

RecBCD enzyme is a bipolar DNA helicase

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Supplementary information

Supplementary Figure Legend

The ATP analogues, AMP-PNP and ATP γ S, are competitive inhibitors of the ^{his}RecD ATPase. Steady state ATPase rates were determined in the presence of increasing quantities of AMP-PNP (a) or ATP γ S (b) as described in Supplementary Methods. The lines represent a fit of the data to $V = V_0 - V_0 \cdot [I] / (K_{app} + [I])$. For AMP-PNP, V_0 was $2.4 \pm 0.1 \text{ s}^{-1}$, and K_{app} was $1.09 \pm 0.16 \text{ mM}$; for ATP γ S, V_0 was $2.4 \pm 0.1 \text{ s}^{-1}$, and K_{app} was $0.06 \pm 0.007 \text{ mM}$.

Supplementary Methods

^{his}RecD protein cloning, expression and purification. ^{his}RecD protein was expressed using the pET vector system (Novagen). The entire *recD* gene was amplified by polymerase chain reaction from a pPB100 template plasmid¹ using primers flanked by *Nde*I and *Bam*HI sites and the Expand Hi-Fi PCR system (Roche Molecular). The PCR product was cut and ligated into the pET24a vector. The gene was subsequently subcloned into a pET15b construct by moving the complete gene using the *Nde*I and *Bam*HI sites. This construct contains a 20 amino acid N-terminal fusion that includes a histidine tag and a thrombin cleavage site. The entire coding region of the pET15b construct and its promoter were sequenced to confirm the integrity of the gene after PCR (DBS DNA sequencing facility, UC Davis). The *recD* nucleotide sequence was

identical to that found in the *E. coli* K12 complete genome (Accession number NC_000913). The K177Q mutant RecD expression plasmid was produced by site directed mutagenesis of the wild type construct using the QuikChange kit (Stratagene) according to manufacturer's instructions. The sequences of the primers used for mutagenesis were 5'-CCT GGC ACC GGT CAA ACG ACC ACC GTA GCG and its complementary strand, and these were obtained HPSF-purified from MWG-Biotech. The successful introduction of the desired mutation, and the integrity of the remainder of the gene was confirmed by DNA sequencing.

For expression, the *E. coli* BL21 (DE3) cell line (Novagen) was transformed with the pET15b-^{his}RecD construct. Cells were grown at 37°C to mid-log phase, induced with 1 mM IPTG, and then grown for a further three hours. A protein of the expected size (slightly larger than RecD due to the histidine-tag leader) was well expressed in the form of inclusion bodies. Cells were collected by centrifugation and resuspended in 50 mM Tris-Cl pH 7.5, 200 mM NaCl, 2 mM EDTA, 1 mM DTT and 10% sucrose. Cells from a total culture of 12 litres were resuspended in 200 ml of this buffer and stored at -80°C as 4 x 50 ml aliquots. A 50 ml aliquot of cells was thawed and sonicated on ice. An insoluble pellet was recovered by centrifugation and resuspended in isolation buffer (2M urea, 20 mM Tris-Cl, 0.5 M NaCl, 2% Triton X-100, pH 8.0). Sonication was repeated as above. Again an insoluble pellet was collected and washed with isolation buffer followed by sonication. Finally, an insoluble pellet was collected and resuspended in 100 ml of solubilization buffer (6 M guanidinium HCl, 20 mM Tris-Cl, 0.5 M NaCl, 10 mM imidazole, 1 mM BME, pH 8.0). The resuspension was stirred for 60 minutes at room temperature, and then centrifuged at 40K rpm for 40 minutes to remove insoluble matter. The supernatant was passed through a 0.45 micrometer filter

and stored at –80°C until required. RecD protein was purified using a 20ml Fast-Flow Ni²⁺-chelating sepharose column (AP-Biotech). All chromatographic steps were performed at 4°C. The column was equilibrated in solubilization buffer, the sample loaded, and the column washed with several column volumes of solubilization buffer. The column was then washed with ~5 column volumes of refolding buffer (6 M urea, 20 mM Tris-Cl, 0.5 M NaCl, 5 mM imidazole, 1 mM BME, 0.1% tritonX-100, pH 8.0). RecD was refolded on the column with a 500 ml gradient at 4 ml/min from refolding buffer to wash buffer (20 mM Tris-Cl, 0.5 M NaCl, 10 mM imidazole, 1 mM BME, 0.1% Triton X-100, pH 8.0). RecD was eluted with a 200 ml gradient at 4 ml/min from wash to elution buffer (20 mM Tris-Cl, 0.5 M NaCl, 0.5 M imidazole, 1 mM BME, 0.1% Triton X-100, pH 8.0). Peak fractions were pooled and dialysed extensively against storage buffer (20 mM Tri-Cl pH 7.5, 0.5 M NaCl, 0.1% tritonX-100, 10% glycerol, 0.1 mM DTT). After dialysis, the dialysate was slightly cloudy. To remove insoluble aggregates, the dialysate was centrifuged at 40K rpm for 40 minutes. The supernatant was aliquoted, snap frozen in liquid nitrogen, and stored at –80°C. Triton X-100 and glycerol resulted in a high storage buffer OD₂₈₀ and so ^{his}RecD concentration was determined with BCA and BioRad protein assay kits using BSA (Pierce) as a standard. These estimates were in good agreement with each other and with those from SDS-PAGE analysis.

ATP hydrolysis competition assays. ATP hydrolysis was measured spectrophotometrically by coupling to NADH oxidation². Assays were performed using 50 nM ^{his}RecD at 20 °C in a buffer containing 20 mM Tris-Cl (pH 7.5), 50 mM NaCl, 3 mM MgCl₂, 4 mM DTT, 35 U/ml pyruvate kinase, 20 U/ml lactate dehydrogenase, 2 mM phosphoenolpyruvate, 0.2 mg/ml NADH, 2 mM (saturating) ATP, 100 μM

(nucleotides) poly(dT) ssDNA, and the indicated concentration of either AMP-PNP or ATP γ S. Data were analysed using the program GraphPad Prism 3.02 (GraphPad Software). The rates of ATP hydrolysis were plotted as a function of the inhibitor concentration, and the data were fit, using GraphPad Prism 3.02 (GraphPad Software), to the following equation:

$$V = V_0 - V_0 \cdot [I] / (K_{app} + [I])$$

where V_0 is ATPase rate in the absence of the inhibitor, $[I]$ is the inhibitor concentration (AMP-PNP or ATP γ S), and K_{app} is the observed inhibition constant at the given ATP concentration.

Supplementary Methods References

1. Boehmer, P. E. and Emmerson, P. T. *Escherichia coli* RecBCD enzyme: inducible overproduction and reconstitution of the ATP-dependent deoxyribonuclease from purified subunits. *Gene* **102**, 1-6 (1991).
2. Kreuzer, K.N. and Jongeneel, C.V. *Escherichia coli* phage T4 topoisomerase. *Methods Enzymol.* **100**, 144-160 (1983).