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Crystallization and preliminary X-ray crystallographic and electron microscopic study of a bacterial DNA helicase (RSF1010 RepA)

DIETMAR RÖLEKE,[a] HELGA HOIER,[a]* CORNELIA BARTSCH,[a] PATRICK UMBACH,[a] EBERHARD SCHERZINGER,[b] RUDI LURZ[b] AND WOLFRAM SAENGER[a] at [a]*Institut für Kristallographie, Freie Universität Berlin, Takustrasse 6, 14195 Berlin, Germany, and* [b]*Max-Planck-Institut für Molekulare Genetik, Ihnestrasse 73, 14195 Berlin, Germany*

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Abstract

Helicases are ATP-driven enzymes essential for DNA unwinding. The broad host range plasmid RSF1010 harbours a gene (*repA*) encoding for one of the smallest known oligomeric helicases, RepA, a homo-hexamer with 30 kDa subunits. Electron micrographs indicate that the overall shape of RepA resembles a hexagon with globular monomers at the corners, diameter 140 Å, and a central channel. Below pH 6, the molecules aggregate into tubular structures. The enzyme has been purified and crystallized using the hanging-drop vapour-diffusion method with polyethyleneglycol monomethylether as precipitating agent. The crystals exhibit the monoclinic space group $P2_1$ with unit-cell parameters $a = 105.8$, $b = 180.3$, $c = 115.4$ Å, $\beta = 95.2°$, and diffract to 3.5 Å resolution using rotating-anode Cu $K\alpha$ radiation. Assuming two 180 kDa molecules per asymmetric unit, the volume per unit weight is $V_m = 3.06$ Å^3 Da^{-1}, equivalent to a solvent content of 60%. A self-rotation search indicates that the sixfold axis of the hexamer is parallel to the *ac* plane and inclined at about 2° to the *c* axis. The two hexamers are oriented head-to-head with point-group symmetry D_6.

1. Introduction

Helicases are a family of enzymes involved in DNA and RNA unwinding processes (Matson & Kaiser-Rogers, 1990) and consequently play an essential role in replication, transcription, recombination and DNA repair. Since these are basic biological processes, the helicases are present in all organisms. The unwinding of ds-DNA to ss-DNA by hydrogen-bond breaking is fuelled by ATP hydrolysis. In general, single-stranded regions of DNA are required for helicase binding and initiation of unidirectional unwinding of the double helix. For most helicases the unwinding is strictly processive in the 5′ to 3′ direction. In a few cases, however, a reverse directionality has been observed (Matson & Kaiser-Rogers, 1990).

Insight into oligomer association and stabilization was obtained by protein cross-linking, sedimentation equilibration using analytical ultracentrifugation, fluorescence spectroscopy to follow the binding of ligand molecules, and small-angle X-ray scattering to study the association state as a function of protein concentration (Geiselmann, Yager, Gill, Calmettes & von Hippel, 1992; Patel & Hingorani, 1993; Bujalowski, Klonowska & Jezewska, 1994).

The three-dimensional structures of several helicases have been investigated by electron microscopy. In all these studies the enzyme was found to be comprised of either six or twelve identical subunits. In the case of *E. coli* DnaB, the protein is present as a triangle-shaped oligomer, composed of a trimer of dimers with point-group symmetry D_3 (San Martin, Stamford, Dammerova, Dixon & Carazo, 1995), while bacteriophage T7 gene 4 (Egelman, Yu, Wild, Hingorani & Patel, 1995), bacteriophage T4 (gp41) (Dong, Gogol & von Hippel, 1995) and RSF1010 RepA helicases (Scherzinger, in preparation) are ring-shaped hexamers with a central channel and point group C_6. RuvB (Stasiak *et al.*, 1994) is constituted by two hexameric rings in a bipolar directionality, point group D_6. In all these structures ss-DNA is assumed to pass through the central channels of the protein complexes. Helicase Rho unwinds RNA–DNA hybrids and occurs as a homo-hexameric structure with D_3 symmetry. Although the monomers are identical, they bind ATP and RNA with different affinities, three high and three low, suggestive of anticooperativity (Geiselmann, Wang, Seifried & von Hippel, 1993).

Despite extensive biochemical investigations of helicases in both eukaryotes and prokaryotes, the unwinding mechanism is not yet known in detail. Binding studies with the dimeric Rep helicase of *E. coli* (Amaratunga & Lohmann, 1993) favour an active rolling mechanism for DNA unwinding in which the dimeric protein is simultaneously bound to the 3′ ss-DNA and the ds-DNA. Different mechanisms with DNA passing through the central channel and RNA binding at the circumference have been proposed for the hexameric DnaB (San Martin *et al.*, 1995) and Rho (Geiselmann *et al.*, 1993) helicases, respectively.

Here, we report the purification, electron-microscopy study, crystallization and preliminary crystallographic characterization of RepA, a DNA helicase coded for by plasmid RSF1010 (Scherzinger, Haring & Otto, 1991). It is a homo-hexameric protein required for plasmid replication, and, with a molecular mass of 30 kDa per subunit, is one of the smallest helicases found so far. Elucidation of the three-dimensional structure by X-ray crystallography will give insight into the architecture of this enzyme at atomic level and provide a basis to investigate the mechanism of DNA unwinding by a combination of structural, biochemical and molecular biological studies.

2. Experimental

2.1. *Expression and purification*

RepA helicase was purified from *E. coli* XL1-Blue (Stratagene) cells harboring ptacREP-A, a multicopy plasmid in which the coding sequences of RepA are expressed from promoter P*tac* (Scholz *et al.*, 1989). Expression of RepA protein was induced by addition of isopropyl β-D-thiogalactoside (IPTG) to 0.4 m*M*. Cells were harvested 12 h after induction and suspended in lysozyme (0.5 mg ml^{-1}) containing buffer [10 m*M* Tris–HCl, pH 8.0, 200 m*M* NaCl, 5 m*M* EDTA, 100 µl saturated PMSF (phenylmethylsulfonyl fluoride) solution]. After sonication, cell debris was removed

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by centrifugation at 30 000 rev min^{-1} at 278 K for 90 min. The supernatant was loaded on a Pharmacia DEAE-Sephacel column and eluted with a linear salt gradient of buffer systems *A* (20 m*M* Tris–HCl, pH 8.0, 10% glycerol, 0.1 m*M* EDTA, 50 m*M* NaCl) and *B* (20 m*M* Tris–HCl, pH 8.0, 10% glycerol, 0.1 m*M* EDTA, 2 *M* NaCl). After dialysis against buffer *A* the sample was loaded on a Sigma Heparin–agarose column and eluted with buffer systems *A* and *B* as described above, and further purified by gel filtration on Pharmacia Sephacryl S200. In the crystallization experiments the protein was concentrated in a centriprep concentrator tube to 10 mg ml^{-1} in 20 m*M* Tris–HCl, pH 8.0, 10% glycerol, 0.1 m*M* EDTA and 150 m*M* NaCl.

For electron microscopy, the RepA protein concentration was adjusted to 50 µg ml^{-1} in 40 m*M* MES buffer containing 10 m*M* NaCl, 10 m*M* $MgCl_2$, 1 m*M* DTT and 5 µg ml^{-1} BSA; the pH ranged from 5.1 to 7.0. Samples were negatively stained with 2% uranyl acetate according to Valentine, Shapiro & Stadtman (1968).

2.2. *Crystallization*

Crystals were grown by the hanging-drop vapour-diffusion method (McPherson, 1982). In the drops, 3 µl protein stock solution were mixed with 3 µl reservoir solution containing 20–22%(*w*/*v*) polyethyleneglycol monomethylether 5000, 2–5%(*v*/*v*) 2-propanol, 0.1 *M* citrate buffer, pH 6.0. Prismatic crystals, 0.4 × 0.5 × 0.2 mm, grew within one week at 291 K (Fig. 1).

X-ray diffraction data have been collected at room temperature on a MAR Research image-plate area detector using graphite-monochromated Cu $K\alpha$ radiation produced with an Enraf–Nonius FR-571 rotating-anode generator running at 45 kV, 90 mA, using a 3 mm focus cup.

3. Results and discussion

The electron micrographs of RSF1010 RepA molecules (Fig. 2) indicate that at neutral pH the helicase has the form of a hexamer, in agreement with gel filtration and cross-linking studies of the native enzyme (Scherzinger, in preparation). The hexamer, with longest diameter about 140 Å, is composed of globular subunits and appears to have a central channel. At pH 5.6, the hexamers aggregate like coins in a roll into tubular structures. This permits the height of the hexamer to be determined as 57 Å. There is no indication whether the hexamers in the tubules are oriented head-to-head or head-to-tail.

RepA crystallizes in the monoclinic space group $P2_1$ with unit-cell dimensions $a = 105.8$, $b = 180.3$, $c = 115.4$ Å, $\beta = 95.2°$. On a rotating-anode X-ray source the native crystals diffract to 3.5 Å resolution; using synchrotron radiation (beamline X11, EMBL at DESY, Hamburg) diffraction extends beyond 2.9 Å. A data set to 4.5 Å resolution was collected in-house at room temperature and processed using *DENZO* and *SCALEPACK* (Otwinowski, 1993).

For cryo-experiments the crystals were soaked in reservoir solution containing 20% glycerol, prior to flash-freezing in a nitrogen stream at 100 K. Under these conditions X-ray diffraction data have been collected at the ESRF Grenoble beamline BL19, equipped with a CCD detector. After processing using *DENZO* and *SCALEPACK*, the completeness of the data set was 95% at 3.5 Å resolution with $R_{sym} = 0.094$. Due to cell shrinkage, unit-cell dimensions were $a = 102.5$, $b = 178.9$, $c = 110.6$ Å, $\beta = 95.5°$.

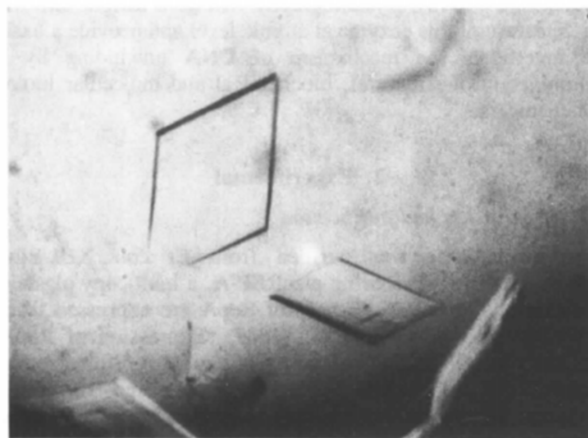
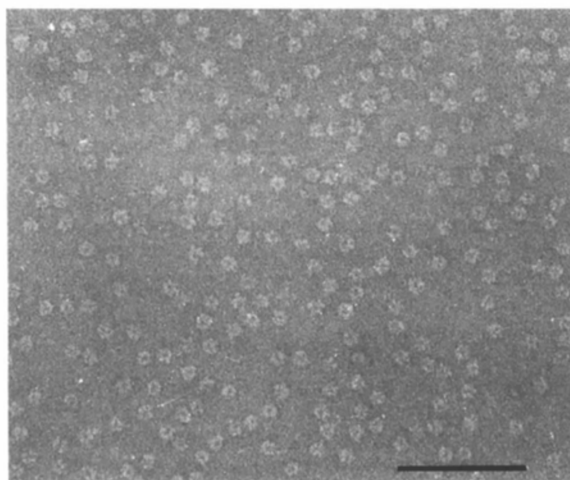


Fig. 1. Crystals of helicase RepA. The long diameter of the largest crystal is 0.6 mm.



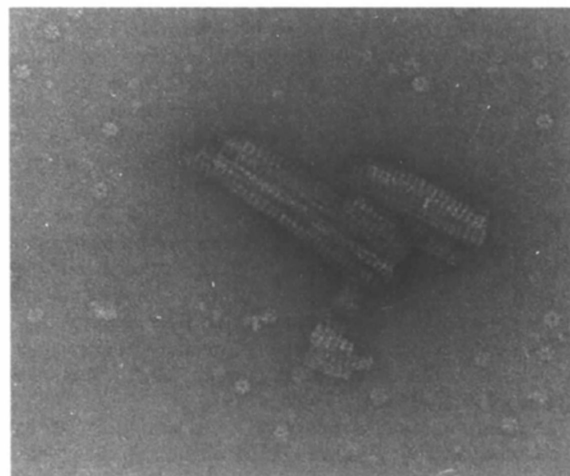


Fig. 2. Electron micrographs of helicase RepA at pH 7.0 showing hexameric structures (top), and at pH 5.6 where tubular arrangements of the protein are formed. The bar represents 100 nm.

Assuming two hexamers per asymmetric unit, the volume per unit weight is 3.06 Å^3 Da^{-1}, which is within the range of values compiled by Matthews (1968). In addition, density measurements by floating crystals in a Ficoll gradient (Westbrook, 1985) yielded $D = 1.175$ g cm^{-3}, which is again consistent with two hexameric molecules per asymmetric unit ($D_{\rm theor} = 1.155$ g cm^{-3} for two hexamers, $D_{\rm theor} = 1.078$ g cm^{-3} for one hexamer).

A self-rotation function was calculated using *POLARRFN* of the *CCP4* package (Collaborative Computational Project, Number 4, 1994) with a radius of integration of 30 Å and data in the resolution shell from 12 to 5 Å. Since we expected a sixfold symmetry, as indicated by the electron micrographs, sections with κ values of 60, 120 and 180° have been inspected. At all three κ sections, peaks appear at $\varphi = 7°$, $\psi = 90°$; in addition the $\kappa = 180°$ section exhibits six peaks along ψ, spaced at regular intervals of 30° (Fig. 3). This arrangement indicates the presence of a molecule with a sixfold rotation axis perpendicular to the crystallographic b axis and inclined at 2° to the c axis, and suggests an overall D_6 symmetry. This means that two RepA hexamers are arranged head-to-head. A head-to-tail orientation would not give rise to the six twofold axes.

It is questionable whether this dimer of hexamers is of biological significance since, under physiological conditions, RepA occurs as a homo-hexamer as shown by gel filtration and chemical cross-linking studies (Scherzinger, in preparation). However, under slightly acidic conditions (pH 5 to 6), the hexamers have a tendency to aggregate, forming the tubular structures shown in Fig. 2. Since crystallization required pH 6.0, the formation of dimers of hexamers might be a result of these conditions.

A schematic description of the possible packing of RepA molecules in the crystal unit cell is given in Fig. 4. The dimer of hexamers occupies the asymmetric unit such that its hexagonal axis is almost (at 2°) colinear with the c axis. The length of the c axis (110.6 Å at 100 K and 115.8 Å at room temperature) closely corresponds to the thickness of two hexamers, $2 \times 57 = 114$ Å, as derived from electron micrographs. We assume that the length of the a axis (102.5 Å at 110 K and 105.8 Å at room temperature) reflects the diameter of the hexagon; this is 25% less than the value of 140 Å estimated from electron micrographs. We associate this difference with artifacts due to negative staining.

At present, attempts to solve the three-dimensional structure by single or multiple isomorphous replacement techniques are in progress.

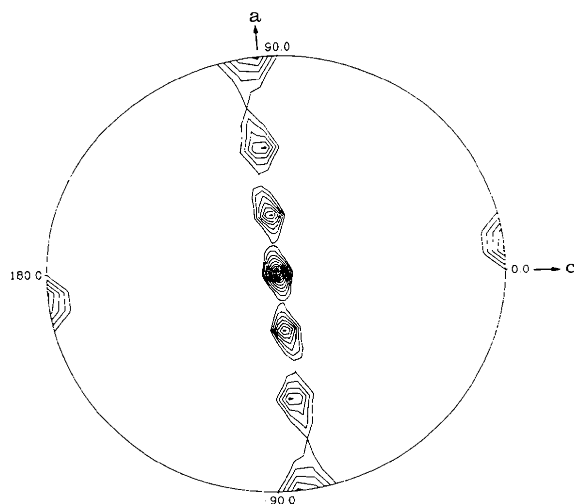


Fig. 3. A plot of the self-rotation function at section $\kappa = 180°$ with the crystallographic b axis perpendicular to the plane of projection.

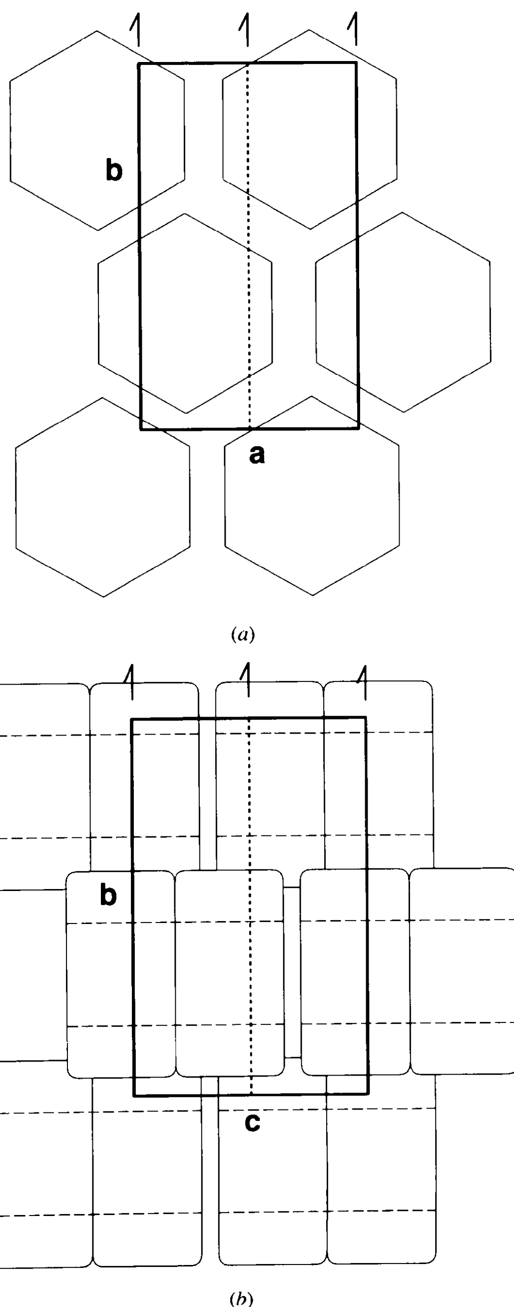


Fig. 4. Possible packing of the dimers of RepA hexamers in the crystal unit cell. Views are on (*a*) the *ab* plane, and (*b*) the *bc* plane; twofold screw axes are indicated.

4. Conclusions

Chemical cross-linking and gel filtration had indicated that RepA occurs as a homo-hexamer at neutral pH, and electron micrographs of RepA molecules showed a hexagonal structure with a central channel, composed of six subunits. It was not clear from these studies whether the organization of the monomers is rotational symmetric or whether RepA consists of a trimer of dimers as observed for helicases DnaB (San Martin *et al.*, 1995) and Rho (Geiselmann *et al.*, 1993). This ambiguity has been resolved by the self-rotation function which clearly indicates the presence of a sixfold rotation axis within the molecule so that the six monomers are identical to each other, in agreement with biochemical data.

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