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Purification and characterization of *Thermus thermophilus* UvrD

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Abstract The DNA helicase UvrD (helicase II) protein plays an important role in nucleotide excision repair, mismatch repair, rolling circular plasmid replication, and in DNA replication. A homologue of the *Escherichia coli uvrD* gene was previously identified in *Thermus thermophilus*; however, to date, a UvrD helicase has not been purified and characterized from a thermophile. Here we report the purification and characterization of a UvrD protein from *Thermus thermophilus* HB8. The purified UvrD has a temperature range from 10° to >65°C, with an optimum of 50°C, within the temperature limits of the assay. The enzyme had a requirement for divalent metal ions and nucleoside triphosphates which related to enzyme activity in the order ATP > dATP > dGTP > GTP > > CTP > dCTP > > UTP. A simple real-time helicase assay was developed that should facilitate detailed kinetic studies of the enzyme. Evaluation of helicase substrates using this assay showed that the enzyme was highly active on a double-stranded DNA with 5′ recessed ends in comparison with substrates with 3′ recessed or blunt ends, and supports enzyme translocation in a 3′–5′ direction relative to the strand bound by the enzyme.

Keywords DNA helicase · DNA repair · *Thermus thermophilus* · UvrD

Introduction

DNA helicases are motor proteins, which bind and hydrolyze nucleoside 5′-triphosphates and translocate along DNA, producing the single-stranded DNA intermediates required for replication and repair of the DNA (Matson 1986; Matson and Kaiser-Rogers 1990; van Brabant et al. 2000). DNA helicases are ubiquitous and there can be a number of different helicases in any particular cell. Some redundancy of function may occur, although loss of any one activity can seriously affect the viability of an organism (Matson and Kaiser-Rogers 1990). One of the best characterized helicases is the 82-kDa product of the *Escherichia coli uvrD* gene, whose product UvrD unwinds both DNA duplexes and DNA/RNA hybrids in an ATP-dependent reaction with 3′–5′ polarity and a "step size" of 4 or 5 base pairs (Ali and Lohman 1997; Matson 1986; Matson and Kaiser-Rogers 1990). Mutations in *uvrD* result in a pleitrophic phenotype. UvrD helicase plays an important role in DNA repair as part of the nucleotide excision repair pathway. UvrD-deficient cells are sensitive to DNA damaging agents and are affected in several aspects of DNA metabolism (Caron et al. 1985). The function of UvrD in nucleotide excision repair is to remove the short section of lesion containing DNA that is delimited by the incision sites created through the action of UvrABC exinuclease (Sancar 1996). UvrD also plays a role in the mismatch repair pathway (Lahue et al. 1989), where, after being loaded on the DNA by MutL (Mechanic et al. 2000), it unwinds the error-containing DNA, which is subsequently digested by exonuclease action (Modrich and Lahue 1996). UvrD also plays an important role in (1) DNA replication as part of a pathway which maintains cell viability in the absence of DNA polymerase I (Moolenaar et al. 2000), (2) transposon excision (Lundblad and Kleckner 1985), and (3) in the replication of rolling-circle plasmids in *E. coli* (Bruand and Ehrlich 2000). While single mutants of *uvrD* are still viable, double mutants of *uvrD* and the related helicase *rep* are not, suggesting that their activities may overlap. Crystallographic studies of the related helicases Rep and PcrA in their apo form, and in complexes with DNA and nucleotides, suggest that members of this family of helicases act as monomers (Soultanas and Wigley 2000);

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however, to date no crystal structure of a UvrD has been resolved, although *E. coli* UvrD has been shown to interact with itself and to form oligomers in solution (Mechanic et al. 1999). A number of homologues of *E. coli uvrD* have been identified in other bacteria and many DNA helicases have been purified and characterized from mesophilic organisms. However, relatively few have been characterized from thermophilic organisms. Although a homologue of the *E. coli uvrD* gene has been identified by Hiramatsu et al. (1997) in *Thermus thermophilus* (Tth) HB8 and was shown to partially complement the UV sensitivity of an *E. coli uvrD* mutant (Hiramatsu et al. 1997), a UvrD protein from a thermophilic organism has not been characterized. Here we report the purification and characterization of the Tth UvrD helicase.

Materials and methods

Cloning, overexpression, and purification of Tth UvrD

Oligonucleotide primers used for amplification of the Tth *uvrD* gene were designed using the published sequence of the Tth *uvrD* (Hiramatsu et al. 1997). The forward primer UvrD-1 (5′-gactctccatatgagcgacgccctcctagccccctcaac-3′) was designed to include a 5′ *Nde*I site, with the ATG of the *Nde*I site acting as the start codon of the open reading frame in the amplified *uvrD* gene. This ATG codon replaces the predicted valine start codon (GTG) of the native gene (Hiramatsu et al. 1997). The reverse primer for the gene UvrD-2 (5′-actatcgctcttccgcatgccggcttaagctccgcgtact-3′) was designed to include a *Sap*I site. The PCR reaction mixture (20ZZZ;µl) contained 40 ng of UvrD-1 and UvrD-2 primers; 0.2 mM each of dATP, dCTP, dGTP, and dTTP; 2 µl of 10× Expand High Fidelity buffer; 2.5 mM $MgCl_2$, 1U Expand High Fidelity DNA polymerase (Roche Molecular Biochemicals) and 100 ng *T. thermophilus* HB8 genomic DNA (ATCC). A "hotstart" PCR was performed and the reaction was cycled as follows (95°C × 1 min, 62.5°C × 1 min, 72°C × 2 min) × 10 cycles; [95°C × 1 min, 62.5°C × 1 min, 72°C × (2 min + 20 s/cycle)] × 25 cycles; 72°C × 10 min. An amplified product of the anticipated size (2,103 bp) was generated and excised from a 1% agarose gel and purified by Geneclean (Bio101). The purified PCR fragment was subcloned into the pGem-T-Easy vector according to the manufacturer's instructions (Promega). The Tth *uvrD* gene was subsequently subcloned from the pGem-T Easy vector into the pTYB1 vector (New England Biolabs) as an *Nde*I/*Sap*I restriction fragment and transformed into *E. coli* ER2566 by electrophoration.

One liter of LB medium containing (50 µg/ml ampicillin) was inoculated with 10 ml of an overnight culture of *E. coli* ER2566 harboring a pTYB1–Tth *uvrD* construct and grown to an OD_{600} of 0.6. Overexpression of the fusion protein was then induced by adding IPTG to a final concentration of 1 mM for 3 h. After induction, cells were isolated by centrifugation at 5,000 *g* × 10 min and resuspended in 20 ml buffer A (20 mM Hepes-KOH, pH 8.0, 500 mM NaCl, 0.1% Triton-X-100). From this point onward, solutions were kept at 4°C. The cells were lysed by sonication (4×10-s bursts). The crude lysate was clarified by centrifugation at 12,000 *g* for 30 min and the supernatant was loaded onto a 5-ml column of chitin beads (New England Biolabs) pre-equilibrated in buffer A. The column was washed with 100 ml of buffer A, followed by 30 ml of buffer B (20 mM Hepes-KOH, pH 8.0, 1 M NaCl). The column was then washed with 10 ml of buffer C (20 mM Hepes-KOH, pH 8.0, 50 mM NaCl) containing 100 mM DTT and plugged; 200 µl of 1 M DTT was then added to the column. Following incubation for 16 h at 4°C, the native Tth UvrD was eluted in 1-ml fractions from the column with 15 ml of buffer C. The concentration of protein in the eluted fractions was estimated. 20 µl of each fraction was analyzed by 7.5% SDS-PAGE. BSA was added to fractions bearing the purified Tth UvrD to a final concentration of 1 mg/ml and aliquots were frozen in liquid nitrogen and stored at –70°C.

Preparation of helicase substrate

A helicase substrate was prepared using a 57-mer oligonucleotide (5′-cggccagtgccaagcttgcatgcctgcaggtcgactctagaggatccccgggtaccg-3′) and a complementary 110-mer oligonucleotide (5′-ctctcactcactcatctactcgggccggtacccggggatcctctagagtcgacctgcaggcatgcaagcttggcactggccgagctcgagttcgtaatcatggtcatagc-3′). Oligonucleotides were gel-purified and, when annealed, produced a partial duplex DNA substrate consisting of a centrally located 57-b-p double-stranded region, a 25-base 5′ overhang and a 28-base 3′ overhang. 1.5 µg of the 57-mer was end-labeled using 20 units of T4-polynucleotide kinase (New England Biolabs), γ-^{32}P ATP (0.66 mM, 2 Ci/l), kinase buffer in a 30 µl reaction at 37°C for 30 min. Nucleotides were then removed from the reaction mix using a centri-spin^{-10} column (Princeton Separations). The labeled 57-mer and the 110-mer were mixed in equimolar quantities in 10 mM Tris-HCl, pH 7.5–8.0, 50 mM NaCl, 1 mM EDTA in a final reaction volume of 100 µl. The reaction was heated to 100°C for 2 min and then cooled to 20°C at a rate of 1°C/min. Substrates with 5′ and 3′ blunt ends, a 5′ overhang at both ends and a 3′ overhang at both ends were prepared as outlined using the following complementary oligonucleotides respectively: 5′-gtgattgccgtataccgctatctgccaagtacagtctcgatctcagcaaccatgtc-3′ and 5′-gacatggttgctgagatcgagactgtacttggcagatagcggtatacggcaatcac-3′; 5′-ttttttttttttttttttttttttttgtgattgccgtataccgctatctgccaagtacagtctcgatctcagcaaccatgtc-3′ and 5′-ttttttttttttttttttttttttttttgacatggttgctgagatcgagactgtacttggcagatagcggtatacggcaatcac-3′; 5′-gtgattgccgtataccgctatctgccaagtacagtctcgatctcagcaaccatgtctttttttttttttttttttttttt-3′ and 5′-gacatggttgctgagatcgagactgtacttggcagatagcggtatacggcaatcacttttttttttttttttttttttttttt-3′.

Helicase assay using radiolabeled substrate

Helicase assays contained 25 mM Tris-HCl, pH8.9, 1.5 mM $MgCl_2$, 2.5 mM ATP, 1 ng (13 fmol) purified Tth UvrD, 40–50 ng (850 fmol) radiolabeled substrate in a total volume of 20 µl. The reactions were overlaid with mineral oil and incubated at specified temperatures for 15 min. The positive control reaction was incubated for a further 2 min at 95°C to completely denature the substrate. Reactions were stopped by transferring them to ice and adding a half volume of 3× stop buffer (3% SDS, 120 mM EDTA, 60% glycerol, 0.3% xylene cyanol). Reactions were frozen immediately in liquid nitrogen and stored at –20°C until they were analyzed by nondenaturing electrophoresis on 15% polyacrylamide gels and phosphorimaging (Molecular Dynamics Storm 850) The band intensities were quantified using ImageQuant software. The percentage 57-mer displaced was calculated as follows: %57-mer displaced = (%test – %–ve)/(% + ve – %–ve) where %test is the percentage of labeled DNA in the lane of interest which is single stranded; %–ve is the percentage of labeled DNA in the negative control lane (incubated at the specified temperature and time in the absence of Tth UvrD) which is single stranded; and % + ve is the percentage of labeled DNA in the positive control lane (incubated at the specified temperature and time in the absence of Tth UvrD, and then heated to 95°C for 2 min) which is single stranded.

Real-time fluorescent helicase assay

The constituents of the helicase reactions were the same as outlined for the assay using radiolabeled substrate except that the substrate was not radiolabeled and SYBR Green I (Molecular Probes, catalog no S-7567) was added. Glass capillary tubes (Roche Molecular Biochemicals) were used instead of 0.5-ml reaction tubes and fluorescence was monitored continuously while incubating the reactions at 50°C in a Roche light-cycler. To correct for any loss of fluorescence due to bleaching or changes in temperature, the

fluorescence values at equivalent time points for the test reactions were subtracted from those of the controls.

Results and discussion

Overexpression and purification of Tth UvrD

The *uvrD* gene was amplified from *T. thermophilus* genomic DNA using primers designed from the published sequence [(Hiramatsu et al. 1997) GenBank, EMBL and DDBJ accession no. AB001291]. *Nde*I and *Sap*I restriction sites were incorporated into the upstream and downstream primers, respectively, to allow cloning of the fragment into the pTYB1 expression vector. The first GTG codon of the predicted coding sequence (Hiramatsu et al. 1997) was replaced with an ATG codon, which also formed part of the *Nde*I restriction site. PCR amplification of the *uvrD* open reading frame from *T. thermophilus* genomic DNA yielded the expected 2,103-bp fragment, which was subsequently cloned into pGEM-T Easy vector by TA cloning. The Tth *uvrD* gene was then subcloned into the pTYB1 vector as an *Nde*I/*Sap*I restriction fragment. This placed the *uvrD* gene under the control of the T7 promoter and directly upstream and in frame with the vector sequence coding for the intein–chitin binding domain (ICBD). In this system, the *uvrD* is expressed as a 132.4-kDa fusion protein carrying a C-terminal ICBD tag facilitating affinity purification by chitin affinity chromatography. The design was such that the native Tth UvrD protein could be produced when the intein domain was removed. Following overexpression of the fusion protein, samples from each stage of the purification were analyzed by SDS-PAGE. The 132.4-kDa fusion protein was visible after induction. Following affinity chromatography using chitin beads and removal of the ICBD, the eluted 77.4-kDa Tth UvrD was >95% pure (data not shown).

Helicase substrate design and confirmation of helicase activity of Tth UvrD

The basic helicase substrate consisted of two gel-purified oligonucleotides, an end-labeled 57-mer and a 110-mer, which, when annealed, produced a partial duplex DNA substrate consisting of a centrally located 57-bp double-stranded region. The substrate had both 3′ and 5′ overhangs to allow for either 3′–5′ or 5′–3′ directionality in the enzyme's activity. The conversion of the 5′ end-labeled 57-mer in duplex form to a single-stranded form was monitored by nondenaturing polyacrylamide gel electrophoresis and phosphorimaging. Analysis of the substrate using nondenaturing gel electrophoresis conditions showed that the substrate was stable at higher temperatures. At 70°C or above, magnesium was required to stabilize the substrate, whereas at lower temperatures this effect was diminished or not observed (data not shown). A concentration of 1.5 mM was sufficient to stabilize the substrate without inhibiting the helicase activity. Although the substrate oligonucleotides were gel-purified, the labeled 57/110-mer substrate ran as a main band and a diffuse smear, which probably arose from multiple conformations of the substrate forming that were not resolved under the nondenaturing gel electrophoresis conditions used (Fig. 1A).

To investigate whether the purified protein had helicase activity, the partial duplex 57/110-mer substrate was incubated in helicase buffer in the presence of the purified Tth UvrD at 50°C (Fig. 1A). The percentage displacement of the radiolabeled 57-mer was calculated and plotted versus time (Fig. 1B). The purified enzyme displaced the labeled 57-mer in a dose-dependent manner, reaching a peak displacement of ~65% after ~20 min (Fig. 1A).

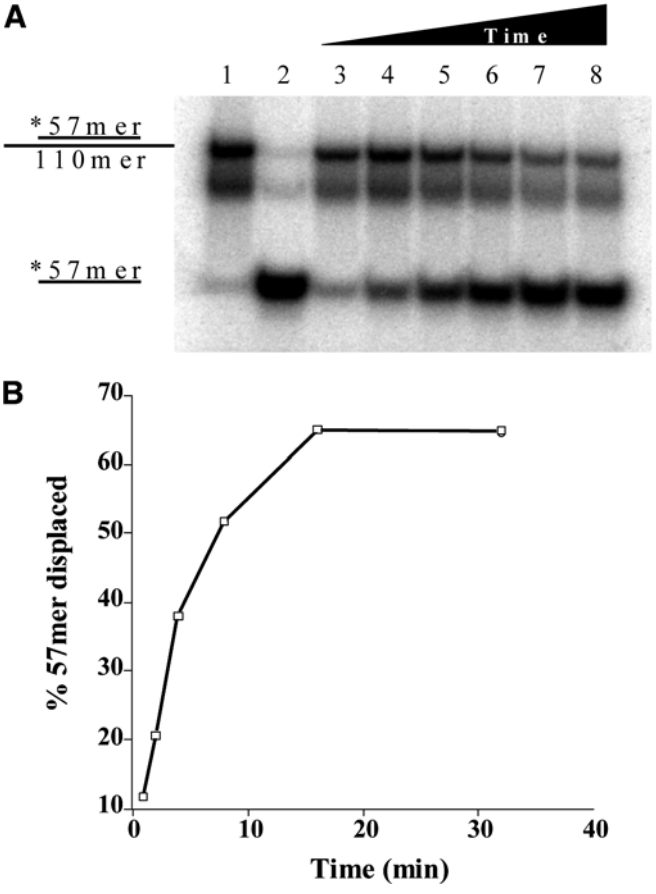


Fig. 1A, B Assay of purified Tth UvrD helicase activity. **A** Purified Tth UvrD (13 fmol) was incubated with the end-labeled 57/110-mer substrate in helicase reaction buffer. Following incubation at 50°C, stop buffer was added and reaction products were analyzed by electrophoresis on a 15% nondenaturing polyacrylamide gel and the end-labeled 57-mer was detected by phosphorimaging. *Lane 1*, reaction mix incubated for 32 min in the absence of Tth UvrD; *lane 2*, as lane 1 but reaction mix was incubated at 95°C for 2 min following the 32-min incubation; *lanes 3–8*, reaction mix incubated with Tth UvrD for 1, 2, 4, 8, 16, and 32 min, respectively. **B** The fraction of labeled 57-mer displaced from the partial duplex 57/110-mer substrate by the Tth UvrD was determined as described, and plotted with respect to time

Temperature optimum of Tth UvrD

To determine the temperature optimum of the Tth UvrD helicase activity, helicase reactions were carried out at temperatures ranging from 0° to 80°C (Fig. 2). The average amount of 57-mer displaced was plotted versus temperature. Maximum and minimum displacement control reactions were carried out at each temperature. Displacement of the labeled 57-mer due to thermal denaturation of the duplex substrate was consistently low at temperatures below 60°C. An increasing amount of denaturation was observed as the temperature was elevated above 60°C. At 75°C the denaturation of the substrate was close to 100%. Thus it was not possible to accurately measure the helicase activity above 70°C. Analysis of the temperature profile of the Tth UvrD under the conditions used showed that its temperature optimum was between 45° and 55°C. However, it is possible that the enzyme has a second temperature optimum at a temperature >70°C but a more stable substrate will be required in order to investigate this possibility. Using the *E. coli* nucleotide excision repair system as a model, the size of the excised fragment of DNA produced by nucleotide excision repair would be expected to be approximately 12–13 nucleotides in length (Sancar 1996). As the melting temperature of such a fragment of DNA would be expected to be well below 75°C, it raises the question as to whether the UvrD is redundant in displacement of the excised DNA in nucleotide excision repair in thermophiles. However, the *E. coli* UvrD also acts to increase the rate of dissociation of UvrC from the repair site and has been found to interact with the UvrB protein. It is possible that these properties of UvrD in thermostable organisms may be more functionally important than its ability to displace DNA in short patch repair per se. The requirement for UvrD activity in mismatch repair may be greater in thermophiles, as the section of DNA excised is large (Modrich and Lahue 1996).

Inhibition by single-stranded DNA

Since the *E. coli* homologue of Tth UvrD has been shown to bind single-stranded DNA, the ability of an excess of single-stranded DNA to inhibit helicase activity was investigated. A single-stranded 20-mer oligonucleotide was used as the competing single-stranded DNA. The percentage 57-mer displaced was plotted versus concentration of the single-stranded 20-mer. The presence of the single-stranded DNA has the effect of lowering the helicase activity on the labeled substrate consistent with the notion that UvrD binds single-stranded DNA transiently (Fig. 3). The inhibition of the helicase activity by ssDNA reaches saturation after a decrease in displacement of ~25%. This suggests that the ssDNA is binding to a site on the helicase, which can potentially reduce the helicase activity to a limited extent. Such a site is likely to be distinct from the active site.

Optimization of helicase buffer

In general, helicases require a divalent metal ion available in solution and also bind and hydrolyze a nucleotide triphosphate. In order to find the optimal conditions for helicase activity, the effect of key reaction mix components on the activity of the enzyme were investigated. To determine the effect of salt concentration on the enzymes activity, the helicase buffer was supplemented with either NaCl or KCl at concentrations ranging from 2.5 to 640 mM. The enzyme tolerated salt concentrations below 40 mM; however, at concentrations above this, the enzyme activity dropped rapidly, with less than 10%

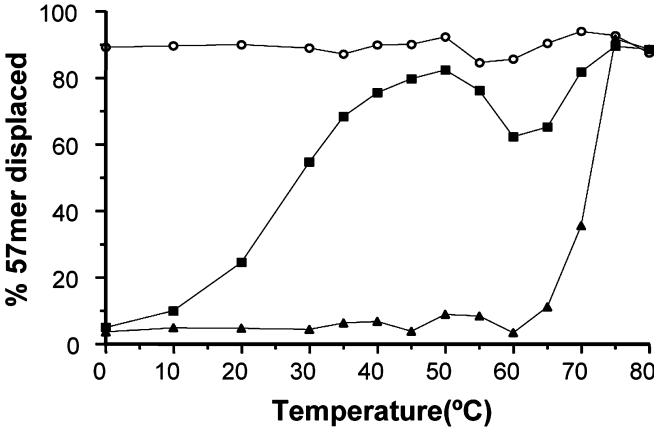


Fig. 2 Temperature profile of Tth UvrD helicase activity. Purified Tth UvrD (13 fmol) was incubated with the partial duplex 57/110-mer substrate in helicase reaction buffer at different temperatures. Percentage displacement of the end-labeled 57-mer was calculated as described. *Filled triangle*, no helicase added; *open circle*, no helicase added but substrate was incubated at 95°C for 2 min (maximum displacement); *filled square*, Tth UvrD helicase added

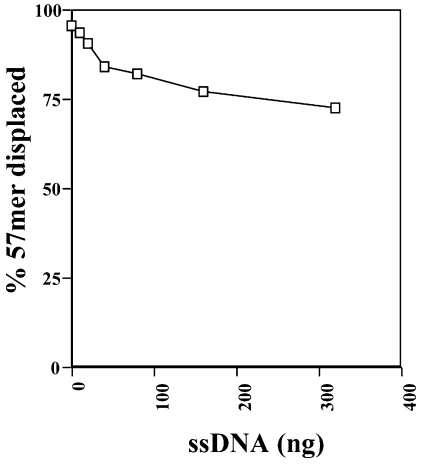
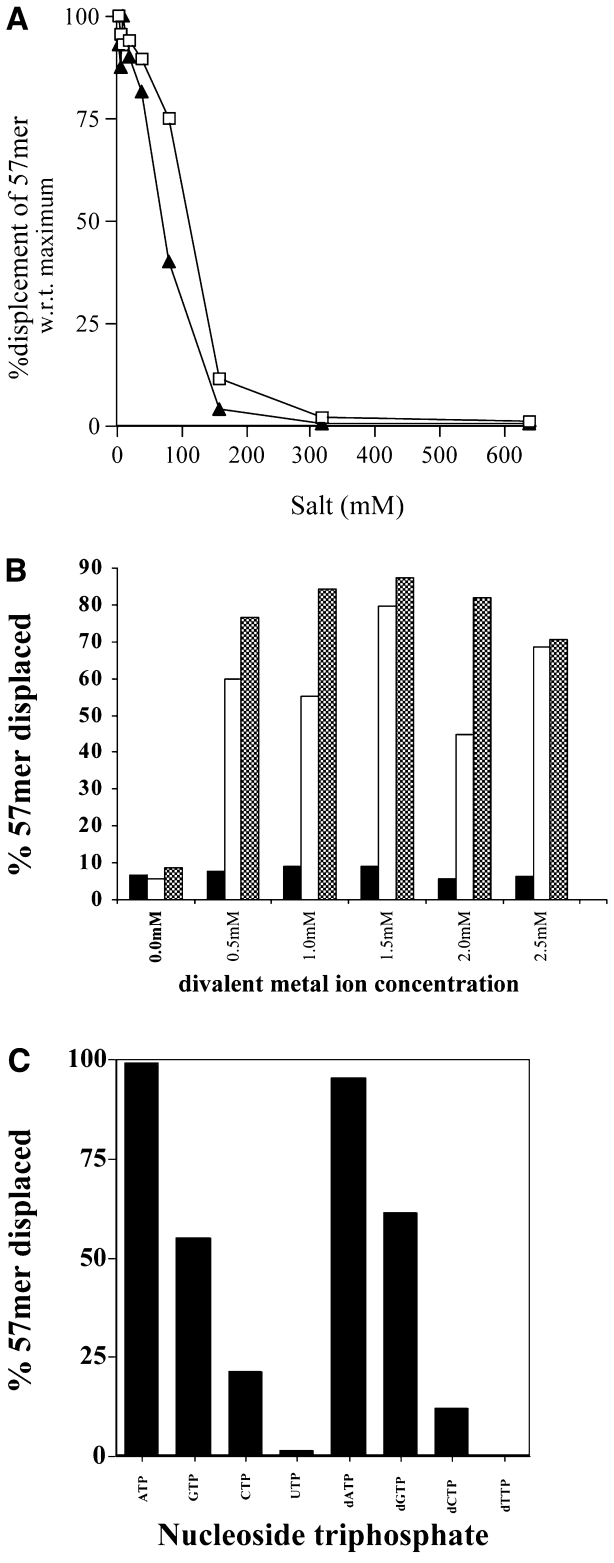


Fig. 3 Effect of single-stranded DNA on TthUvrD activity. Purified Tth UvrD (13 fmol) was incubated with the partial duplex 57/110-mer substrate in helicase reaction buffer at 50°C for 15 min in the presence of increasing amounts of single-stranded 20-mer. The percentage displacement of the end-labeled 57-mer was calculated as described

of activity remaining at 160 mM NaCl. While the enzyme was more tolerant of KCl at lower concentrations, the helicase activity dropped more rapidly at concentrations of KCl above 40 mM than it did for equivalent concentrations of NaCl (Fig. 4A).



To determine the optimal divalent metal ion concentration for the Tth UvrD helicase activity, $MgCl_2$, $MnCl_2$, or $CaCl_2$ was included in helicase reactions at concentrations ranging from 0.0 mM to 2.5 mM (Fig. 4B.). Helicase activity was stimulated most by $MgCl_2$ at a concentration of 1.5 mM. $MnCl_2$ stimulated activity, though not as well as the $MgCl_2$, while $CaCl_2$ had very little effect on the enzyme activity.

To determine the optimal nucleoside triphosphate for Tth UvrD's helicase activity, ATP, GTP, CTP, UTP, dATP, dGTP, dCTP, or dTTP were included in helicase assays at a concentration of 2.5 mM (Fig. 4C). ATP and dATP stimulated the helicase activity most, with ATP being marginally better than dATP. A 40%–50% drop from maximum activity was observed using GTP and dGTP. Both CTP and dCTP stimulated activity poorly. Stimulation by UTP was marginal, while stimulation by dTTP was not significantly evident. This is similar to *E. coli* UvrD, which prefers either ATP or dATP, with the other nucleotides giving much lower stimulation (Matson and Kaiser-Rogers 1990). It was also found that the Tth UvrD's helicase activity in the presence of ATP was not affected by the presence of other nucleotide triphosphates (results not shown).

"Real-time" helicase assay

Traditional helicase assays are monitored by stopping the reaction at specific points and examining the ratio of conversion of double-stranded substrate to single-stranded form by conventional nondenaturing electrophoresis. Detection of the products usually depends on radiolabeling at least one of the strands of DNA. For kinetic studies, acquisition of multiple data points for a single reaction, enabling the profiling of the course of the helicase reaction from start to completion, is desirable. In order to acquire multiple data points for a single reaction, we investigated the possibility of using a double-strand-specific fluorescent dye for development of a real-time helicase assay. A number of real-time helicase assays have been developed (Bjornson et al. 1994; Eggleston et al. 1996; Houston and Kodadek 1994; Raney et al. 1994). Here, we investigated the ability of the fluorescent DNA dye SYBR Green I to track the progress of a helicase assay. SYBR Green I is a non-sequence-specific double-stranded-specific dye, which is

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Fig. 4A–C Reagent requirements for the helicase reaction. Tth UvrD (13 fmol) was incubated with the partial duplex 57/110-mer substrate at 50°C for 15 min in helicase reaction buffer. **A** The reaction buffer was supplemented with either NaCl (*open square*) or KCl (*filled triangle*) over a 2.5–640 mM range. **B** The reaction buffer without $MgCl_2$ was supplemented with 0.0, 0.5, 1.0, 1.5, 2.0, or 2.5 mM of $CaCl_2$ (*black*), $MnCl_2$ (*open*) or $MgCl_2$ (*gray*). **C** The reaction buffer without ATP was supplemented with 2.5 mM of ATP, GTP, CTP, UTP, dATP, dGTP, dCTP, or dTTP. The percentage displacement of the end-labeled 57-mer was calculated as described

thought to bind DNA in the minor groove. Because of the ability of SYBR Green I to specifically detect very low quantities of double-stranded DNA, without causing any apparent inhibition of helicase activity, it compares favorably with other previously described real-time fluorescent helicase assays. SYBR Green I is one of the most sensitive stains available for detection of double-stranded DNA and has a much reduced sensitivity for detection of single-stranded DNA. A large increase in fluorescence is exhibited on DNA binding and the stain has been used extensively for the detection of double-stranded DNA in real-time PCR (Singer and Haugland 1999) (www.probes.com).

In order to evaluate whether SYBR Green I significantly affects helicase activity, helicase assays were performed using radiolabeled 57/110-mer substrate in the presence of varying concentrations of SYBR Green I. The dye had no apparent effect on helicase activity under the conditions investigated (Fig. 5). A 1/10,000 dilution of the commercial stock of SYBR Green I was chosen for use in the real-time helicase assays and a real time helicase reaction was carried out in triplicate. Fluorescence readings were taken at 0.9 s intervals and plotted versus time (Fig. 6A). In the control reaction, a sharp loss of fluorescence was observed as the temperature was increased to reaction temperature (50°C). After this initial sharp drop, the fluorescence intensity value of the control reaction dropped a small amount (1.9 units) during the course of the 21-min reaction. This may be due to bleaching of the dye during this period. By contrast, in the reactions containing UvrD, the fluorescence signal intensity decreased in a dose-dependent manner (Fig. 6A, B). The three reactions, containing equal quantities of Tth UvrD, produced almost identical reaction profiles (Fig. 6A), demonstrating the reproducibility of the protocol.

Substrate preference of Tth UvrD

Helicases can display directionality or polarity in binding single-stranded DNA. If the helicase translocates unidirectionally with respect to the bound DNA, the polarity manifests itself as a preference for a particular form of substrate end i.e., 3′ overhang, 5′ overhang or blunt-ended DNA. To investigate whether Tth UvrD displays such a preference for a particular form of substrate end, three substrates were designed which consisted of two annealed oligonucleotides that formed a 56-bp duplex region with either 25-poly-dT 3′ overhangs, 25-poly-dT 5′ overhangs or blunt ends. Real-time assays were performed using these substrates. Control reactions containing each of the substrates but

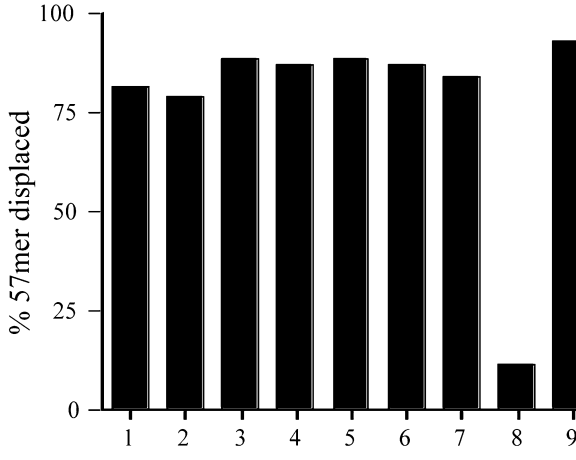

Fig. 5 DNA helicase assay in presence of SYBR Green I. Purified Tth UvrD (13 fmol) was incubated with the partial duplex 57/110-mer substrate in helicase reaction buffer. Following incubation for 10 min at 50°C, stop buffer was added and reaction products were analyzed by electrophoresis on a 15% nondenaturing polyacrylamide gel and detected by phosphorimaging. Reactions were supplemented with SYBR Green I; *1* no SYBR Green I, *2* 1/1000 dilution, *3* 1/5000 dilution, *4* 1/10,000 dilution, *5* 1/15,000 dilution, *6* 1/20,000 dilution, *7* 1/100,000 dilution, *8* no Tth UvrD or SYBR Green I, *9* no Tth UvrD or SYBR Green I and heated to 95°C

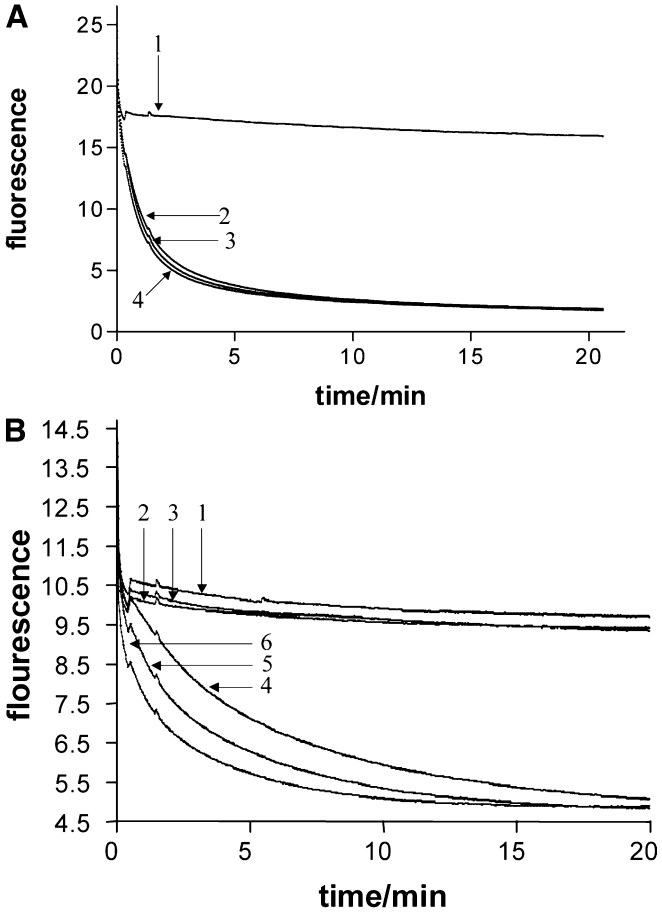

Fig. 6A, B Real-time DNA helicase assay. The partial duplex 57/110-mer substrate was incubated with Tth UvrD in helicase buffer in the presence of a 1/10,000 dilution of SYBR Green I at 50°C. Fluorescence values were measured at 0.9-s intervals for 21 min. **A** *Line 1*, no Tth UvrD added; *lines 2, 3, and 4*, 13 fmol Tth UvrD added. **B** *Lines 1, 2, and 3*, no Tth UvrD added; *lines 4, 5, and 6*, 13, 26, and 45 fmol Tth UvrD added, respectively. Reactions containing no Tth UvrD contained an equivalent volume of Tth UvrD storage buffer without the enzyme

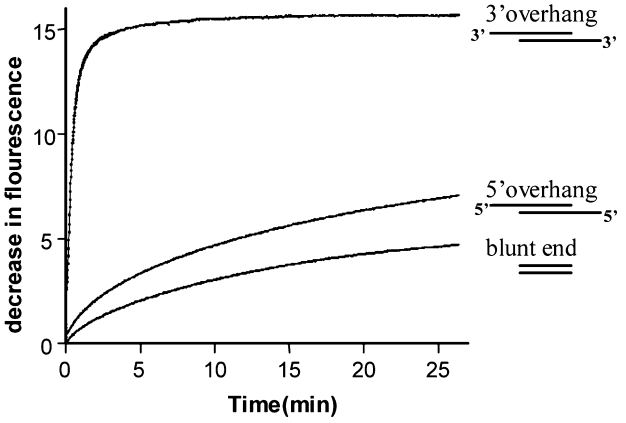

Fig. 7 Effect of DNA substrate ends on Tth UvrD helicase activity. DNA substrates consisting of a 56-bp double-stranded region with a 25-base 3′ overhang, a 25-base 5′ overhang, or blunt ends were incubated with or without Tth UvrD (13 fmol) in helicase buffer in the presence of SYBR Green I (1/10,000 dilution) at 50°C in a light cycler. Fluorescence values were measured at 0.9-s intervals for 26 min. For each substrate type, fluorescence values for reactions containing Tth UvrD were subtracted from the equivalent reactions containing no Tth UvrD, giving a value for the decrease in fluorescence in the presence of Tth UvrD

with an equivalent volume of storage buffer instead of Tth UvrD were also carried out. The fluorescence values for the test reactions containing Tth UvrD were subtracted from those of the control reactions at the respective time points, giving values for the decrease in fluorescence intensity due to Tth UvrD helicase activity. These values were plotted versus time (Fig. 7). Tth UvrD showed a strong preference for the substrate with 3′ overhangs, indicating the enzyme has a 3′ –5′ polarity with respect to the bound DNA strand.

The proteins of thermophiles are in general very stable to denaturants such as heat and solvents, and lend themselves well to biochemical characterization and crystallization studies. The thermostable UvrD protein reported here should prove useful in this regard in refining our knowledge of this family of helicases and DNA repair in *T. thermophilus* and thermophiles in general.

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