indexing and recording of reflection intensities was carried out with *DENZO* and the data were scaled and merged in *SCALEPACK* from the *HKL*-2000 program suite (Otwinowski & Minor, 1997). Data-collection and processing statistics are summarized in Table 1.

3. Results and discussion

The crystal structures of both free RTP and RTP in complex with B-site DNA have fuelled debate over how the apparently symmetric RTP–TerI complex is assembled and how it can function in a polar manner (Bussiere *et al.*, 1995; Wilce *et al.*, 2001). As yet, no structure of the functional RTP–TerI complex exists and attempts to produce it have been complicated by the instability in solution of RTP at high concentration. In this study, we have utilized an RTP C110S mutant that has been shown to be more stable in solution at the concentration required for crystallization (Vivian *et al.*, 2003). As in previous preparations of RTP–DNA complexes, the formation of the RTP C110S–TerI complex was to be tracked by an HSQC NMR titration experiment in order to determine the molar ratio at which the complex was fully formed (Vivian *et al.*, 2001; Hastings *et al.*, 2005). The cross-peaks on the resultant NMR spectra were too broad to be accurately interpreted, as may be expected in the formation of this ∼80 kDa species (Fig. 1). Hence, a ratio of RTP C110S to duplex TerI DNA of 2:1 was chosen as an estimate of the fully bound complex. Although the NMR spectra were not useful in determining the ratio of RTP to DNA required, they did reveal that RTP was interacting with TerI.

The best diffracting crystals were produced from a drop comprising 2 µl protein solution and 1 µl reservoir. Combined with the addition of glycerol to 10%, this had the effect of producing fewer yet larger crystals per drop. The crystals diffracted very weakly and data could not be collected using an in-house rotating-anode X-ray generator, instead requiring a synchrotron source. A data set was collected to 3.9 Å resolution from a single crystal to 97.0% completeness. The overall R_{sym} was 12%. The diffraction data were consistent with the space group *P*622, with unit-cell parameters $a = b = 118.8$, $c = 142.6$ Å.

The functional terminator complex is composed of two homodimers of RTP bound to two overlapping sites on Ter. An estimate of the solvent content assuming the asymmetric unit to contain the complete 37-base-pair TerI with one dimer of RTP is ∼54% (V_M = 2.7 Å^3 Da^{-1}) and with two dimers bound is ∼32% (V_M = 1.8 Å^3 Da^{-1}) (Matthews, 1968). Both these values fall into the range observed for protein crystals (Kantardjieff & Rupp, 2003). Molecular-replacement methods will be used to determine the structure of the RTP–TerI complex.

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