Structure of the DNA Repair Helicase Hel308 Reveals DNA Binding and Autoinhibitory Domains*

Received for publication, September 10, 2007, and in revised form, November 2, 2007. Published, JBC Papers in Press, December 4, 2007, DOI 10.1074/jbc.M707548200

Jodi D. Richards 1, Kenneth A. Johnson 1, Huanting Liu, Anne-Marie McRobbie, Stephen McMahon, Muse Oke, Lester Carter, James H. Naismith 2, and Malcolm F. White 3

From the Centre for Biomolecular Sciences, University of St. Andrews, North Haugh, St. Andrews, Fife KY16 9ST, Scotland

Hel308 is a superfamly 2 helicase conserved in eukaryotes and archaea. It is thought to function in the early stages of recombination following replication fork arrest and has a specificity for removal of the lagging strand in model replication forks. A homologous helicase constitutes the N-terminal domain of human DNA polymerase Q. The Drosophila homologue mus301 is implicated in double strand break repair and meiotic recombination. We have solved the high resolution crystal structure of Hel308 from the crenarchaeote Sulfolobus solfataricus, revealing a five-domain structure with a central pore lined with essential DNA binding residues. The fifth domain is shown to act as an autoinhibitory domain or molecular brake, clamping the single-stranded DNA extruded through the central pore of the helicase structure to limit the helicase activity of the enzyme. This provides an elegant mechanism to tune the processivity of the enzyme to its functional role. Hel308 can displace streptavidin from a biotinylated DNA molecule, and this activity is only partially inhibited when the DNA is pre-bound with abundant DNA-binding proteins RPA or Alba1, whereas pre-binding with the recombinase RadA has no effect on activity. These data suggest that one function of the enzyme may be in the removal of bound proteins at stalled replication forks and recombination intermediates.

DNA helicases unwind duplex DNA and are essential components of the DNA replication, recombination, and repair machinery in all cellular organisms and many viruses. DNA helicases utilize the energy released by ATP hydrolysis to undergo conformational cycling and translocate along single-stranded DNA (ssDNA), displacing a duplex DNA strand in the process. Many helicases belong to one of three superfamilies (SF1, 2, and 3), classified according to the conservation of specific sequence motifs (1). SF1 and SF2 helicases possess two motor domains with RecA-like folds that couple ATP hydrolysis to DNA translocation (2). SF2 DNA helicases include RecG in bacteria, hepatitis C virus NS5, and the RecQ family helicases, which all translocate along ssDNA with a 3′ to 5′ polarity (3). The RecQ helicases play a key role in maintaining genomic integrity by stabilizing stalled replication forks and removing intermediates of DNA recombination (4). Previous studies have shown that RecQ proteins target specialized DNA structures, specifically branched substrates that mimic replication forks and Holliday junctions. In humans, RecQ family helicases include the BLM and WRN proteins, mutated in certain rare inherited diseases in humans (5).

The Hel308 family SF2 helicases, like RecQ, are implicated in DNA repair, recombination, and genome stability. The founding member, Mus308 from Drosophila melanogaster, was identified in a screen for mutations conferring hypersensitivity to DNA cross-linking reagents (6). Mus308 consists of an N-terminal SF2 helicase fused to a C-terminal DNA polymerase. The human ortholog, PolQ, has the same arrangement (7), and the polymerase domain has been shown to function efficiently in the bypass of damaged DNA templates, consistent with a role in DNA repair (8, 9). In addition to this helicase-polymerase fusion protein, metazoans also encode an ortholog of the helicase alone. This protein, known as Hel308 in Homo sapiens, has been characterized biochemically and shown to function as a typical SF2, 3′ to 5′ DNA helicase with limited processivity (10). The ortholog from D. melanogaster, Mus301, has been shown to function in double strand break repair and meiotic recombination (11). The Hel308 family helicases therefore have RecQ-like properties.

Bacteria and fungi lack orthologs of the Hel308 family, but clear homologs are present in archaea. Hel308 proteins from Pyrococcus furiosus (also known as Hjm helicase) and Methanothermobacter thermautotrophicum have been cloned and studied biochemically (12, 13). Both proteins have RecQ-like activities in vitro, targeting branched DNA substrates that are models for stalled replication forks and unwinding lagging strands. Hel308 from M. thermautotrophicum functions like RecQ in a genetic screen for synthetic lethality in Escherichia coli, reinforcing the impression that the two proteins may have related functions (13).

Here we report the high resolution crystal structure of Hel308 from the crenarchaeote Sulfolobus solfataricus strain PBL2025. The structure reveals a five-domain organization
with the first four domains, including the two motor domains, a winged-helix domain 3, and domain 4, forming a ring structure with a central cavity for ssDNA. The fifth domain adopts a helix-loop-helix structure known to function in DNA binding in many other proteins. Site-directed mutagenesis of conserved arginine residues confirms the path of ssDNA through the central cavity, and mutant helicases with domain 5 removed or mutated have significantly higher processivity than the wild-type enzyme. This suggests that domain 5 acts as an autoinhibitory domain to control the helicase activity of the enzyme in vivo. Hel308 displaces streptavidin from a biotinylated oligonucleotide efficiently, consistent with a role in the removal of bound proteins from stalled replication forks or recombination intermediates. Together with the recent report of the crystal structure of Hel308 from Archaeoglobus fulgidus bound to a DNA substrate (14), these observations provide considerable new information on the molecular basis for the function of this important class of DNA helicases.

**EXPERIMENTAL PROCEDURES**

Overexpression and Purification of Recombinant Hel308—
The *S. solfataricus* strain PBL2025 Hel308 gene was cloned into a pDEST14 vector using the Gateway cloning system (Invitrogen) and provided by Scottish Structural Proteomics Facility, St. Andrews University. The gene was sequenced and submitted to the EMBL database with accession number AM778123. For expression of domain 5 of Hel308, the domain was amplified by PCR using the primers 5'-AAGGAAGAGCTATTG and 5'-CTAATGAAATCTATTA-CTAATGAAATCTATTA to control the helicase activity of the enzyme in vivo. Hel308 displaces streptavidin from a biotinylated oligonucleotide efficiently, consistent with a role in the removal of bound proteins from stalled replication forks or recombination intermediates. Together with the recent report of the crystal structure of Hel308 from Archaeoglobus fulgidus bound to a DNA substrate (14), these observations provide considerable new information on the molecular basis for the function of this important class of DNA helicases.

AAGGAAGAGCTATTG and 5'-CAAAGAAGAGCTATTG and 5'-CTAATGAAATCTATTA-CTAATGAAATCTATTA cloned into the pET151/D TOPO vector using the Compani pET directional TOPO expression kit (Invitrogen) according to the manufacturer's instructions.

Recombinant Hel308 was expressed with a His tag in C43 cells; the cultures were grown at 37 °C to an *A*₆₀₀ of ~0.8 in Luria Bertani broth containing ampicillin at a final concentration of 100 µg/ml. Protein expression was induced by addition of 0.1 mM isopropyl-1-thio-β-D-galactopyranoside, and the cells were incubated for a further 3 h at 30 °C before being harvested. Selenomethionine-labeled Hel308 protein was produced in BL21 (DE3) cells using a published procedure that employs a simplified selenomethionine medium (15). An overnight culture in 100 ml of Luria broth supplemented with 100 µg/ml of ampicillin was harvested and the pellet washed gently three times in the simple selenomethionine medium, resulting in a final volume of 10 ml of cells that was used to inoculate 1 liter of the same medium supplemented with 100 µg/ml of ampicillin. The cells were grown in shaker flasks at 200 rpm and 37 °C to an optical density of 0.6, whereupon the temperature was reduced to 25 °C and expression was induced with 0.2 mM isopropyl-1-thio-β-D-galactopyranoside. The cells were harvested the following day.

For purification of native and seleno-labeled protein, cell pellets with overexpressed PBL2025 Hel308 or domain 5 protein were resuspended in lysis buffer (50 mM sodium phosphate, pH 7.5, 500 mM NaCl, 1 mg ml⁻¹ lysozyme) with appropriate protease inhibitors and lysed on ice using a Constant Systems cell disrupter at 207 mega paseals. The crude lysate was centrifuged (15,000 × g, 30 min, 277 K), and the cleared lysate was filtered through a 0.22-μm filter. Protein was purified from cleared lysate by two-step nickel-affinity chromatography. The lysate was batch bound to nickel-Sepharose 6 fast flow medium (GE Healthcare), poured into a column, and washed with 50 column volumes of lysis buffer plus 20 mM imidazole. The His-tagged protein was eluted in ten column volumes of lysis buffer plus 500 mM imidazole and immediately desalted into 50 mM Tris-HCl, 500 mM NaCl, pH 7.5, on a HiPrep 26/10 desalting column (GE Healthcare) to remove imidazole. His-tagged tobacco etch virus protease was added to the protein at a 1:10 mass ratio to remove the N-terminal His tag, leaving the native protein. The protease/target protein mixture was incubated at room temperature for 15 h. Cleaved Hel308 was separated from His-tagged tobacco etch virus by difference purification on nickel resin and polished by a HiLoad superdex 16/60 S-200 gel filtration column (GE Healthcare) equilibrated and eluted with 10 mM Tris-chloride, pH 7.5, 150 mM sodium chloride. The purified protein was characterized by SDS-PAGE and mass spectrometry. For crystallization trials, the protein was concentrated to 10 mg⁻¹ ml⁻¹.

Crystallization—Initial hits for crystallization were found by performing sitting drop experiments of 1 µl of protein solution (10 mg·ml⁻¹ in 10 mM Tris-Cl, pH 7.5, 150 mM NaCl) plus 1 µl of well solution against the following four 96 condition crystallization screens: the Classics, PEGs and JCSG+ suites (Qiagen) and JMAC, a homemade PEG-based screen. The crystals used for data collection were grown from hanging drops containing 1 µl of the 10 mg·ml⁻¹ protein solution mixed with 0.5 µl of the 0.5 ml of well solution containing, for native protein crystals, 15.3% PEG8000, 0.1 M sodium cacodylate, pH 6.5, 0.13 M ammonium sulfate, and 0.03 M magnesium chloride and for selenomethionine-labeled protein, 13.6% PEG 8000, 0.1 M sodium cacodylate, pH 6.5, 0.12 M ammonium sulfate, and 0.03 M magnesium chloride. Crystals were prepared for data collection by soaking hanging drops containing 1 µl of the 10 mg·ml⁻¹ protein solution with 0.5 µl of the 0.5 ml of well solution containing, for native protein crystals, 15.3% PEG8000, 0.1 M sodium cacodylate, pH 6.5, 0.13 M ammonium sulfate, and 0.03 M magnesium chloride and for selenomethionine-labeled protein, 13.6% PEG 8000, 0.1 M sodium cacodylate, pH 6.5, 0.12 M ammonium sulfate, and 0.03 M magnesium chloride. Crystals were prepared for data collection by soaking in a cryoprotecting solution containing 16% PEG 8000 (w/v), 0.1 M sodium cacodylate, pH 6.5, 0.1 M ammonium sulfate, 0.05 M magnesium chloride, and 20% PEG 400 (w/v). The cryoprotected crystals were flash-frozen in liquid nitrogen and stored at ~80 °C for shipment to the European Synchrotron Radiation Facility.

Data Collection, Structure Solution, and Refinement—Data from the crystal of the native protein for refinement were collected to 2.3 Å in 0.5° slices at x-ray wavelength of 0.934 Å on beamline ID14-1 at the European Synchrotron Radiation Facility using an ADSC Q210 detector. The native data were processed using MOSFLM and scaled using Scala from the CCP4 suite (16) (CCP4 no. 4, 1994; Table 1). Data from the crystal of the selenomethionine-labeled protein were collected to 2.6 Å in 0.5° slices at the selenium peak (0.979 Å) on beamline BM14 at the European Synchrotron Radiation Facility using a MAR225 detector. The selenium peak data were processed using the HKL2000 interface (17) to Denzo and scaled using Scalepack with the “no merge original index” flag set. Scaling statistics for Table 1 were recalculated using Scala.

The 24 selenium sites were readily located and confirmed using the ShelxC, ShelxD, and ShelxE programs (18) as imple-

---

5 S. McMahon and J. Maclean, unpublished.
bond distances were corrected and the structure refined in an (26). Side chain rotamers, main chain bond angles, and C share” motif that physically separates the two DNA strands (358–370) in monomer B, where should be found the “plow-14 residues (358–371) in monomer A and one of 13 residues of Hel308 comprising 1388 amino acid residues, 7 sulfate ions, large difference peaks near positively charged side chains were 107.5. Refinement

Using the program Sequoia (25) to help with the building of less ordered loops. Transport layer security parameters were refined for each domain, giving ten in total. Seven

Simulated annealing was performed (24) and analyzed by Refmac5 (23) from the CCP4 suite and iteratively rebuilt using Xfit. Phases were improved by slow cooling, followed by purification on 12% native acrylamide: Tris borate-EDTA (TBE) gels, as described previously (27). The oligonucleotide sequences were as described previously (28). The shorter 3’-overhang substrate with a 25-bp duplex was made by annealing oligo x50 with oligo r26–50. The 3’-overhang with the 50-bp duplex was made by annealing oligo b50 with a complementary sequence that included a 25T tail (a single-stranded DNA sequence of 25 deoxythymidine nucleotides at the 3’-end. The stalled replication fork model substrate was made by annealing oligos b50, x50, and r26–50.

Fluorescence Anisotropy—The DNA binding affinity of Hel308 was investigated using fluorescence anisotropy using a Varian Cary Eclipse fluorometer with automatic polarizers. All experiments were carried out under temperature control at 20 °C. For direct titration, a 15-mer oligonucleotide (5’-TCG-GAGTACAGTGGG) with a 5’-fluorescein label was used at a final concentration of 20 nM in 150 μl of anisotropy buffer (20 mM HEPES, pH 7.6, 100 mM NaCl, 1 mM dithiothreitol, 0.01% Triton X-100). The first measurement was taken prior to the addition of protein; this was subtracted from the data as a blank. The protein concentration was increased cumulatively and further readings were taken after each addition, with corrections made for dilution. Changes in fluorescence intensity were also recorded; to avoid anisotropy effects on fluorescence intensity, “magic angle” conditions were used (29). Decreases in fluorescence intensity of up to 30% were observed, which is not unusual given the pH-sensitive nature of the fluorophore (30). Measurements were also taken for a double-stranded 15 mer (15-mer oligonucleotide described above, annealed to its complement). Data were fitted, using Kaleidagraph, to the following equation, where A is the total protein concentration, and D represents the total DNA concentration. A(48.75) (maximum anisotropy) is the anisotropy of the DNA-protein complex, and A(5) is the dissociation constant (29).

For the competition assays, Hel308 was added to 100 nM fluorescein-labeled 15-mer DNA in anisotropy buffer to obtain ~80% saturation, and then unlabeled 15-mer oligonucleotide was added progressively, with measurements taken as described above. The titration curves were fitted as described in Reid et al. (29) assuming a 1:1 interaction between the protein and the DNA. The Kd values calculated from direct and competitive titrations were in good agreement.

Helicase Assay—The helicase reactions were set up containing 1X helicase buffer (100 mM MES, pH 6.0, 5 mM dithiothreitol, 100 mM NaCl, 0.1 mg/ml bovine serum albumin), 10 mM 32P-labeled DNA substrates, and 0.5 μM protein. The assay was carried out at either 60 or 45 °C, as stated, in a final volume of 60 μl. Reactions were equilibrated at this temperature for 5 min

| TABLE 1 Crystallographic statistics for data collected on native and selenomethionine Hel308 |
|---------------------------------------------|-------------|-------------|
|                                | Native      | Selenium peak |
|---------------------------------------------|-------------|-------------|
| **Data collection**                      |             |             |
| Space group                               | P22         | P22         |
| Unit cell (Å, °)                           | a = 61.7    | a = 61.8    |
|                                            | b = 138.1   | b = 138.0   |
|                                            | c = 107.6   | c = 107.5   |
|                                            | β = 94.7    | β = 95.2    |
| **Resolution (Å)**                        | 30.2-2.3    | 30.2-2.6    |
| **Wavelength (Å)**                        | 0.934       | 0.979       |
| **Unique reflections**                    | 79222       | 58476       |
| **Multiplicity**                          | 6.0         | 3.7         |
| **Completeness (%)**                      | 99.8        | 99.5        |
| **Rmerge (%)**                            | 7.8         | 8.3         |
| **I/σ(I)**                                | 14.7 (4.2)  | 11.2 (4.3)  |
| **Structure solution**                    | Monomers    | 2           |
|                                            | Selenium sites | 24         |
| **Refinement**                            | Protein atoms | 11116      |
|                                            | Sulfate ions  | 7           |
|                                            | Waters       | 146         |
| **Average B-factors (Å²)**                | Chain A     | 45.76       |
|                                            | Chain B     | 45.92       |
|                                            | Sulfate ions | 48.75       |
|                                            | Waters       | 39.58       |
| **Monomer superposition rmsd (Å)**        | Domains 1–5 | 0.874 (690 residues) |
|                                            | Domains 1–4 | 0.446 (630 residues) |
|                                            | Domain 5    | 0.389 (61 residues) |
| **r.m.s.d. bond lengths (Å)**             | 0.011       |
| **Angles (°)**                            | 1.18        |
| **Ramachandran angles**                   | Favored (%) | 97.5        |
|                                            | Disallowed (%) | 0.07      |
to bind to the DNA. 6 μM free biotin was then added as a streptavidin trap, and the reaction was initiated by the addition of 0.5 μM Hel308 (final volume 60 μl). At the indicated time points, 10-μl aliquots of the reaction mixture were removed and added to 10 μl of STOP solution (1 M NaCl, 100 mM Tris-HCl, pH 8.0, 200 mM EDTA, pH 8.0) together with 10 μM non-biotinylated b50 oligonucleotide to bind to the protein and reduce band shifting. In experiments with competitor proteins, recombinant S. solfataricus Alba1, RPA and RadA purified as described (33–35) were added at a final concentration of 10 μM and incubated with the DNA for 5 min prior to addition of 0.5 μM Hel308 as described above. Samples were collected at 0.5, 1, 2, 3, 5, 10, and 15 min and separated on a 12% native acrylamide-TBE gel as described for the helicase assays above.

RESULTS

Gene Cloning, Site-directed Mutagenesis, and Protein Expression—The hel308 gene from the crenarchaeote S. solfataricus strain PBL2025 (36) was amplified by PCR, cloned into the expression vector pDEST14, and expressed in E. coli with an N-terminal His tag. The recombinant protein was purified using immobilized metal affinity chromatography and gel filtration as described under “Experimental Procedures.” For crystallization, the tag was removed by cleavage with the tobacco etch virus protease during the purification process. A K52A variant corresponding to a mutation in the canonical Walker A motif was constructed as a control lacking ATPase and helicase activity. Two conserved arginine residues expected to have a role in DNA binding were mutated to produce the R255A and R320A mutants. A further two mutants targeted the C-terminal domain 5 of ssHel308. The first arginine of the conserved RAR motif was mutated to alanine (R662A), and the entire domain 5 was removed by truncation of the protein in another mutant (K646-stop). Site-directed mutant versions of the protein were expressed and purified as for the wild type.

Structure of Hel308—The asymmetric unit contains two monomers of the protein. Analysis using PISA (37) indicates the protein is a monomer, consistent with gel filtration results. The monomer can be decomposed into five domains, as reported previously (14) (Fig. 1). Domain 1 (residues 1–197) and initiated by the addition of 1 mM Mg-ATP. 10-μl samples were taken at relevant time points and added to 20 μl of chilled STOP Solution (100 mM Tris, pH 8.0, 50 mM EDTA, 0.5% SDS, 1 mg/ml Proteinase K, 300 mM NaCl, and 5 μM of a competitor DNA designed to bind to the displaced strand to prevent reannealing). Gel loading dye was added, and samples were analyzed on 12% native acrylamide-TBE gels. The gels were phosphorimaged and quantified as described previously (32).

Streptavidin Displacement Assay—To monitor the displacement of streptavidin from a biotinylated oligonucleotide, a 32p-labeled 50-mer DNA oligonucleotide (oligo b50) (28), with a biotin label on the 5’-end was incubated at a final concentration of 10 nM with 300 nM streptavidin (Sigma) in 1× helicase buffer with 1 mM Mg-ATP at 45 °C for 5 min to allow the streptavidin
and domain 2 (200–416) are the classical ATP binding motor domains seen in all SF1 and SF2 helicase structures. These two domains share the same core α/β fold; the rmsd of 106 superimposable Ca atoms is 2.6 Å. The interface between these two domains forms the ATP binding site, and the conformation changes induced during the complete cycle of ATP hydrolysis are thought to drive duplex unwinding (reviewed in Ref. (3)). Domain 3 (426–501) is a winged helix domain commonly seen in nucleic acid-binding proteins and closely matches Histone H5 (1.5 Å rmsd for 62 overlapping carbons). A simple search of structural similarity using SSM reveals over 400 such domains sharing the same core α/β sheet; the rmsd of 106 superimposable Ca atoms the rmsd is 2.0 Å; the main differences are in the positions of some secondary structure elements and loops. Our apo structure superimposes equally well on the apo and DNA-bound forms of A. fulgidus Hel308, suggesting that this helicase adopts quite a rigid structure that is not perturbed significantly by DNA binding. This has allowed us to model the DNA from the A. fulgidus complex into our apo S. solfataricus structure (Fig. 1B). The DNA threads through the central pore, forming interactions with domains 3 and 4, and engages domain 5 on the opposite side. The interactions with domain 3 and domain 4 are extensive. Loss of these domains uncouples ATP hydrolysis from helicase activity, and domain 4 has been proposed as the ratchet powered by ATP hydrolysis that unwinds the DNA (14). Four sulfates present in our crystal structure coincide with the likely positions of phosphate residues in the DNA backbone. In the Hel308-DNA co-crystal structure, the DNA duplex is split by a loop almost at the entrance to the central hole that forms a small two-stranded β sheet (residues 347–359). In our apo structure this loop is disordered, as was observed in the apo form of A. fulgidus Hel308 (14). Three conserved arginine residues (Arg-255, Arg-320, and Arg-662) mutated in this study clearly adopt positions consistent with a role in DNA binding (Fig. 1C).

**DNA Binding by Wild-type and Mutant Hel308 Proteins—** ssDNA and dsDNA binding by the wild-type and mutant versions of Hel308 was assessed by fluorescence anisotropy, using a 15-mer oligonucleotide with a 5′-fluorescein dye as a reporter. Equilibrium dissociation constants were obtained by plotting the change in fluorescence anisotropy in response to increasing concentrations of Hel308 (Fig. 2A). Wild-type

---

**FIGURE 2. Affinity of DNA binding by wild-type and mutant Hel308.** A, ssDNA binding affinities of wild-type and mutant forms of Hel308 measured by change in fluorescence anisotropy at 20 °C. Direct titration of increasing concentrations of Hel308 into a solution containing a 15-mer oligonucleotide with a 5′-fluorescein fluorescent reporter. Protein binding to the DNA leads to an increase in the fluorescence anisotropy, from which dissociation constants can be calculated. The means of triplicate measurements were plotted, and the standard errors are shown. Wild-type Hel308, open circles; K646STOP, closed triangles; K52A, closed circles; R320A, closed squares; R255A, open triangles; R662A, open squares; domain 5 alone, closed diamonds. B, competition analysis of Hel308 binding to ssDNA. Competitor DNA (unlabeled) was titrated into the assay containing Hel308 bound to fluorescently labeled DNA. Assays were carried out at 20 °C. Each data set was carried out in triplicate, and the standard errors are shown. C, SDS-PAGE analysis of purified recombinant proteins. D, table summarizing the dissociation constants measured for wild-type and mutant Hel308 binding to ssDNA and dsDNA. Standard errors derived from the curve fitting in Kaleidagraph are shown.
Hel308 bound the single-stranded and duplex DNA ligands with $K_D$ of 0.14 and 5.3 μM, respectively. The strong preference for binding to ssDNA is consistent with the function of the helicase, which must track along ssDNA and displace a duplex DNA strand. To ensure that the DNA binding affinity was not affected by protein-dye interactions, competition assays were performed by first forming a complex of Hel308 with the fluorescent oligo and then titrating an unlabeled competitor ssDNA of the same sequence (29). The decrease in anisotropy observed in Fig. 2B yielded a $K_D$ of 0.13 μM, in good agreement with that calculated from the forward titration, suggesting that the influence of the fluorescein was minimal. As expected, the K52A mutant bound ssDNA and dsDNA with similar affinities to the wild-type protein. Both the R255A and R320A mutants had significantly reduced binding affinities, with the R255A mutant showing the largest effect, a 25-fold increase in the $K_D$ for ssDNA. These data confirm an important role in ssDNA binding for Arg-320 and particularly Arg-255, which line the central pore predicted as the path for ssDNA (Fig. 1). The truncated protein lacking domain 5 and the R662A mutant both had ssDNA binding affinities comparable with or only slightly weaker than the wild-type enzyme for this short oligonucleotide. A longer oligonucleotide might be expected to engage more fully with domain 5. A role for ssDNA binding by domain 5 was confirmed by expressing this domain independently and measuring its affinity for ssDNA. Domain 5 adopts a stable, autonomous folded structure in isolation and binds ssDNA with a dissociation constant of 1.3 μM, ~10-fold weaker than the intact protein but more tightly than the R255A mutant.

Helicase Activity of Wild-type and Mutant Hel308 Proteins—
The 3′-5′ helicase activity of Hel308 was assayed using a minimal substrate: a 3′-overhang with a 25-nt ssDNA region and a 25-bp duplex region. Helicase assays were carried out at 60 °C over a 3-min time course and analyzed by gel electrophoresis (Fig. 3). The wild-type protein displaced the duplex strand efficiently, as observed previously for the homologues from archaea and H. sapiens (10, 13). As expected, the K52A mutant was unable to function as a helicase and the R255A and R320A mutants had significantly reduced helicase activity, consistent with a defect in ssDNA binding. Surprisingly, the K646-stop mutant lacking domain 5 and the R662A mutant lacking the first arginine of the RAR motif in domain 5 both showed significantly faster rates of DNA unwinding than the wild-type protein. To follow up this observation, we tested the helicase activity of the wild-type and truncated proteins using a DNA substrate with a longer duplex region of 50 bp (Fig. 3). Hel308 has been shown previously to have a limited processivity and a limited ability to displace longer DNA duplex strands (13). Consistent with these observations, the wild-type ssHel308 protein showed only a very weak helicase activity against the larger substrate. However, significantly faster unwinding was observed with the truncated mutant, consistent with the effects observed for the shorter substrate.

Previously, biochemical studies have identified branched DNA structures, and specifically substrates resembling a stalled DNA replication fork, as the preferred substrates of Hel308 (13). Accordingly, we compared the activities of the wild-type and truncation mutant using a model replication fork substrate with a 25-bp duplex region corresponding to the lagging strand of a replication fork (Fig. 4). These assays were carried out at the reduced temperature of 45 °C to allow more accurate determination of reaction progress. The transient production and then disappearance of the 3′-overhang intermediate at early time points suggested that unwinding may proceed via displacement of the top strand, yielding a 3′-overhang substrate intermediate that is then unwound to yield the final products. This conclusion was strengthened by the observation that a simple 3′-overhang substrate was unwound at the same rate as the fork substrate (Fig. 4). Once again, the truncation mutant lacking domain 5 unwound the fork structure much more quickly than the wild-type protein, disassembling ~80% of the substrate within 30 s, whereas the wild-type enzyme unwound only ~15% in the same period. As for the wild-type protein, the truncation mutant showed no difference in the rates of unwinding of the fork and a simple 3′-overhang (Fig. 4).

Taken together, these observations suggest that domain 5, which is conserved in all Hel308 proteins, functions as an autoinhibitory domain, limiting the activity of the helicase, possibly by binding the emergent ssDNA product and functioning as a "molecular brake." Deletion of this domain does not perturb the relative rates of unwinding of fork versus overhang substrates,
Hel308 Displaces Streptavidin and DNA-binding Proteins from a Biotinylated Oligonucleotide

The translocation of Hel308 along ssDNA was investigated by its ability to displace streptavidin bound to a biotinylated oligonucleotide. Wild-type and mutant Hel308 proteins were incubated with the DNA substrate in the presence of ATP at 45 °C followed by electrophoresis to separate the free biotinylated probes from those bound by streptavidin (38). Wild-type Hel308 was able to displace >80% of the streptavidin in 10 min (Fig. 5). The ability of Hel308 to translocates along ssDNA with a force sufficient to dissociate a tightly bound biotin:streptavidin linkage suggests that Hel308 could act to displace proteins bound to ssDNA in vivo (39). We therefore investigated the effect of adding the S. solfataricus DNA-binding proteins RPA (the canonical ssDNA-binding protein), Alba1 (the major chromatin protein that binds both dsDNA and ssDNA), and RadA (the archaeal Rad51 family recombinase) to the streptavidin-bound DNA at a concentration of 10 μM before addition of the helicase at 0.5 μM (Fig. 5). Streptavidin displacement by Hel308 was not inhibited by RadA, whereas reduced activity was observed in the presence of Alba1 and RPA. By comparison, the repli-cative helicase MCM from S. solfataricus was completely inhibited by a 2-fold molar excess of Alba1 (40). These data are consistent with a role for Hel308 in remodeling stalled replication forks by protein as well as DNA displacement.

The truncation mutant retained the ability to displace streptavidin (Fig. 5) with activity comparable with the wild-type protein. The finding that the truncated enzyme is a better helicase but no better at displacing bound proteins than the wild-type protein probably relates to the difference between the two types of assay, as the streptavidin displacement assay reflects the force exerted by the helicase as it translocates along the ssDNA and allows helicase binding anywhere on the ssDNA molecule.

DISCUSSION

The high resolution crystal structure of Hel308 presented here, together with the site-directed mutagenesis and biochem-

as both rates are enhanced to the same extent in the truncated species.

Hel308 Displaces Streptavidin and DNA-binding Proteins from a Biotinylated Oligonucleotide—The translocation of Hel308 along ssDNA was investigated by its ability to displace streptavidin bound to a biotinylated oligonucleotide. Wild-type and mutant Hel308 proteins were incubated with the DNA substrate in the presence of ATP at 45 °C followed by electrophoresis to separate the free biotinylated probes from those bound by streptavidin (38). Wild-type Hel308 was able to displace >80% of the streptavidin in 10 min (Fig. 5). The ability of Hel308 to translocate along ssDNA with a force sufficient to dissociate a tightly bound biotin:streptavidin linkage suggests that Hel308 could act to displace proteins bound to ssDNA in vivo (39). We therefore investigated the effect of adding the S. solfataricus DNA-binding proteins RPA (the canonical ssDNA-binding protein), Alba1 (the major chromatin protein that binds both dsDNA and ssDNA), and RadA (the archaeal Rad51 family recombinase) to the streptavidin-bound DNA at a concentration of 10 μM before addition of the helicase at 0.5 μM (Fig. 5). Streptavidin displacement by Hel308 was not inhibited by RadA, whereas reduced activity was observed in the presence of Alba1 and RPA. By comparison, the repli-cative helicase MCM from S. solfataricus was completely inhibited by a 2-fold molar excess of Alba1 (40). These data are consistent with a role for Hel308 in remodeling stalled replication forks by protein as well as DNA displacement.

The truncation mutant retained the ability to displace streptavidin (Fig. 5) with activity comparable with the wild-type protein. The finding that the truncated enzyme is a better helicase but no better at displacing bound proteins than the wild-type protein probably relates to the difference between the two types of assay, as the streptavidin displacement assay reflects the force exerted by the helicase as it translocates along the ssDNA and allows helicase binding anywhere on the ssDNA molecule.

DISCUSSION

The high resolution crystal structure of Hel308 presented here, together with the site-directed mutagenesis and biochem-

as both rates are enhanced to the same extent in the truncated species.

Hel308 Displaces Streptavidin and DNA-binding Proteins from a Biotinylated Oligonucleotide—The translocation of Hel308 along ssDNA was investigated by its ability to displace streptavidin bound to a biotinylated oligonucleotide. Wild-type and mutant Hel308 proteins were incubated with the DNA substrate in the presence of ATP at 45 °C followed by electrophoresis to separate the free biotinylated probes from those bound by streptavidin (38). Wild-type Hel308 was able to displace >80% of the streptavidin in 10 min (Fig. 5). The ability of Hel308 to translocate along ssDNA with a force sufficient to dissociate a tightly bound biotin:streptavidin linkage suggests that Hel308 could act to displace proteins bound to ssDNA in vivo (39). We therefore investigated the effect of adding the S. solfataricus DNA-binding proteins RPA (the canonical ssDNA-binding protein), Alba1 (the major chromatin protein that binds both dsDNA and ssDNA), and RadA (the archaeal Rad51 family recombinase) to the streptavidin-bound DNA at a concentration of 10 μM before addition of the helicase at 0.5 μM (Fig. 5). Streptavidin displacement by Hel308 was not inhibited by RadA, whereas reduced activity was observed in the presence of Alba1 and RPA. By comparison, the repli-cative helicase MCM from S. solfataricus was completely inhibited by a 2-fold molar excess of Alba1 (40). These data are consistent with a role for Hel308 in remodeling stalled replication forks by protein as well as DNA displacement.

The truncation mutant retained the ability to displace streptavidin (Fig. 5) with activity comparable with the wild-type protein. The finding that the truncated enzyme is a better helicase but no better at displacing bound proteins than the wild-type protein probably relates to the difference between the two types of assay, as the streptavidin displacement assay reflects the force exerted by the helicase as it translocates along the ssDNA and allows helicase binding anywhere on the ssDNA molecule.

DISCUSSION

The high resolution crystal structure of Hel308 presented here, together with the site-directed mutagenesis and biochem-

as both rates are enhanced to the same extent in the truncated species.

Hel308 Displaces Streptavidin and DNA-binding Proteins from a Biotinylated Oligonucleotide—The translocation of Hel308 along ssDNA was investigated by its ability to displace streptavidin bound to a biotinylated oligonucleotide. Wild-type and mutant Hel308 proteins were incubated with the DNA substrate in the presence of ATP at 45 °C followed by electrophoresis to separate the free biotinylated probes from those bound by streptavidin (38). Wild-type Hel308 was able to displace >80% of the streptavidin in 10 min (Fig. 5). The ability of Hel308 to translocate along ssDNA with a force sufficient to dissociate a tightly bound biotin:streptavidin linkage suggests that Hel308 could act to displace proteins bound to ssDNA in vivo (39). We therefore investigated the effect of adding the S. solfataricus DNA-binding proteins RPA (the canonical ssDNA-binding protein), Alba1 (the major chromatin protein that binds both dsDNA and ssDNA), and RadA (the archaeal Rad51 family recombinase) to the streptavidin-bound DNA at a concentration of 10 μM before addition of the helicase at 0.5 μM (Fig. 5). Streptavidin displacement by Hel308 was not inhibited by RadA, whereas reduced activity was observed in the presence of Alba1 and RPA. By comparison, the repli-cative helicase MCM from S. solfataricus was completely inhibited by a 2-fold molar excess of Alba1 (40). These data are consistent with a role for Hel308 in remodeling stalled replication forks by protein as well as DNA displacement.

The truncation mutant retained the ability to displace streptavidin (Fig. 5) with activity comparable with the wild-type protein. The finding that the truncated enzyme is a better helicase but no better at displacing bound proteins than the wild-type protein probably relates to the difference between the two types of assay, as the streptavidin displacement assay reflects the force exerted by the helicase as it translocates along the ssDNA and allows helicase binding anywhere on the ssDNA molecule.

DISCUSSION

The high resolution crystal structure of Hel308 presented here, together with the site-directed mutagenesis and biochem-
ical data, provide several new insights into the molecular mechanism of this conserved SF2 DNA helicase. The helicase is organized into five domains. The first four form a ring with a central pore large enough for the passage of single-stranded, but not double-stranded, DNA, consistent with the recently published structure of Hel308 from *A. fulgidus* in the presence and absence of a DNA substrate (14). The crystal structure of Hel308 suggests an ancillary role for domain 5, which sits perpendicular to the ring formed by the first four domains and has been shown to engage the 3′-DNA end that extrudes through the central pore as the helicase translocates DNA. Hopfner and coworkers (14) have suggested a role as a specificity domain for domain 5, predicting that it confers binding specificity for the branched DNA substrates preferred by the enzyme. However, our data show clearly that either deletion of domain 5 or abrogation of its DNA binding affinity in the R662A mutant results in an increase in activity of the helicase. Importantly, the truncated mutant retains a faster helicase activity than the wild-type enzyme and can displace longer DNA strands more effectively. The effect is equally pronounced with the minimal 3′-overhang substrate and the preferred lagging strand substrate, which are unwound with equal rates, arguing against a role for domain 5 in determining substrate specificity. Altogether, our data suggest strongly that the function of domain 5 in *in vitro* is to act as a molecular brake, limiting the extent of DNA unwinding by Hel308, rather than a specificity domain.

Autoinhibitory domains have been observed in other helicases. The SF1 helicase Rep, which functions in replication restart, in inhibited by its 2B subdomain, which is thought to block the Rep helicase from invading the DNA duplex (41). This inhibition is alleviated by dimerization of the Rep protein. Recently, the C-terminal D7 domain of the transcription-coupled repair protein Mfd from *E. coli* has also been shown to inhibit the helicase activity of Mfd in *in vitro* (42). In this case, the interaction of Mfd with a stalled RNA polymerase molecule is thought to result in a conformational change in the D7 domain, alleviating the repression of the helicase. Finally, domain 4 of the bacterial DNA repair helicase UvrB has also been shown to function as an autoinhibitory domain in *in vitro*, where it is proposed to regulate the repair activity of the UvrABC system and prevent spurious incision events (43). For all these examples, inhibition is thought to be "on" by default and to be relieved by the formation of cognate protein-protein interactions. Hel308 domain 5 differs from all these examples in binding ssDNA directly, thus binding the product of the DNA helicase reaction. This represents an elegant mechanism with the potential to limit or tune helicase activity in *in vivo*. It remains to be determined whether the autoinhibitory function of Hel308 domain 5 is alleviated by interactions with other proteins.

The limited helicase activity of Hel308 is consistent with the proposed function in *in vivo* in the removal of short sections of the lagging strand next to a stalled replication fork to clear an ssDNA binding site for proteins that can restart replication or initiate recombination (13) (Fig. 6). In *E. coli*, the 3′-5′ helicase activities of PriA or Rep carry out this role (reviewed in Ref. (44)). We have also shown that Hel308 can displace streptavidin from biotinylated ssDNA, as has been demonstrated previously for a number of helicases, such as the replicative helicase Dda (45). The ability of helicases to disrupt protein-nucleic acid complexes may be relevant in *in vivo*, where abundant DNA-binding proteins are associated with dsDNA and ssDNA under most conditions. In the case of Hel308, we see no significant reduction of streptavidin displacement activity when using a DNA substrate pre-bound with RadA and significant residual activity in the presence of 20-fold molar excess of the abundant DNA-binding proteins RPA and Alba1. These assays were all carried out at 45 °C, well below the *in vitro* operating temperature of 80 °C where one would expect significantly more robust helicase and protein displacement activity. A plausible additional function of Hel308 may therefore be to displace DNA-binding proteins from the site of a stalled replication fork, allowing binding of Rad51 (RadA in archaea) and the initiation of recombination, or the binding of other proteins for DNA replication (Fig. 6). This is analogous to the role proposed for RecFOR in the displacement of single-stranded DNA-binding protein from stalled forks to allow RecA-mediated recombination (46).

Equally, Hel308 could function like bacterial UvrD, another 3′-5′ helicase that has been shown to disrupt RecA nucleoprotein filaments and thus limit recombination (47). In this context, it is intriguing to note that Ishino and coworkers (12) report a direct interaction between Hel308 and RadA from *P. furiosus*.

**Acknowledgment**—We thank Georg Lipps for the kind donation of PBL2025 chromosomal DNA.

**REFERENCES**

1. Gorbalenya, A. E., and Koonin, E. V. (1993) *Curr. Opin. Struct. Biol.* 3, 419–429
2. Singleton, M. R., and Wigley, D. B. (2002) *J. Bacteriol.* 184, 1819–1826
3. Singleton, M. R., Dillingham, M. S., and Wigley, D. B. (2007) *Annu. Rev.*

**FIGURE 6. Schematic model for possible roles of Hel308 at stalled replication forks.** Hel308 can translocate in a 3′ to 5′ direction along ssDNA and displace either bound protein or a duplex DNA strand. In *in vivo*, the protein could function to generate an area of ssDNA on the lagging strand to allow replication restart or the initiation of recombination. This could involve the removal of bound proteins such as RPA or RadA (archaeal Rad51), as shown on the left, or the displacement of a short section of the lagging strand, as shown on the right.
Hel308 Helicase Structure and Autoinhibition

Biochem. 76, 23–50
4. Sharma, S., Doherty, K. M., and Brosh, R. M., Jr. (2006) Biochem. J. 398, 319–337
5. Hanada, K., and Hickson, I. D. (2007) Cell Mol. Life Sci. 64, 2306–2333
6. Boyd, J. B., Golino, M. D., Nguyen, T. D., and Green, M. M. (1976) Genetics 84, 485–506
7. Seki, M., Marini, F., and Wood, R. D. (2003) Nucleic Acids Res. 31, 6117–6126
8. Yoshimura, M., Kohzaki, M., Nakamura, J., Asagoshi, K., Sonoda, E., Hou, E., Prasad, R., Wilson, S. H., Tano, K., Yasui, A., Lan, L., Seki, M., Wood, R. D., Arakawa, H., Buerstedde, J. M., Hochemger, H., Okada, T., Hiraoka, M., and Takeda, S. (2006) Mol. Cell 24, 115–125
9. Takata, K., Shimizu, T., Iwai, S., and Wood, R. D. (2006) J. Biol. Chem. 281, 23445–23455
10. Marini, F., and Wood, R. D. (2003) Mol. Endocrinol. 10, 607–612
11. McCaffrey, R., St. Johnston, D., and Gonzalez-Reyes, A. (2006) Genetics 174, 1273–1285
12. Fujiikane, R., Shinagawa, H., and Ishino, Y. (2006) Genes Cells 11, 99–110
13. Guy, C. P., and Bolt, E. L. (2005) Nucleic Acids Res. 33, 3678–3690
14. Buttner, K., Nehring, S., and Hopfner, K. P. (2007) Nat. Struct. Mol. Biol. 14, 647–652
15. Guerrero, S. A., Hecht, H. J., Hofmann, B., Biebl, H., and Singh, M. (2001) Appl. Microbiol. Biotechnol. 56, 718–723
16. Potterson, L., McNicholas, S., Krissinel, E., Gruber, J., Cowtan, K., Emsley, P., Marshudov, G. N., Cohen, S., Perrakis, A., and Noble, M. (2004) Acta Crystallogr. Sect. D Biol. Crystallogr. 60, Pt. 12 Pt. 1, 2288–2294
17. Pape, T., and Schneider, T. R. (2004) J. Appl. Crystallogr. 37, 843–844
18. Schneider, T. R., and Sheldrick, G. M. (2002) Acta Crystallogr. Sect. D Biol. Crystallogr. 58, Pt. 10 Pt. 2, 1772–1779
19. Terwilliger, T. C., and Berendzen, J. (1999) Acta Crystallogr. Sect. D Biol. Crystallogr. 55, Pt. 4, 849–861
20. Terwilliger, T. C. (2000) Acta Crystallogr. Sect. D Biol. Crystallogr. 56, Pt. 8, 965–972
21. Cowtan, K. (2001) Acta Crystallogr. Sect. D Biol. Crystallogr. 57, Pt. 10, 1435–1444
22. McPhee, D. E. (1999) J. Struct. Biol. 125, 156–165
23. Mursudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) Acta Crystallogr. Sect. D Biol. Crystallogr. 53, Pt. 3, 240–255
24. Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Acta Crystallogr. Sect. D Biol. Crystallogr. 54, Pt. 5, 905–921
25. Bruns, C. M., Hubatsch, I., Riderstrom, M., Mannervik, B., and Tainer, J. A. (1999) J. Mol. Biol. 288, 427–439
26. Davis, I. W., Leaver-Fay, A., Chen, V. B., Block, J. N., Kapral, G. J., Wang, X., Murray, L. W., Arendall, W. B., III, Snoeyink, J., Richardson, J. S., and Richardson, D. C. (2007) Nucleic Acids Res. 35, W375–W383
27. Kvaratskhelia, M., and White, M. F. (2000) J. Mol. Biol. 295, 193–202
28. Roberts, J., and White, M. F. (2005) J. Biol. Chem. 280, 5924–5928
29. Reid, S. L., Parry, D., Liu, H. H., and Connolly, B. A. (2001) Biochemistry 40, 2484–2494
30. Lundblad, J. R., Laurance, M., and Goodman, R. H. (1996) Mol. Endocrinol. 10, 607–612
31. Deleted in proof
32. Rudolf, J., Makrantoni, V., Ineglewde, W. J., Stark, M. J., and White, M. F. (2006) Mol. Cell 23, 801–808
33. Ariza, A., Richard, D. J., White, M. F., and Bond, C. S. (2005) Nucleic Acids Res. 33, 1465–1473
34. Jelinska, C., Conroy, M. J., Craven, C. J., Hounslow, A. M., Bullough, P. A., Waltho, J. P., Taylor, G. L., and White, M. F. (2005) Structure 13, 963–971
35. Wadsworth, R. I., and White, M. F. (2001) Nucleic Acids Res. 29, 914–920
36. Schelert, J., Dixit, V., Hoang, V., Simbahan, J., Drozdz, M., and Blum, P. (2004) J. Bacteriol. 186, 427–437
37. Krissinel, E., and Henrick, K. (2007) J. Mol. Biol. 372, 774–797
38. Morris, P. D., and Raney, K. D. (1999) Biochemistry 38, 5161–5171
39. Byrd, A. K., and Raney, K. D. (2004) Nat. Struct. Mol. Biol. 11, 531–538
40. Marsh, V. L., McGeoch, A. T., and Bell, S. D. (2006) J. Mol. Biol. 357, 1345–1350
41. Brendza, K. M., Cheng, W., Fischer, C. J., Chesnik, M. A., Niedziela-Majka, A., and Lohman, T. M. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 10076–10081
42. Smith, A. J., Szczelkun, M. D., and Savery, N. J. (2007) Nucleic Acids Res. 35, 1802–1811
43. Waliho, J. W., Della-Viella, M., Skovraga, M., Croteau, D. L., Erie, D. A., and Van Houten, B. (2006) J. Biol. Chem. 281, 15227–15237
44. Keller, R. C., and Marians, K. J. (2006) J. Biol. Chem. 281, 15227–15237
45. Byrd, A. K., and Raney, K. D. (2006) Nucleic Acids Res. 34, 3020–3029
46. Michel, B., Grompone, G., Flores, M. J., and Bidnenko, V. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 12783–12788
47. Veaute, X., Delmas, S., Selva, M., Jeusset, J., Le Cam, E., Matic, I., Fabre, F., and Petit, M. A. (2005) EMBO J. 24, 180–189