A Partially Functional DNA Helicase II Mutant Defective in Forming Stable Binary Complexes with ATP and DNA

A ROLE FOR HELICASE MOTIF III*

(Received for publication, May 10, 1996, and in revised form, July 24, 1996)

Robert M. Brosh, Jr.‡ and Steven W. Matson¶¶

From the Department of Biology and the Curriculum in Genetics and Molecular Biology, University of North Carolina, Chapel Hill, North Carolina 27599-3280

To address the functional significance of motif III in *Escherichia coli* DNA helicase II, the conserved aspartic acid at position 248 was changed to asparagine. UvrDD248N failed to form stable binary complexes with either DNA or ATP. However, UvrDD248N was capable of forming an active ternary complex when both ATP and single-stranded DNA were present. The DNA-stimulated ATPase activity of UvrDD248N was reduced relative to that of wild-type UvrD with no significant change in the apparent *Km* for ATP. The mutant protein also demonstrated a reduced DNA unwinding activity. The requirement for high concentrations of UvrDD248N to achieve unwinding of long duplex substrates likely reflects the reduced stability of various binary and ternary complexes that must exist in the catalytic cycle of a helicase. The data suggest that motif III may act as an interface between the ATP binding and DNA binding domains of a helicase.

The *uvrD*248N allele was also characterized in genetic assays. The D248N protein complemented the UV-sensitive phenotype of a *uvrD* deletion strain to levels nearly equivalent to wild-type helicase II. In contrast, the mutant protein only partially complemented the mutant phenotype. A correlation between the level of genetic complementation and the helicase activity of UvrDD248N is discussed.

The unwinding of double-stranded DNA (dsDNA)† is essential for the complex processes of DNA replication, repair, and recombination (Lohman, 1992; Lohman, 1993; Matson et al., 1994; Matson and Kaiser-Rogers, 1990). The unwinding of duplex RNA and DNA-RNA hybrids is equally important in transcription (Brennan et al., 1987), RNA processing (Schmid and Linder, 1992; Wassarman and Steitz, 1991), and translation (Ray et al., 1985; Schmid and Linder, 1992; Wassarman and Steitz, 1991). Helicases catalyze these reactions by disrupting the hydrogen bonds between the complementary base pairs of duplex nucleic acid in an NTP hydrolysis-dependent reaction.

Helicases of prokaryotic, eukaryotic, and viral origin have been isolated and described (Lohman, 1992; Lohman, 1993; Matson, 1991; Matson et al., 1994; Matson and Kaiser-Rogers, 1990; Thommes and Hubscher, 1992). However, the mechanism of helicase-catalyzed nucleic acid unwinding is not fully understood. It has been proposed that most, perhaps all, helicases function as oligomers to provide for multiple DNA binding sites (Lohman, 1992; Lohman, 1993). In support of this notion, the assembly state of all DNA helicases examined thus far is a dimer or hexamer (Lohman, 1992; Lohman, 1993). A rolling model for helicase-catalyzed DNA unwinding has been proposed for Rep protein (Wong et al., 1993). Conformational changes in the protein, induced by ligand binding, allow the protein complex to alternate between states in which subunits interact with single-stranded DNA (ssDNA) or dsDNA. Evidence for such a mechanism has not yet been provided for other helicases.

Sequence analysis of helicases and putative helicases from a wide array of sources has revealed seven distinct regions of homology (Gorbalenya et al., 1988; Gorbalenya et al., 1989; Hodgman, 1988). These regions are referred to as motifs I, Ia, and II–VI. Presumably, these motifs partially define functional domains relevant to the known biochemical activities of these proteins. For example, motifs I and II are known to define a nucleotide binding/hydrolysis domain (Brosh and Matson, 1995; George et al., 1994; Fry et al., 1986; Jindal et al., 1994; Pause and Sonenberg, 1992; Walker et al., 1982; Washburn and Kushner, 1993; Zavitz and Marians, 1992). The functional significance of the remaining five motifs remains to be established but likely relates to activities required for polynucleotide unwinding.

To continue to address the structure-function relationship of a DNA helicase, we have replaced a highly conserved aspartic acid residue with asparagine in motif III of UvrDp (DNA helicase II). Biochemical characterization of the UvrDD248N protein has been performed to further our understanding of the functional significance of motif III in the mechanism of helicase-catalyzed DNA unwinding. *Escherichia coli* DNA helicase II is an excellent model enzyme for these studies because it has been extensively characterized *in vitro*. Helicase II is a DNA-stimulated ATPase (Richet and Kohiyama, 1976) that prefers to unwind a dsDNA substrate on which a 3'-ssDNA tail is available for binding to initiate unwinding (Matson, 1986). At higher protein concentrations the enzyme can also initiate unwinding from a blunt end or nick (Runyon et al., 1990; Runyon and Lohman, 1989; Runyon and Lohman, 1993). Helicase II unwinds DNA in a protein concentration-dependent manner (Abdel-Monem et al., 1977; Kuhn et al., 1979; Matson and George, 1987) and may act as a helix-destabilizing protein remaining bound to the ssDNA generated during an unwinding reaction (Runyon et al., 1990; Wessel et al., 1990).

Biochemical and genetic studies have demonstrated a role for helicase II in two DNA repair pathways, methyl-directed...
mismatch repair (Grillley et al., 1990; Lahue et al., 1989; Modrich, 1989) and UvrABC-mediated nucleotide excision repair (Caron et al., 1985; Husain et al., 1985; Orren et al., 1991). The uvrDDD248N allele was assessed for its ability to complement in DNA repair in an effort to characterize the functional significance of the conserved aspartic acid as it relates to the defined roles of helicase II in the cell.

**EXPERIMENTAL PROCEDURES**

**Materials**

*Bacterial Strains—*E. coli* strain BL21(DE3) (ΔDE3 omyA T rbs-mua*) was obtained from Novagen, Inc. JH137 (K91 lacZ dinD1::Mud1 (Ap’/lac)) was obtained from P. Model (Rockefeller University). BL21(DE3)ΔuvrD and JH137ΔuvrD were previously constructed in this laboratory (George et al., 1994).*DNA and Nucleotides—Bacteriophage M13mp18 and M13mp7 ssDNAs and their derivatives were prepared as described (Lechner and Richardson, 1985). All unlabeled nucleotides were from U.S. Biological Corp. except ATP-γ-S, which was from Boehringer Mannheim. Plasmid pET81F1+ was kindly provided by Dr. P. J. Laipis (University of Florida). Plasmid pET81 was purchased from Novagen, Inc. Concentrations of DNA and nucleotides were determined by UV spectrophotometry using published extinction coefficients and are expressed as nucleotide equivalents.

*Enzymes—*Restriction endonucleases, DNA polymerase I (large fragment), phage T7 DNA polymerase, and phage T4 polynuclease kinase were purchased from New England Biolabs, Inc., or U.S. Biological Corp. The reaction conditions used were those suggested by the supplier.

*E. coli* DNA helicase II was purified from BL21(DE3)/pLysS cells containing the pET9d-H2wt expression plasmid (George et al., 1994). The UvrDDD248N protein was purified from BL21(DE3)ΔuvrD/pLysS cells containing the pET81-H2D248N expression plasmid. Ten liters of cells grown in LB media (Miller, 1972) plus ampicillin (200 μg/ml) were harvested 4 h after induction. The procedure of Runyon et al. (1993) was used to purify both the wild-type and mutant proteins except for the following modifications in the purification of UvrDDD248N. After pooling the peak fractions containing UvrDDD248N from the heparin-agarose column, the pool was dialyzed against buffer A (20 mM KPO4 (pH 7.2), 10% glycerol, 5 mM β-mercaptoethanol, and 0.5 mM EDTA) plus 150 mM NaCl and loaded onto a DEAE-Sepharose A-50 column (4 × 2.5-cm inner diameter) that had been equilibrated in buffer A plus 150 mM KCl. The DEAE-Sepharose column was washed to base line with buffer A and 150 mM KCl, and the protein eluted with a linear 10-column volume KCl gradient (150 mM-500 mM) in buffer A. The fractions containing UvrDDD248N eluted in the range of 300–440 mM KCl. These fractions were pooled, diluted to a protein concentration of 100 μg/ml, and dialyzed against buffer B (20 mM Tris-HCl (pH 8.0), 1% Triton X-100, and 0.5 mM EDTA) plus 150 mM NaCl. The dialysate was loaded on to dsDNA-cellulose and further purified as described previously (Runyon et al., 1993). Final protein concentration was determined using the helicase II extinction coefficient (Runyon et al., 1993).

**Methods**

*Site-directed Mutagenesis and DNA Constructions—*For mutagenesis and expression, the *uvrD* gene was cloned into pET81F1+, a *T7* expression vector with a bacteriophage *φ1* origin of replication (Tanhauser et al., 1992). pET81F1+ was digested to completion with *Bam*II, the 5’-overhang ends were filled in using DNA polymerase I (large fragment), and the DNA was extracted with phenol-chloroform followed by an ethanol precipitation. The DNA was resuspended in TE (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) and digested with *Nco*I. pET9d-H2wt was digested with *Hind*III, the 5’-overhang ends were filled in using DNA polymerase I (large fragment), and the DNA was extracted with phenol-chloroform followed by an ethanol precipitation. The DNA was resuspended in TE and digested with *Nco*I and the 2.5-kilobase pair DNA fragment containing the entire isolated fragment was ligated to agarose gel and extracted using Geneclean (Bio 101, Inc.). The 2.5-kilobase pair DNA fragment was ligated into pET81F1+, prepared as described above, resulting in the construction pET81-H2wt. pET81-H2wt was the target for site-directed mutagenesis using published procedures (Kunkel et al., 1991; Zoller and Smith, 1989). Oligonucleotide 5’-CTGGTGCTGCATTACCCAGCATGAT’-3’ was used to alter codon 248 of *uvrD* from *GAT* (Asp) to *AAT* (Asn). The entire *uvrD* gene in pET81-H2D248N and pET81-H2wt was sequenced on a model 373A DNA Sequencer (Applied Biosystems) using the *Tag* DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems) to confirm the mutation at position 248. A 2.1-kilobase pair *Not*I fragment, containing a portion of the *uvrD* gene containing the uvrDDD248N mutation plus some 3’-flanking sequence was moved from pET81-H2D248N into pET9d-H2wt to yield pET84-H2D248N.

*Genetic Assays—*The viability of bacterial strains exposed to ultraviolet (UV) light was measured as described previously (Brosh and Matson, 1995). The spontaneous mutation frequency for each cell strain was determined as described (George et al., 1994).

*DNA Binding Assays—*A nicotinucleoside filter binding assay was used to measure binding of UvrD protein to DNA (Matson and Richardson, 1986). Binding reaction mixtures (20 μl) contained 25 μM Tris-Cl (pH 7.5), 3 mM MgCl2, 20 μM NaCl, 5 mM 2-mercaptoethanol, the 92-base pair (bp) partial duplex helicase substrate (approximately 2 μM nucleotide phosphate) (3.26 × 106 cpm μmol−1), and the indicated amount of helicase II. To determine the effect of nucleotide on DNA binding, reaction mixtures were altered to contain either 3 mM ATP-γ-S, 3 mM ADP, or no nucleotide as indicated in the appropriate figure legend. The reaction mixture was incubated at 37 °C for 10 min, diluted with 1 ml of 37 °C prewarmed reaction buffer containing 50 μg/ml bovine serum albumin, and passed through a nitrocellulose filter (0.45 μm; Whatman) at a flow rate of 4 ml/min. The filters were washed with an additional 2 ml of prewarmed reaction buffer. The dried filters were counted in a liquid scintillation counter. Background radioactivity bound in the absence of UvrD protein was subtracted from total radioactivity bound to the filter. Nicotinucleoside filters were pretreated by boiling in deionized distilled water for 20 min and stored in reaction buffer containing 50 μg/ml bovine serum albumin.

*Data Analysis—*The fraction of the 92-bp partial duplex DNA substrate specifically bound by UvrD protein was determined from the nicotinucleoside filter binding assays. A Hill plot was used to analyze the data (Yong and Romano, 1995).

\[ K_d \left( 1 - f \right)/f = K_1 \] (Eq. 1)

\[ \log [P] = \log \left( 1 - f \right) + \log K_d \] (Eq. 2)

*K* is the dissociation constant of the DNA-protein complex, [Pt] is the total concentration of UvrD protein present in the reaction, and 1/f is the ratio of the amount of the bound DNA over the total amount of DNA present in the reaction. The logarithm of ([1 − f]) and the intercept represented the logarithm of *K*.

*ATP Binding Assays—*Gel filtration was used to measure binding of ATP to UvrD proteins (Sung et al., 1988). Binding reaction mixtures (30 μl) contained 10 mM Tris-HCl (pH 7.5), 4 mM MgCl2, 206 μM [*γ*]ATP (465 cpm/μmol), and either 2.2 μM UvrD (monomer) or 2.2 μM UvrDD248N (monomer). The reaction mixture was incubated at room temperature for 25 min and then applied, at room temperature, onto a 12.5-mL Sephadex G-50 column equilibrated in 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 4 mM MgCl2, and 50 μg/ml bovine serum albumin. Four drop (110-μl) fractions were collected. A 20-μl aliquot of each fraction was mixed with 3 μl of Eosinfect A (National Diagnostics) and counted in a liquid scintillation counter. Background counts were determined by performing the same experiment in the absence of UvrD protein and subtracted from experimental data. Protein elution from the column was determined by immunoblotting.

*Proteolytic Digestions of Helicase II Protein—*UvrD and UvrDD248N proteins were lightly digested with a-chymotrypsin (Sigma) as described previously (Chao and Lahman, 1990). Reaction mixtures (15 μl) contained either 1.8 μM UvrD (monomer) or 1.5 μM UvrDD248N (monomer). DNA fragmentation was determined by electrophoresis of helicase II, reaction mixtures contained 2 μM ATP-γ-S, 3 mM MgCl2, and/or 214 μM M13mp7 ssDNA (nucleotide phosphate). One μl of freshly prepared trypsin (5 ng/μl) was added to each reaction mixture, and the proteolysis reaction was incubated at 37 °C for 2 min. The reactions were quenched by adding 15 μl of 2 × SDS gel loading buffer (Laemmli, 1970), and the samples were boiled for 2 min prior to electrophoresis on a 12% polyacrylamide gel run in the presence of SDS. The gel was stained with Coomassie Blue and destained.

*Glutaraldehyde Cross-linking—*Protein cross-linking reactions were performed at room temperature at a final concentration of UvrD or UvrDD248N of 2 μM (monomer). Cross-linking reaction mixtures (20 μl) contained 20 mM Tricine (pH 8.3), 50 mM NaCl, 20% glycerol, 5 mM
Biochemical Characterization of UvrDD248N

The procedure used to purify UvrDD248N was altered slightly (see “Experimental Procedures”) as compared with that used to purify the wild-type protein, since the mutant protein failed to bind to a ssDNA-cellulose column. The modified procedure resulted in purification of the mutant protein to apparent homogeneity as judged by the presence of a single band of protein on an SDS-polyacrylamide gel (see Fig. 3, lane F).

DNA Binding—The marked reduction in binding to ssDNA-cellulose prompted us to examine the DNA binding properties

\[\begin{align*}
\text{DNA} & \text{binding} & \text{by} & \text{UvrDD248N} & \text{and} & \text{UvrD}.
\end{align*}\]

FIG. 2. DNA binding by UvrDD248N and UvrD. Binding assays were performed as described under “Experimental Procedures” using the indicated amounts of UvrD (□) or UvrDD248N (○). DNA binding incubations were performed in the presence of no nucleotide (A), 3 mM ADP (B), or 3 mM ATP-γS (C). Background values were typically less than 2% and have been subtracted from the reported data. These data represent the average of at least three independent determinations.

**RESULTS**

The conserved primary sequence and relative position of motif III in a number of *E. coli* and *Sacccharomyces cerevisiae* DNA helicases (Fig. 1) suggest that this region of the protein may have functional significance in the mechanism of helicase-catalyzed unwinding. To begin to address the functional role of motif III in DNA helicases the highly conserved aspartic acid in motif III of the well-characterized UvrD protein from *E. coli* was altered by site-directed mutagenesis. The negatively charged aspartic acid was replaced by a neutral asparagine (Fig. 1). The resulting mutant, designated UvrDD248N, was expressed, purified, and biochemically characterized.

**Biochemical Characterization of UvrDD248N**

The D248N amino acid substitution in motif III of UvrD.

**The relative positions of the seven previously described helicase motifs are shown with the conserved sequence of motif III (Gorbalenya et al., 1989), and Pif1p (Foury and Lahaye, 1994) were realigned with the other proteins in this study. Shown below the alignment is the sequence of UvrDD248N, in which the highly conserved aspartic acid is replaced with an asparagine.**

**FIG. 1.** The D248N amino acid substitution in motif III of UvrD.

The conserved primary sequence and relative position of motif III in a number of *E. coli* and *Sacccharomyces cerevisiae* DNA helicases (Fig. 1) suggest that this region of the protein may have functional significance in the mechanism of helicase-catalyzed unwinding. To begin to address the functional role of motif III in DNA helicases the highly conserved aspartic acid in motif III of the well-characterized UvrD protein from *E. coli* was altered by site-directed mutagenesis. The negatively charged aspartic acid was replaced by a neutral asparagine (Fig. 1). The resulting mutant, designated UvrDD248N, was expressed, purified, and biochemically characterized.

**RESULTS**

The conserved primary sequence and relative position of motif III in a number of *E. coli* and *Sacccharomyces cerevisiae* DNA helicases (Fig. 1) suggest that this region of the protein may have functional significance in the mechanism of helicase-catalyzed unwinding. To begin to address the functional role of motif III in DNA helicases the highly conserved aspartic acid in motif III of the well-characterized UvrD protein from *E. coli* was altered by site-directed mutagenesis. The negatively charged aspartic acid was replaced by a neutral asparagine (Fig. 1). The resulting mutant, designated UvrDD248N, was expressed, purified, and biochemically characterized.

**Biochemical Characterization of UvrDD248N**

The procedure used to purify UvrDD248N was altered slightly (see “Experimental Procedures”) as compared with that used to purify the wild-type protein, since the mutant protein failed to bind to a ssDNA-cellulose column. The modified procedure resulted in purification of the mutant protein to apparent homogeneity as judged by the presence of a single band of protein on an SDS-polyacrylamide gel (see Fig. 3, lane F).

**DNA Binding**—The marked reduction in binding to ssDNA-cellulose prompted us to examine the DNA binding properties
of the mutant protein. Nitrocellulose filter binding assays using the 92-bp partial duplex DNA as a ligand were performed with both the UvrDD248N and the wild-type proteins (Fig. 2).

The mutant enzyme was dramatically compromised in its ability to bind DNA in the absence of nucleotide or in the presence of ADP (Fig. 2, A and B). However, in the presence of the poorly hydrolyzed ATP analog ATPγS, UvrDD248N demonstrated a binding isotherm similar to that measured for the wild-type enzyme (Fig. 2C). Moreover, the D248N mutant protein was able to bind ssDNA-cellulose when the resin was equilibrated in buffer containing 2 mM ATP (data not shown).

To determine the apparent dissociation constants ($K_D$) for the protein-DNA complexes, the data were analyzed by a Hill plot as described under “Experimental Procedures.” In the absence of nucleotide, the apparent $K_D$ values were $7 \pm 1$ nM (monomer) and $165 \pm 10$ nM (monomer), respectively (Table I). In the presence of ADP the apparent $K_D$ values were $9 \pm 1$ nM (monomer) and $340 \pm 10$ nM (monomer), respectively. Thus, the stability of the UvrDD248N-protein-DNA complex formed in the absence of nucleotide or in the presence of ADP was significantly reduced compared with the corresponding wild-type protein-DNA complex. In the presence of ATPγS, the apparent $K_D$ values for the wild-type and mutant proteins were $3.8 \pm 1$ nM (monomer) and $1.7 \pm 1$ nM (monomer), respectively. In contrast to the results presented above, the wild-type and the mutant protein-DNA complexes, formed in the presence of ATPγS, are nearly equal in stability.

Partial Proteolytic Cleavage—To probe further the interactions of UvrDD248N with ssDNA and ATP we examined the pattern of proteolytic cleavage by chymotrypsin in the presence of various ligands. Chao and Lohman (1990) have demonstrated that helicase II is protected from cleavage by chymotrypsin when ATPγS and/or ssDNA is present and bound by the protein. We performed a set of limited proteolysis experiments with the wild-type and UvrDD248N proteins to test for the ability of these proteins to bind ATPγS and/or M13 ssDNA (Fig. 3). In the absence of ATPγS or ssDNA, UvrDD248N is cleaved by chymotrypsin into two fragments with approximate molecular masses of 53 and 29 kDa (lane G). The same cleavage pattern is obtained in the presence of ATPγS or ssDNA (lane H). Thus, the mutation has not drastically altered the conformation of the protein. Wild-type UvrD protein is protected from cleavage when ssDNA is present as expected (lane D). In contrast, UvrDD248N is not protected from limited chymotrypsin cleavage when ssDNA is added (lane I). This result suggests that the mutant protein failed to bind stably to ssDNA in agreement with the nitrocellulose filter binding assay results. Binding of ATPγS to wild-type helicase II also rendered the protein resistant to chymotrypsin cleavage (lane C). In contrast, UvrDD248N failed to be protected from cleavage by ATPγS (lane H), suggesting that the mutant is also defective in its interaction with nucleoside triphosphate in the absence of DNA. When both ATPγS and ssDNA are present, both UvrDD248N (lane J) and the wild-type protein (lane E) are protected from proteolytic cleavage. Thus, the mutant protein was capable of forming a ternary complex with ATP and ssDNA when both ligands were present. However, the above results indicate that UvrDD248N exhibited a defect in binding ssDNA in the absence of ATP as well as a defect in binding ATP in the absence of ssDNA. Similar results were obtained using trypsin as the source of protease, although the cleavage pattern was different (data not shown).

ATP Binding—The observation that UvrDD248N was susceptible to cleavage by chymotrypsin in the presence of ATPγS led us to directly determine if the mutant protein was compromised in its ability to form a binary complex with ATP. [3H]ATP binding by UvrD and UvrDD248N was measured using a gel filtration assay (see “Experimental Procedures”). The results are shown in Table II. ATP binding by UvrDD248N was reduced to less than 15% that of wild-type helicase II as measured by this assay. This result is consistent with the results from the proteolytic cleavage protection experiments in which the presence of ATP failed to render UvrDD248N resistant to chymotrypsin cleavage. Taken together, these data demonstrate that Asp248 is required for stable binding of ATP to the protein in the absence of DNA.

ATPase and Helicase Activity—To further characterize the UvrDD248N mutant protein we measured the ssDNA-stimulated ATPase activity of the mutant protein and compared this activity with that of the wild-type protein. The ATP hydrolysis constants $k_{cat}$ and $K_m$ are shown in Table III. The $k_{cat}$ value of UvrDD248N was 5.0 s$^{-1}$, approximately 4% that of the wild-type protein. No significant change in the apparent $K_m$ for ATP was detected for the mutant protein compared with wild-type UvrD. These reactions are performed in the presence of excess ssDNA and thus must reflect the interaction of ATP with the protein in the ternary enzyme-DNA-ATP complex. The specificity constant ($k_{cat}/K_m$) of UvrDD248N was reduced 19-fold.

### Table I

| Protein       | Nucleotide cofactor | $K_D$ (nM) |
|---------------|---------------------|------------|
| UvrD          | ATPγS               | 3.8        |
| UvrD          | ADP                 | 9.2        |
| UvrD          | None                | 7.2        |
| UvrDD248N     | ATPγS               | 1.7        |
| UvrDD248N     | ADP                 | 340        |
| UvrDD248N     | None                | 165        |
Table II
Binding of ATP by UvrD and UvrDD248N

| Protein  | ATP bound/UvrDp | fmol/pmol monomer |
|----------|----------------|------------------|
| UvrD     | 43.8           |                  |
| UvrDD248N| 5.7            |                  |

Table III

The $k_{cat}$ and $K_m$ for DNA-stimulated ATP hydrolysis catalyzed by UvrD and UvrDD248N

| Enzyme   | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ |
|----------|-----------|-------|---------------|
| UvrD     | 123 ± 7.6 | 92.7 ± 35 | 1.3          |
| UvrDD248N| 5 ± 2.3   | 74.7 ± 22 | 0.067        |

Compared with wild-type UvrD, reflecting the decrease in $k_{cat}$ with little change in $K_m$. Therefore, the replacement of Asp248 with an asparagine negatively impacts the ATP hydrolysis reaction catalyzed by helicase II but does not significantly alter the interaction of ATP with the enzyme in the presence of ssDNA.

To determine the impact of the D248N mutation on helicase activity, we examined the unwinding activity of the mutant protein using both partial duplex and blunt duplex DNA substrates. Titrations of the UvrD and UvrDD248N proteins with partial duplex substrates are shown in Fig. 4. Wild-type helicase II, at a concentration of 0.68 nM (monomer), unwound 73% of the 20-bp partial duplex DNA substrate in a 10-min reaction (Fig. 4A). The UvrDD248N mutant protein achieved a comparable level of unwinding (72%) but at a considerably higher protein concentration (9.36 nM monomer). Wild-type helicase II unwound nearly 75% of the 92-bp partial duplex substrate at a concentration of 34 nM (monomer) (Fig. 4B). The D248N mutant required a concentration of 187 nM (monomer) to unwind approximately 70% of the 92-bp partial duplex. Thus the unwinding efficiency of the D248N mutant is moderately compromised on relatively short duplexes of 20 and 92 bp in length. However, the UvrDD248N mutant retained unwinding activity on short partial duplex substrates even at low concentrations of protein.

The unwinding reactions catalyzed by the wild-type and D248N mutant proteins were distinctly different as the length of the duplex region was increased to 346 bp (Fig. 4C). At 93.6 nM (monomer), UvrDD248N unwound only 2% of the 346-bp partial duplex, whereas wild-type enzyme unwound more than 75% of the partial duplex substrate at 90.5 nM (monomer). However, substantial levels of unwinding were attained using the UvrDD248N mutant at higher protein concentrations in a markedly biphasic titration curve. In contrast, the wild-type protein unwinds increasing amounts of the 346-mer at each incremental protein concentration. The discrepancy between the helicase activity of the wild-type and D248N mutant proteins is yet more dramatic on the 851-bp partial duplex DNA substrate (Fig. 4D). At a UvrDD248N concentration of 187 nM (monomer), 3% of the 851-mer is unwound. In contrast, the wild-type protein unwound more than 80% of the 851-bp substrate at 181 nM (monomer). The wild-type UvrD protein exhibited a typical protein concentration-dependent displacement of the 851-nucleotide fragment. The UvrDD248N mutant protein only achieved significant unwinding of the 851-bp partial duplex at the highest concentration of protein tested, again showing a pronounced biphasic protein titration in these unwinding reactions. Clearly, much higher concentrations of UvrDD248N were required to achieve unwinding of relatively long partial duplex substrates of 343 and 851 bp in length. However, at these high protein concentrations a significant fraction of the substrate was unwound.

Helicase II has also been shown to unwind blunt-ended duplex DNA substrates (Runyon et al., 1990; Runyon and Lohman, 1989). We tested the ability of the UvrDD248N mutant protein to unwind a 346-bp blunt duplex DNA substrate (Fig. 5). Wild-type helicase II, at a concentration of 136 nM (monomer), unwound about 50% of the 346-bp fragment in a 10-min reaction (Fig. 5A). Less than 0.5% of the 346-bp blunt duplex was unwound by the UvrDD248N mutant at a comparable concentration of protein (141 nM monomer). An increase in wild-type helicase II concentration to 543 nM (monomer) resulted in the unwinding of 95% of the 346-bp duplex. At a comparable mutant enzyme concentration (562 nM monomer) only 1.7% of the 346-bp blunt duplex was unwound. We also examined the blunt duplex unwinding reaction catalyzed by...
Helicase II has been shown to self-assemble to form a dimer or higher order oligomer in solution (Runyon et al., 1993). Upon binding DNA, the dimeric form of helicase II is stabilized. To test the ability of UvrDD248N to oligomerize, we examined protein dimer formation by treating the UvrDD248N mutant protein with glutaraldehyde and detecting cross-linked dimers on denaturing SDS-polyacrylamide gels as described (Runyon et al., 1993). At a concentration of 2 μM (monomer), both UvrDD248N and UvrD formed dimers and higher order oligomers in solution as previously reported for Uvrd (Runyon et al., 1993) (data not shown). In the presence of 2 mM ATPγS and 4.2 mM (dT)10, the extent of dimer formation was enhanced, as expected, for both proteins. These results demonstrate that UvrDD248N has the ability to form dimers and that these dimers are stabilized upon binding DNA and nucleotide, similar to results previously reported for wild-type helicase II.

**Genetic Characterization of the uvrDD248N Allele**

We have genetically characterized the uvrDD248N allele by assessing its ability to complement the loss of the wild-type protein in two DNA repair pathways. For these studies plasmids containing uvrD (pET9d-H2wt) or uvrDD248N (pET9d-H2D248N) were transformed into *E. coli* JH137 or JH137ΔuvrD. Immunoblot data have demonstrated that helicase II expression from the pET9d-H2wt plasmid (in the absence of induction) is slightly less than that produced from the chromosome of JH137 (George et al., 1994).

To assess the ability of UvrDD248N to function in UvraABC-mediated nucleotide excision repair, we determined the relative UV sensitivity of strains expressing the mutant or wild-type proteins (Fig. 6). UvrDD248N, supplied on a plasmid, complemented the UV-sensitive phenotype of the *uvrD* deletion strain JH137ΔuvrD to nearly the same level as the wild-type *uvrD* allele. Thus, the UvrDD248N protein retains its ability to function in UvraABC-mediated nucleotide excision repair, although this protein is not as efficient as the wild-type protein.

The UvrDD248N mutant protein was also examined for its ability to complement the loss of helicase II in methyl-directed mismatch repair. The mutant allele was introduced into a *uvrD* deletion strain on a plasmid, and the spontaneous mutation frequency at the rpoB locus was measured and compared with relevant strains as shown in Table IV. The relative mutability values of JH137ΔuvrD, JH137ΔuvrDpET9dΔH2D248N, and JH137ΔuvrDpET9dΔH2wt were found to be 185, 16.7, and 0.94, respectively. Thus, *uvrD248N* partially complemented JH137ΔuvrD in methyl-directed mismatch repair. The wild-type allele, expressed from the same plasmid, exhibited full complementation of the repair deficiency. The partial genetic complementation of UvrDD248N in mismatch repair suggests that the mutant protein can function in unwinding at least some of the hemimethylated duplexes containing a mismatch. The mutation frequencies of JH137 (with a wild-type copy of *uvrD* on the chromosome) transformed with pET9d-H2wt and pET9d-H2D248N were also measured and found to be equivalent to that of JH137. Thus, *uvrD248N* is recessive to the wild-type allele in methyl-directed mismatch repair.

**DISCUSSION**

To explore the functional significance of motif III in superfamily 1 DNA helicases we have mutagenized a very highly conserved amino acid residue within this region of *E. coli* DNA helicase II (see Fig. 1). Motif III is separated by 16–18 amino acids from motif II, which is known to be involved in nucleotide binding/hydrolysis, and is separated from motif IV, whose function is still unknown, by 24–26 amino acid residues (Hodgman, 1988). Thus, in addition to amino acid sequence conservation, there is conservation of the spacing between motifs II and III.
Relativemutabilityvalueswereobtainedbydividingthemutationfrequencyofthecellstraininquestionbythefrequencyofthewild-typestrain.

ThisinterpretationderivesfromthefactthatthemotifsIand IIofthedomaininDNAhelicaseII.

...andbetweenmotifsIIIand IV.

TherehavebeeninonobiochemicalstudiestodateregardingthefunctionofmotifIIIinsuperfamily1DNAhelicases. However,mutationalofthehighlyconservedglycineinmotifIII(seeFig.1)oftheherpessimplexvirusUL5proteintoaserinederesultedinthefailureofthemutantproteintonoviralDNAreplication(ZhuandWeller,1992).AlthoughtheUL5gene productisonlyonecomponentofthethree-protein-helicaseprimasecomplex,thistheresultsdemonstratethatmotifIIIhasasignificantcellularfunction. Tocontinue to define thefunctional significanceofmotifIIIwehavemutatedahighlyconervedmotifIIIresidueinE.coliDNAhelicaseII,anenzyme thathasbeenextensivelycharacterizedbothbiochemicallyandgenetically. Thespecificmutantcharacterizedhere, UvrDD248N, lackstheneutralchargeontheconservedasparticacidresiduethatwasthrough nuanced asparagin. Limitedproteolysis studies with both trypsin and chymotrypsin have shown thatthisisoetric change does not grossly alter thestructureoftheUvDprotein.

ThemostsignificantdefectwehaveneedintheUvrDD248N mutantproteinistheinabilityofthisproventoformstablenonary complexes with either ATP or ssDNA. Thefault of UvrDD248N to stably bind ssDNAwasinitiallynotedduri ngpurificationofthemutantprotein. Typically, helicase II is purified usingssDNA-cellulose column. TheUvrDD248N mutant failed to bind ssDNA-celluloseunderthestandard conditionsusedforpurification. However, ifthecolumnwas equilibratedwithabuffercontainingATP,theproteinboundwith apparentlynormalaffinity. Nitrocellulosefilterbindingassays andlimitedproteolysis protectionexperiments have confirmed thattheUvrDD248NmutantdoesnotstablybindDNAinthe absenceofATP. However, theproteinexhibitsanormal interaction with ssDNAwhenATP istpresent, as evidencedbya bindingisomershallow to thatofthewildtypeproteininin trocelulosefilterbindingassays. Thus, anary complex consisting of enzyme-ATP-ssDNAcan beformed andis enzymaticallyas activeas anATPaseandaDNahelicase. Forthis reason, motif III doesnotappear to bethessDNA binding domain inDNA helicase II.

Wealsenote thatthemitmutantproteinisnotabletostably bind ATP intheabsenceofssDNAas demonstratedusing limitedproteolysis protectionexperiments andgelfiltration analysis ofATP-enzyme complexes. Thisresult is surprisingin view ofthe commonlyheldnotion thatmotifs I and II ofthe DNA helicasesareinvolvedinATP binding/hydrolysis. We suggest thattheaspartic acid residue inmotifIIIisinvolved instabilizingtheinteractionbetweenATPandtheenzymeandisnotlikelytobedirectlyinvolvedinATP binding. Supportforthisinterpretationderivesfromthefactthatthe$K_m$ for ATP inthessDNA-stimulatedATPase reaction catalyzed by UvrDD248Nisessentiallythesameasthatofthewildtype enzyme. Moreover, there is evidence ofaweak interaction with ATP inthegelfiltration experiments (see Table II).

Takentogether, theresultspresentedhere suggestthatthe aspartic acid residue inmotifIIIofDNA helicase II is involved

| Strain          | Relevant genotype | Mutation frequency ($\times 10^{-3}$) | Relative mutability |
|-----------------|-------------------|--------------------------------------|--------------------|
| JH137           | uvrd                 | 2.6 ± 1.2                          | 1                  |
| JH137Δuvrd      | Δuvrd               | 488 ± 61.4                          | 185                |
| JH137ΔuvrdΔET9d-H2wt | uvrd              | 2.5 ± 1.4                          | 0.94               |
| JH137ΔuvrdΔET9d-H2D248N | uvrdDD248N | 44.1 ± 12.4                        | 16.7               |
| JH137ΔET9d-H2D248N | uvrdD' / uvrdDD248N | 2.1 ± 1.1                          | 0.80               |

---

4. M. Brosh and S. W. Matson, unpublished results.
be expected due to the decrease in tantisals are compromised. A decrease in helicase activity might catalytic efficiency are impaired in this mutant enzyme.

plexes. It is possible that both binary complex stability and main, which in turn impairs the stability of the binary complexes. It is possible that both binary complex stability and catalytic efficiency are impaired in this mutant enzyme.

The unwinding reaction catalyzed by the UvrDD248N mutant is also compromised. A decrease in helicase activity might be expected due to the decrease in $k_{cat}$ for ATP hydrolysis, since ATP hydrolysis is coupled to the unwinding reaction. However, it is not clear how tightly coupled these two reactions might be; therefore, it is possible that a decreased $k_{cat}$ for ATPase activity would result in only a small change in helicase activity. In the case of the UvrDD248N mutant, the helicase reaction is significantly reduced, and relatively high concentrations of mutant enzyme compared with wild-type helicase II are required to achieve substantial unwinding of partial duplex substrates. Moreover, the unwinding titration curves obtained using the mutant protein are markedly biphasic as compared with the relatively smooth hyperbolic curves obtained using the wild-type protein. The mutant enzyme exhibits a very low rate of unwinding at low protein concentrations and a rate of unwinding that approaches that exhibited by the wild-type protein at relatively high concentrations of the UvrDD248N protein dissociating from partially unwound DNA is significantly increased. In this case, substantially more of the mutant enzyme may be required to unwind a long duplex region, since helicase II molecules constantly dissociate from the partially unwound substrate after they hydrolyze ATP. In support of this interpretation we have shown that the addition of SSB stimulated the unwinding reaction catalyzed by UvrDD248N. Moreover, this interpretation is consistent with the notion that helicase II serves as its own helix-stabilizing protein during an unwinding reaction and underscores the importance of this property of helicase II.

We also note that the UvrDD248N mutant exhibits a reduced ability to unwind the 346-bp blunt duplex DNA substrate. Even at very high concentrations, the mutant enzyme performed poorly in a 10-min reaction. However, the mutant enzyme was able to catalyze significant unwinding of the blunt duplex substrate at high protein concentrations with long periods of incubation. The reduction in unwinding from a blunt duplex end may be the result of a defect in either initiation or propagation of the unwinding reaction. It is clear that the enzyme is compromised in propagation of the unwinding reaction, particularly on longer duplex regions. A defect in initiating an unwinding reaction from a blunt duplex end would further diminish overall unwinding of the substrate. Runyon and Lohman (1993) have provided evidence to suggest that the initiation of unwinding on blunt duplex DNA is a rate-limiting step. The substantial reduction in blunt duplex unwinding by UvrDD248N would suggest that the mutant enzyme is compromised in both the initiation and propagation steps. This would be consistent with the instability of the binary complex formed by the enzyme with DNA or ATP.

Despite the fact that the ssDNA-dependent ATPase and helicase reactions catalyzed by UvrDD248N are significantly reduced relative to the wild-type protein, this mutant is able to complement the absence of UvrDp in both excision repair and methyl-directed mismatch repair. Complementation provided by the UvrDD248N protein is at nearly wild-type levels in UvrABC-mediated excision repair of UV-induced damage. The fact that the UvrDD248N protein retained substantial activity with the 20-bp partial duplex substrate would indicate that the mutant enzyme should be capable of excising the 12–13-mer released in excision repair. A second requirement of UvrDp in this pathway is the turnover of UvrCp (Husain et al., 1985; Orren et al., 1991). The level of genetic complementation provided by the UvrDD248N mutant suggests that the mutant enzyme functions in this capacity.

Complementation of the helicase II deficit in methyl-directed mismatch repair is apparently not as robust as that observed for repair of UV-induced DNA damage, although it is difficult to directly compare results from the two different assays. It is possible that the complementation we observe is due to repair requiring short track length excision and resynthesis (i.e., the GATC is located close to the base pair mismatch), while repair requiring long track length excision and resynthesis is inefficient. This is consistent with the results of in vitro helicase assays indicating a more pronounced reduction in helicase activity on longer partial duplex substrates. We also note that the uvrDD248N allele is recessive to the wild-type allele in complementation of the defect in methyl-directed mismatch repair. Assuming that helicase II functions as a dimer and that the UvrDD248N protein can interact with the wild-type protein, this interaction must not dramatically interfere with the ability of the wild-type enzyme to function in the mismatch repair pathway. Alternatively, a population of wild-type helicase II dimers may exist, since expression of UvrDD248N using the pET9d expression vector is expected to be slightly less than expression of the wild-type protein from the chromosome (George et al., 1994).

Several studies that have begun to address the functional significance of motif III in members of a distantly related group of proteins (which include DNA and RNA helicases) designated

FIG. 7. Proposed kinetic mechanism for ATP hydrolysis by DNA helicase II (H2). See “Discussion” for details.

### Motif III Mutant of E. coli DNA Helicase II

#### FIG. 7. Proposed kinetic mechanism for ATP hydrolysis by DNA helicase II (H2). See “Discussion” for details.

[Diagram of the proposed kinetic mechanism for ATP hydrolysis by DNA helicase II (H2).]
superfamily 2 (Gorbaleny et al., 1988) are consistent with the results reported here. A motif III mutation in mammalian translation initiation factor (eukaryotic initiation factor 4A) eliminated the RNA-unwinding activity of the enzyme yet enhanced the ATPase activity by 3-fold, suggesting that the ATPase and helicase activities of the mutant protein had become uncoupled (Pause and Sonenberg, 1992). A dominant negative mutation in motif III of the splicing factor PRP2 slightly reduced the RNA-stimulated ATPase activity of the mutant protein and resulted in the accumulation of stalled splicing complexes, leading the authors to propose that the dominant negative phenotype was due primarily to a defect in the putative RNA helicase activity of PRP2 protein (Plumpton et al., 1994). A mutation in motif III of E. coli RecG protein significantly reduced the ATPase activity of the mutant protein relative to wild-type protein and abolished the enzyme’s ability to catalyze branch migration of Holliday junction intermediates (Sharples et al., 1994). Finally, mutations in motif III of herpesvirus protein UL9 decrease viral DNA replication (Martinez et al., 1992), suggesting that motif III of superfamily 2 is functionally significant.

We suggest that the aspartic acid residue in motif III of DNA helicase II, and perhaps in other helicases, is important in stabilizing the binary complex between protein and ssDNA or protein and ATP. Motif III is unlikely to be the primary binding site for either ligand. It is more likely that motif III serves as an interface between the ATP binding domain and the ssDNA binding domain of a DNA helicase and is important in maintaining the proper alignment between the two domains.

Acknowledgments—We thank Jeff Doyle for help in site-directed mutagenesis and preliminary biochemical characterization of UvrDD248N mutant protein. We also thank Dr. Dan Bean for the motif III alignment with yeast DNA helicases. We thank Dr. Dan Bean, Mark Hall, and Leah Mechanic for critical reading of the manuscript. We are grateful to Susan Whitfield for preparation of the artwork.

REFERENCES

Abdul-Moneim, M., Durwald, H., and Hoffman-Berling, H. (1977) Eur. J. Biochem. 79, 39–45
Aboussekhra, A., Chanet, R., Zegaga, Z., Cassier-Chauvat, C., Heude, M., and Fabre, P. (1988) Nucleic Acid Res. 17, 7211–7219
Brennan, C. A., Dombroski, A. J., and Platt, T. (1987) Cell 48, 945–952
Brush, R. M., Jr., and Matson, S. W. (1995) J. Bacteriol. 177, 5612–5621
Budd, M. E. and Campbell, J. L. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7642–7646
Caron, P. R., Kushner, S. R., and Grossman, L. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 4925–4929
Chao, K., and Lehman, T. M. (1990) J. Biol. Chem. 265, 1067–1076
Foury, F., and Lahaye, A. (1987) EMBO J. 6, 1441–1449
Frost, L. S., Ippen-Ihler, K., and Skurray, R. A. (1994) Microbiol. Rev. 58, 162–210
Fry, D. C., Ruby, S. A., and Mildran, A. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 907–911
George, J. W., Brush, R. M., Jr., and Matson, S. W. (1994) J. Mol. Biol. 235, 424–455
Gorbalenya, A. E., Koonin, E. V., Donchenko, A. P., and Blinov, V. M. (1988) FEBS 235, 16–24
Gorbalenya, A. E., Koonin, E. V., Donchenko, A. P., and Blinov, V. M. (1989) EMBO J. 8, 4713–4719
Grilly, M., Holmes, L., Yashar, B., and Modrich, P. (1990) Mutat. Res. 236, 253–267
Hodgman, T. C. (1988) Nature 333, 22–23
Husain, I., Van Houten, B., Thomas, D. C., and Abdul-Moneem, M. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 6774–6778
Jindal, H. K., Yang, C. B., Wilson, G. M., Tam, P., and Astell, C. R. (1994) J. Biol. Chem. 269, 3283–3289
Kuhn, B., Abdul-Moneem, M., Krell, H., and Hoffman-Berling, H. (1979) J. Biol. Chem. 254, 11343–11350
Kunkel, T. A., Bebenek, K., and McClary, J. (1991) Methods Enzymol. 204, 125–139
Laemmli, U. K. (1970) Nature 227, 680–685
Lahue, R. S., Au, K. G., and Modrich, P. (1989) Science 245, 160–164
Lehner, R. L., and Richardson, C. C. (1983) J. Biol. Chem. 258, 11185–11196
Lohman, T. M. (1993) J. Biol. Chem. 268, 2269–2272
Matson, S. W. (1991) Proc. Natl. Acad. Sci. Rev. 40, 289–326
Matson, S. W., and George, J. W. (1987) J. Biol. Chem. 262, 2066–2076
Matson, S. W., and Kaiser-Rogers, K. A. (1990) Annu. Rev. Biochem. 59, 289–329
Matson, S. W., and Richardson, C. C. (1987) J. Biol. Chem. 258, 14009–14016
Matson, S. W., and Richardson, C. C. (1985) J. Biol. Chem. 260, 2281–2287
Matson, S. W., Bean, D. W., and George, J. W. (1994) BioEssays 16, 13–22
Miller, J. H. (1972) Experiments in Molecular Genetics, pp. 431–433, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Modrich, P. (1989) J. Biol. Chem. 264, 6597–6600
Orren, D. K., Selby, C. P., Hearst, J. E., and Sancar, A. (1991) J. Biol. Chem. 267, 785–788
Pulse, A., and Sonenberg, N. (1992) EMBO J. 11, 2643–2654
Plumpton, M., McGarvey, M., and Beggs, J. D. (1994) EMBO J. 13, 879–887
Ray, B. K., Lawson, T. G., Kramer, J. C., Black, A., Abrahams, R. D., Merrick, W. C., Thach, R. E. (1985) J. Biol. Chem. 260, 7651–7658
Richet, E., and Kohiyama, M. (1976) J. Biol. Chem. 251, 808–812
Runyon, G. T., Bear, D. G., and Lohman, T. M. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 6383–6387
Runyon, G. T., and Lohman, T. M. (1989) J. Biol. Chem. 264, 17502–17512
Runyon, G. T., and Lohman, T. M. (1993) Biochemistry 32, 4128–4138
Runyon, G. T., Weng, L., and Lohman, T. M. (1993) Biochemistry 32, 602–612
Sharples, G. T., Whitby, M. C., Ryder, L., and Lloyd, R. G. (1994) Nucleic Acids Res. 22, 308–313
Sung, P., Higgins, D., Prakash, L., and Prakash, S. (1988) EMBO J. 7, 3263–3269
Tanhauser, S. M., Jewell, D. A., Tu, C. K., Silverman, D. N., and Laips, P. J. (1992) Gene 117, 113–117
Thommes, P., and Hubercher, U. (1992) Chromosoma 101, 467–473
Walker, J. M., Sarsate, M., Runswick, M. J., and Gay, N. J. (1982) EMBO J. 1, 945–951
Washburn, B. K., and Kushner, S. R. (1993) J. Bacteriol. 175, 341–350
Wassarman, D. A., and Steitz, J. A. (1981) Nature 348, 463–464
Wessel, R., Muller, H., and Hoffmann-Berling, H. (1990) Eur. J. Biochem. 256, 695–701
Weng, L., Amaratunga, M., and Lohman, T. M. (1993) J. Biol. Chem. 268, 20386–20391
Wood, E. R., and Matson, S. W. (1989) J. Biol. Chem. 264, 8297–8303
Yung, Y., and Romano, L. J. (1995) J. Biol. Chem. 270, 24509–24517
Zavitz, K. H., and Marians, K. J. (1994) J. Biol. Chem. 269, 6933–6940
Zhu, L., and Weller, S. R. (1992) J. Virol. 66, 469–479
Zoller, M. J., and Smith, M. (1991) Recombinant DNA Methodology (Wu, R., Grossman, L., and Moldave, K., eds) p. 537, Academic Press, Inc., San Diego