A site-directed mutation in motif IV of Escherichia coli DNA helicase II (UvrD) was generated to examine the functional significance of this region. The highly conserved arginine at position 284 was replaced with alanine to construct UvrD-R284A. The ability of the mutant allele to function in methyl-directed mismatch repair and UvrABC-mediated nucleotide excision repair was examined by genetic complementation assays. The R284A substitution abolished function in both DNA repair pathways. To identify the biochemical defects responsible for the loss of biological function, UvrD-R284A was purified to apparent homogeneity, and its biochemical properties were compared with wild-type UvrD. UvrD-R284A failed to unwind a 92-base pair duplex region and was severely compromised in unwinding a 20-base pair duplex region. The $K_m$ of UvrD-R284A for ATP was significantly greater than 3 m$\text{M}$ compared with 80 m$\text{M}$ for UvrD. A large decrease in ATP binding was confirmed using a nitrocellulose filter binding assay. These data suggested that the R284A mutation severely reduced the affinity of helicase II for ATP. The reduced unwinding activity and loss of biological function of UvrD-R284A was probably the result of decreased affinity for ATP. These results implicate motif IV of superfamily I helicases in nucleotide binding and represent the first characterization of a helicase mutation outside motifs I and II that severely impacted the $K_m$ for ATP.

Helicase-catalyzed unwinding of double-stranded nucleic acid molecules is required in all aspects of DNA and RNA metabolism including replication, DNA repair, recombination, transcription, translation, RNA processing, and bacterial conjugation (1–10). Helicases couple the energy derived from hydrolysis of nucleoside 5'-triphosphates (NTPs)\(^1\) to the disruption of hydrogen bonds between the complementary bases of a double helix. The mechanism of unwinding is not known although models have been proposed and are currently being tested (7, 11, 12). These models are based on the formation of active oligomers, which provide multiple DNA binding sites for the helicase. Most, if not all, helicases appear capable of forming either a dimer or hexamer, and evidence suggests that the oligomer is an active species (7).

Helicases are ubiquitous in nature, with numerous examples in viral, prokaryotic, and eukaryotic organisms. Extensive computer-aided sequence analysis of numerous helicases has uncovered a series of short, conserved amino acid motifs (13–15). This has allowed grouping of helicases into four families based on the extent of amino acid similarity and on the organization of these conserved regions. These families presumably represent evolutionary relationships. Superfamilies I and II are the largest and most closely related groups, whereas superfamily III and family IV have unique motif compositions that differ considerably from those in superfamilies I and II and from each other (16).

Analysis of helicases with mutations in highly conserved residues in several of the so-called “helicase motifs” has suggested a biochemical role of some of these regions in helicase function. For example, motifs I and II, first described as the Walker A and B sequences in a large family of NTP binding proteins (17), have been directly implicated in NTP binding and/or hydrolysis (18–22). The function of the remaining helicase motifs is less clear. Roles for motif VI in nucleic acid binding and ATP hydrolysis have been proposed for various superfamily II RNA helicases (23–25). Motif V has been implicated in single-stranded DNA (ssDNA) binding (26) and motif III in coordination of ATP and ssDNA binding (27) for superfamily I helicases. The recent crystal structure of PcrA, a superfamily I DNA helicase from Bacillus steatorrhoeus, suggested that all seven of the conserved helicase motifs are clustered together in the vicinity of the ATP binding site (28). Thus, all of the motifs may be involved in ATP binding and/or hydrolysis, at least for those enzymes with structures similar to PcrA.

Escherichia coli DNA helicase II, the product of the uvrD gene, is a well characterized DNA helicase. This enzyme is a required component of the UvrABC-mediated nucleotide excision repair pathway (29, 30) and the methyl-directed mismatch repair pathway (31). Less defined roles in recombination and DNA replication have also been suggested (18, 19, 32–40). The purified enzyme unwinds DNA with 3′ to 5′ polarity (41) and is capable of initiating unwinding from a nick, its presumed biological substrate in the repair pathways (42–44). UvrD belongs to helicase superfamily I along with other E. coli helicases such as Rep, RecB, RecD, TraI, and helicase IV (helD gene product) (13, 16), as well as a large number of eukaryotic viral helicases and several yeast helicases.

UvrD has previously been the subject of biochemical and genetic studies involving mutation of highly conserved residues in motifs I, II, and III (18, 19, 27, 44). Currently, very little biochemical information exists on the specific role of motif IV in superfamily I and II helicases although its importance for biological function has been demonstrated in genetic studies (45–47). In this report, the functional significance of motif IV in E. coli DNA helicase II was addressed by site-directed mu-
EXPERIMENTAL PROCEDURES

Materials

Bacterial Strains—E. coli BL21(DE3) (F ompT [lon] hsdS r m gal dcm DE3) was from Novagen, Inc. E. coli JH137 (K91 lacZ dinD1 [Mudl (Ap’ lac)] ) was obtained from Dr. P. Model (Rockefeller University). BL21(DE3) uvrD and JH137 uvrD were constructed previously (18).

DNA and Nucleotides—pET81F1 was from Dr. P. J. Laips (University of Florida), and pET9d, pET11d, and pLysS were from Novagen, Inc. M13mp7 ssDNA was prepared as described previously (48). Unlabeled nucleotides were from U. S. Biochemicals Corp. Radioactively labeled nucleotides were from Amersham Corp. pET9d-UvrD and pET11d-UvrD were constructed previously in this laboratory (18).

Enzymes—Restriction endonuclease EcoRI was from New England Biolabs Inc. and was used as recommended by the supplier.

To overexpress helicase II prior to purification, a 10-liter culture of mid-log phase BL21(DE3)/pET81F1-UvrD or a 2-liter culture of mid-log phase BL21(DE3)/pET11d-UvrD cells containing pET9d-UvrD (27), con- 
sidered the first description of a mutation that results in the absence of enzyme replication less than 5% of the total signal and was subtracted from the experimental data. The nitrocellulose filters used in the DNA and ATP binding experiments (0.45 μ type HA, Millipore Corp.) were pre-treated by soaking in 0.1 M KOH for 40 min followed by extensive washing with deionized, distilled water.

Gluutaraldehyde Cross-linking—The presence of a dimeric helicase II species was monitored by glutaraldehyde cross-linking as described previously (27, 49). Reactions (20 μl) contained 20 μM Tricine (pH 8.3), 50 μM NaCl, 5 μM MgCl2, 5 μM 2-mercaptoethanol, 18% glycerol, and either 1.5 μM UvrD (monomer) or 1.4 μM UvrD-R284A (monomer). When present, the oligonucleotide (dT)9, and ATP. S were included at a concentration of 1.7 μM and 3 μM, respectively. Cross-linking was initiated by the addition of 1 μM Electron glutaraldehyde (EM grade, Electron Microscopy Sciences) to a final concentration of 0.01%. Reactions were incubated at room temperature for 30 min and then quenched with 21 of 100 μM lysis extract and 20 μl of gel-loading buffer (see above). Samples were boiled for 2 min, and the products were resolved on a 9.6% polyacrylamide gel (32:1 cross-linking ratio) in the presence of 0.1% SDS.

DNA sequences were determined by sequencing dideoxynucleotide primer reactions (20 μl) using 2-mercaptoethanol, 20% glycerol, 0.01% bromphenol blue) and boiled for 2 min. Products were resolved on a 12% polyacrylamide gel (32:1 cross-linking ratio) in the presence of 0.1% SDS and visualized by staining with Coomassie Brilliant Blue R-250 (Sigma).

Nitrocellular Filter Binding Assays—The binding of UvrD and UvrD-R284A to ssDNA was determined by measuring the retention of [32P]DNA on nitrocellulose filters as described previously (52). Reactions (20 μl) contained 25 μM Tris-HCl (pH 7.5), 3 μM MgCl2, 20 mM NaCl, 5 mM 2-mercaptoethanol, 50 μM bovine serum albumin, and a [32P]-labeled 92-bp partial duplex helicase substrate (approximately 1.3 μM nucleotide phosphate) subjected to liquid scintillation counting. Background binding in the absence of enzyme represented less than 5% of the total signal and was subtracted from the experimental data. Apparent K0 values were calculated as described previously (27, 53).

Binding of [3H]ATP to UvrD was also examined by nitrocellulose filter binding. Reactions (20 μl) contained 25 μM Tris-HCl (pH 7.5), 3 μM MgCl2, 50 mM NaCl, 6.3 μM 2-mercaptoethanol, 100 μM bovine serum albumin, 12.5% glycerol, and 2.3 μM UvrD or UvrD-R284A (monomer). Binding was initiated by addition of [3H]ATP (1.1 Ci/mmol) to a final concentration of 200 nM at 0 °C. After 4 min, 15 μl of each reaction was applied directly to a nitrocellulose filter presoaked in reaction buffer at 4 °C. Filters were rinsed once with 750 μl of reaction buffer at a flow rate of 4 ml/min, dried, and subjected to liquid scintillation counting. Background binding in the absence of enzyme represented less than 1% of the total signal and was subtracted from the experimental data. The nitrocellulose filters used in the DNA and ATP binding experiments (0.45 μ type HA, Millipore Corp.) were pre-treated by soaking in 0.4 M KOH for 40 min followed by extensive washing with deionized, distilled water.

Gluutaraldehyde Cross-linking—The presence of a dimeric helicase II species was monitored by glutaraldehyde cross-linking as described previously (27, 49). Reactions (20 μl) contained 20 μM Tricine (pH 8.3), 50 μM NaCl, 5 μM MgCl2, 5 μM 2-mercaptoethanol, 18% glycerol, and either 1.5 μM UvrD (monomer) or 1.4 μM UvrD-R284A (monomer). When present, the oligonucleotide (dT)9, and ATP. S were included at a concentration of 1.7 μM and 3 μM, respectively. Cross-linking was initiated by the addition of 1 μM Electron glutaraldehyde (EM grade, Electron Microscopy Sciences) to a final concentration of 0.01%. Reactions were incubated at room temperature for 30 min and then quenched with 21 of 100 μM lysis extract and 20 μl of gel-loading buffer (see above). Samples were boiled for 2 min, and the products were resolved on a 9.6% polyacrylamide gel (32:1 cross-linking ratio) in the presence of 0.1% SDS.

DNA sequences were determined by sequencing dideoxynucleotide primer reactions (20 μl) using 2-mercaptoethanol, 20% glycerol, 0.01% bromphenol blue) and boiled for 2 min. Products were resolved on a 12% polyacrylamide gel (32:1 cross-linking ratio) in the presence of 0.1% SDS and visualized by staining with Coomassie Brilliant Blue R-250 (Sigma).

Nitrocellular Filter Binding Assays—The binding of UvrD and UvrD-R284A to ssDNA was determined by measuring the retention of [32P]DNA on nitrocellulose filters as described previously (52). Reactions (20 μl) contained 25 μM Tris-HCl (pH 7.5), 3 μM MgCl2, 20 mM NaCl, 5 mM 2-mercaptoethanol, 50 μM bovine serum albumin, and a [32P]-labeled 92-bp partial duplex helicase substrate (approximately 1.3 μM nucleotide phosphate) subjected to liquid scintillation counting. Background binding in the absence of enzyme represented less than 5% of the total signal and was subtracted from the experimental data. Apparent K0 values were calculated as described previously (27, 53).

Binding of [3H]ATP to UvrD was also examined by nitrocellulose filter binding. Reactions (20 μl) contained 25 μM Tris-HCl (pH 7.5), 3 μM MgCl2, 50 mM NaCl, 6.3 μM 2-mercaptoethanol, 100 μM bovine serum albumin, 12.5% glycerol, and 2.3 μM UvrD or UvrD-R284A (monomer). Binding was initiated by addition of [3H]ATP (1.1 Ci/mmol) to a final concentration of 200 nM at 0 °C. After 4 min, 15 μl of each reaction was applied directly to a nitrocellulose filter presoaked in reaction buffer at 4 °C. Filters were rinsed once with 750 μl of reaction buffer at a flow rate of 4 ml/min, dried, and subjected to liquid scintillation counting. Background binding in the absence of enzyme represented less than 1% of the total signal and was subtracted from the experimental data. The nitrocellulose filters used in the DNA and ATP binding experiments (0.45 μ type HA, Millipore Corp.) were pre-treated by soaking in 0.4 M KOH for 40 min followed by extensive washing with deionized, distilled water.

Gluutaraldehyde Cross-linking—The presence of a dimeric helicase II species was monitored by glutaraldehyde cross-linking as described previously (27, 49). Reactions (20 μl) contained 20 μM Tricine (pH 8.3), 50 μM NaCl, 5 μM MgCl2, 5 μM 2-mercaptoethanol, 18% glycerol, and either 1.5 μM UvrD (monomer) or 1.4 μM UvrD-R284A (monomer). When present, the oligonucleotide (dT)9, and ATP. S were included at a concentration of 1.7 μM and 3 μM, respectively. Cross-linking was initiated by the addition of 1 μM Electron glutaraldehyde (EM grade, Electron Microscopy Sciences) to a final concentration of 0.01%. Reactions were incubated at room temperature for 30 min and then quenched with 21 of 100 μM lysis extract and 20 μl of gel-loading buffer (see above). Samples were boiled for 2 min, and the products were resolved on a 9.6% polyacrylamide gel (32:1 cross-linking ratio) in the presence of 0.1% SDS.
Presumably, these motifs represent sites of functional significance that have been evolutionarily conserved. The amino acid sequences of motif IV from the E. coli superfamily I helicases, including UvrD, are shown in Fig. 1. To evaluate the functional significance of motif IV, a mutant uvrD allele was constructed containing an arginine to alanine substitution at position 284 (uvrD-R284A). This arginine is the most highly conserved residue in motif IV. It is found in all identified members of superfamily I. The ability of the mutant protein to substitute for the wild-type protein in two DNA repair pathways was examined in genetic complementation studies. In addition, the UvrD-R284A protein was purified and biochemically characterized.

**Genealogical Characterization of UvrD-R284A**

DNA helicase II is an essential component of two DNA repair pathways, methyl-directed mismatch repair and UvrABC-mediated nucleotide excision repair (29–31). The ability of UvrD-R284A to function in each pathway was tested using genetic complementation assays. The frequency of spontaneous mutant formation in E. coli strain JH137 uvrD was 161-fold higher than its parent strain JH137 due to loss of a functional methyl-directed mismatch repair system (55, 56). JH137 uvrD was also highly sensitive to UV light due to loss of UvrABC-mediated nucleotide excision repair (57). Plasmids pET9d-UvrD and pET9d-UvrD-R284A were transformed into JH137 uvrD to examine the ability of UvrD and UvrD-R284A to restore the wild-type spontaneous mutant frequency and UV resistance. The level of uninduced expression of the uvrD gene from pET9d-UvrD in JH137 uvrD was determined previously and was only slightly less than expression from the JH137 uvrD allele in JH137 (15). The frequency of formation of spontaneous mutants at the rpoB locus (Rif\(^+\) phenotype) was determined as described under “Experimental Procedures.” Wild-type uvrD, when introduced on the pET9d plasmid, fully restored methyl-directed mismatch repair function as indicated by a relative mutability of 0.6 compared with 1.0 for the parental strain JH137 (Table I).

**Biochemical Characterization of UvrD-R284A**

To identify the biochemical defects responsible for the loss of biological function of UvrD-R284A, mutant and wild-type helicase II were purified as described previously (49). UvrD was purified to apparent homogeneity as evidenced by the presence of a single protein species on an SDS-polyacrylamide gel (Fig. 3, lane A). Purified UvrD-R284A was contaminated by three faint species migrating slightly faster than UvrD-R284A (Fig. 3, lane D). These species appeared to be proteolytic products of UvrD-R284A because they all reacted strongly with anti-helicase II antibody (data not shown). This suggested that the mutant enzyme was somewhat less stable than the wild-type enzyme. However, UvrD-R284A folded normally as indicated by a chymotrypsin cleavage pattern that was identical to that of UvrD (Fig. 3, lanes B and E). In the presence of ssDNA, both UvrD-
R284A and uvrD were protected from chymotrypsin cleavage (Fig. 3, lanes C and F). Furthermore, proteolytic products did not accumulate with long term enzyme storage. Given these results and the minimal representation of the smaller species in the total protein, it was unlikely that their presence influenced the data presented in this study.

**DNA Binding**—The ability of UvrD-R284A to bind to DNA was tested using a nitrocellulose filter binding assay and the 92-bp partial duplex helicase substrate. Although the substrate contains a short duplex region, the assay measured primarily the affinity of the protein for ssDNA due to the lack of significant binding of helicase II to double-stranded DNA. DNA binding isotherms were generated using a constant ligand concentration and varying concentrations of helicase II (Fig. 4). A Hill plot was used to calculate apparent equilibrium dissociation constants from these data as described previously (27, 53). There was no significant difference between the DNA binding properties of UvrD-R284A and UvrD (apparent $K_d$ of 11 and 17 nM, respectively). Thus, the highly conserved arginine residue in motif IV was not required for nucleic acid binding. This conclusion was further supported by the results of limited proteolysis experiments (see Fig. 3), which demonstrated that both UvrD and UvrD-R284A were protected from chymotrypsin cleavage when an ssDNA ligand was present and presumably bound by the enzyme.

**Dimerization**—A glutaraldehyde cross-linking procedure was used to detect dimerization of UvrD and UvrD-R284A (49). Glutaraldehyde catalyzes the formation of a covalent bond between primary amines in close proximity, providing a simple method for detecting protein-protein interactions in solution. UvrD and UvrD-R284A, at a relatively high concentration, were each exposed to a low concentration of glutaraldehyde in the absence or presence of ATP S or oligonucleotide (dT)$_{15}$. The products were resolved on an SDS-polyacrylamide gel and stained with Coomassie Blue. No difference in the dimerization of UvrD and UvrD-R284A was observed in the absence or presence of ATP S or oligo(dT)$_{15}$ (data not shown).

**ATP Hydrolysis**—Helicase II catalyzes a DNA-stimulated ATP hydrolysis reaction that is coupled to the unwinding reaction. To begin to evaluate the ATPase activity of UvrD-R284A, we attempted to measure a $K_m$ for ATP. Wild-type UvrD exhibited a standard hyperbolic saturation curve as a function of increasing ATP concentration with a $K_m$ of 80 μM (Fig. 5A). The $K_m$ value of UvrD-R284A for ATP was too high to be determined because the maximal velocity could not be approached. The velocity of ATP hydrolysis catalyzed by UvrD-R284A was a linear function of ATP concentration up to 3 mM (Fig. 5B). Apparent substrate inhibition of the wild-type enzyme prevented accurate measurement of ATP hydrolysis velocities at substantially higher ATP concentrations (data not shown). The inability to saturate UvrD-R284A with ATP suggested that it was defective in nucleotide binding.

**ATP Binding**—To directly measure the affinity of helicase II for ATP, an initial attempt was made to determine the equilibrium dissociation constant, $K_d$. Several techniques were used without success, including fluorescence spectroscopy and nitrocellulose filter binding. Failure to determine a $K_d$ was the result of the limited solubility of helicase II and its relatively low affinity for ATP, which made it impossible to generate a complete and reproducible binding curve using these assays. In addition, we did not observe an intrinsic fluorescence change in helicase II upon ATP binding. However, having a $K_d$ for the UvrD-ATP interaction would not be useful for comparative purposes if we were unable to measure this value for UvrD-R284A. Instead, nitrocellulose filter binding assays were used as a qualitative comparison of ATP binding between UvrD and UvrD-R284A by measuring the amount of [3H]ATP bound at a single nucleotide concentration. These assays were performed at 0 °C and in the absence of DNA. Under these conditions, the turnover number for wild-type helicase II was approximately 0.002 s$^{-1}$ (data not shown). Table II shows that at 200 μM [3H]ATP, UvrD bound 0.92 pmol of ATP/pmol of enzyme. This fraction of ATP-bound enzyme was fairly consistent with a $K_m$ of 80 μM ATP. In contrast, UvrD-R284A bound only 0.015 pmol of ATP/pmol of enzyme, 1.6% of the wild-type value. These
The lack of genetic complementation in is equal to the $K$ in the reactions. Data is not equal to if multiple enzyme-bound intermediates are on the $K$ panel A. $K$ was calculated from the Eadie-Hofstee plot. The data in both panels A and B are averages obtained from at least three independent experiments with error bars representing standard deviations.

![Graph](image)

**Fig. 5. ATP hydrolysis by UvrD and UvrD-R284A.** ATP hydrolysis was measured as described under “Experimental Procedures” for UvrD (panel A) and UvrD-R284A (panel B). Reactions containing 2.1 nM UvrD or 229 nM UvrD-R284A (monomer) were initiated with the indicated concentration of $[^{3}H]$ATP and incubated at 37°C for 5 min (UvrD) or 10 min (UvrD-R284A). SigmaPlot (Jandel Scientific) was used to fit the data for UvrD in panel A to a standard rectangular hyperbola and to generate the linear regression for UvrD-R284A in panel B. The inset in panel A represents an Eadie-Hofstee plot of the data shown in panel A. $K_m$ was calculated from the Eadie-Hofstee plot. The data in both panels A and B are averages obtained from at least three independent experiments with error bars representing standard deviations.

**Table II**

| Enzyme     | pmol bound per pmol of enzyme |
|------------|-------------------------------|
| UvrD       | 0.92                          |
| UvrD-R284A | 0.015                         |

results suggested that UvrD-R284A was defective in its association with ATP.

**Helicase Activity**—The lack of genetic complementation in both DNA repair pathways suggested that the mutant protein was severely compromised in its DNA unwinding activity. The unwinding activity of UvrD-R284A was measured using two short partial duplex DNA substrates and was compared with UvrD (Fig. 6). The mutant enzyme failed to catalyze unwinding of a 92-bp duplex region and catalyzed feeble unwinding of a 20-bp duplex region in a 10-min incubation. At a UvrD-R284A concentration of 344 nM, there was no detectable unwinding of the 92-bp partial duplex substrate and less than 10 percent unwinding of the 20-bp partial duplex substrate. In contrast, wild-type helicase II unwound 50% of the 92-bp partial duplex substrate at a protein concentration of 25 nM and 50% of the 20-bp partial duplex substrate at a protein concentration of 0.5 nM. In a 100-min incubation, 197 nM UvrD-R284A unwound 39% of the 20-bp partial duplex. Under the same conditions, 344 nM mutant enzyme did not unwind a detectable fraction of the 92-bp substrate (data not shown).

**DISCUSSION**

The results presented here indicate that arginine 284 is required for high affinity binding of ATP, which suggests that motif IV is an integral part of the nucleotide binding site on DNA helicase II. This was initially surprising since it is generally believed that helicase motifs I and II are involved in NTP binding and/or hydrolysis. This notion stems from the fact that motifs I and II are common to a large population of NTP-binding proteins, including the DNA helicases (17). Moreover, biochemical studies on mutants of a wide range of helicases have generally supported a role for motifs I and II in NTP hydrolysis (18–22). Most of the mutations, however, did not affect NTP binding. Helicase motif IV is not conserved among all NTP binding proteins, which suggested that it might be important for a biochemical property common to helicases. However, UvrD-R284A exhibited a significant defect in ATP binding, and in light of the recent crystal structure of PcrA, this result can be readily explained (see below).

Efforts to define the kinetic parameters $K_m$ and $k_{cat}$ for the DNA-stimulated ATP hydrolysis reaction revealed an inability to saturate UvrD-R284A with ATP. Since the $k_{cat}$ reaction velocity was a linear function of ATP concentration up to 3 mM, the $K_m$ for ATP must be much greater than 3 mM. $K_m$ is defined as the concentration of substrate that provides half-maximal reaction velocity. If it is assumed that the $k_{cat}$ reaction catalyzed by helicase II can be described by simple Michaelis-Menten kinetics and that a minimal reaction is represented by the following scheme, where S is ATP,

$$ E + S = ES \stackrel{k_1}{\longrightarrow} E + P $$

then $K_m$ is equal to $(k_{-1}k_2)/k_1$. If $k_2$ is small compared with the dissociation rate constant $k_{-1}$, then $K_m$ is equal to the equilibrium dissociation constant, $K_m(k_{-1}/k_1)$. However, if $k_2$ is comparable with $k_{-1}$, then ES is not in equilibrium with $E + S$, and $K_m$ is not equal to $K_m$. Furthermore, $K_m$ does not approximate $K_{j}^{P}$ if multiple enzyme-bound intermediates are on the reaction pathway. In the case where multiple intermediates exist, $K_m$ reflects an overall dissociation constant for all of the enzyme-bound species (58). Because the kinetic mechanism for ATP hydrolysis catalyzed by helicase II is not known, it was not possible to unequivocally conclude that the large increase in the $K_m$ value of UvrD-R284A was the direct result of a decreased affinity for ATP. For example, a decrease in the rate of product release or changes in rate constants associated with conformational changes could also affect $K_m$. In fact, it is possible to envision a kinetic mechanism in which an increase in
Motif IV Mutant of E. coli DNA Helicase II

**Fig. 6. Helicase activity of UvrD and UvrD-R284A.** The unwinding of 92-bp (panel A) and 20-bp (panel B) partial duplex DNA substrates by UvrD (○) and UvrD-R284A (■) was measured as described under “Experimental Procedures.” Data represent the average of at least three independent experiments, and error bars are standard deviations.

$K_m$ is not related to ATP binding but, rather, is solely the result of a decrease in the rate of hydrolysis. Such a mechanism is very unlikely for helicase II, however, because several UvrD mutants have been characterized that exhibited a significant decrease in the $k_{cat}$ for ATP hydrolysis without affecting $K_m$ (18, 19, 44). To isolate and evaluate the initial ATP binding event, conditions were sought under which a negligible amount of ATP hydrolysis occurred. At 0 °C in the absence of DNA, the turnover number for ATP by UvrD was approximately 0.002 s$^{-1}$. No hydrolysis was detected under these conditions using UvrD-R284A. Thus, the nitrocellulose filter binding assay likely measured only $E\cdot S \rightleftharpoons ES$, again assuming the simple Michaelis-Menten scheme depicted above. Since the amount of ATP bound by UvrD-R284A was significantly decreased under these conditions, a decreased affinity for ATP was likely the primary defect in the mutant protein. Thus, the large increase in $K_m$ for ATP was the result of a decreased affinity for the substrate.

A defect in ATP binding would seem to be at odds with the results of proteolysis protection experiments (data not shown). The presence of ATP, like ssDNA, protects wild-type helicase II from cleavage by chymotrypsin (51). Based on the decreased binding of ATP by UvrD-R284A, we expected to see a large difference in the amount of ATP required to protect UvrD versus UvrD-R284A from proteolytic cleavage. In fact, the increase in the concentration of ATP required for protection of UvrD-R284A compared with UvrD was relatively small (less than 2-fold, data not shown). However, the structural changes responsible for proteolysis protection when ligands are present are not known. This fact, coupled with the lack of information on the kinetic mechanism for ATP hydrolysis catalyzed by helicase II, makes it possible that the proteolysis protection assay does not directly measure ATP binding. For example, an ATP concentration of 1.5–2.0 mM was required for significant protection of UvrD from cleavage by chymotrypsin. This is clearly inconsistent with a $K_m$ for ATP of 80 μM. This fact alone indicates that this assay measures something more complex than simple ATP binding. Furthermore, when the poorly hydrolyzed ATP analog ATP $S$ was used as a ligand, there was a more substantial increase in the concentration required for proteolytic protection of UvrD-R284A compared with UvrD (approximately 6-fold, data not shown). The results of the proteolysis protection study likely reflected a combination of processes such as ATP hydrolysis and conformational changes in the protein, in addition to nucleotide binding. Thus, the results of this series of experiments, while not fully explained, cannot be interpreted as evidence for a normal interaction with ATP.

Although there was clearly a serious defect in nucleotide binding, it was also possible that an additional defect existed in the hydrolytic mechanism. Such a defect would also have contributed to the inability of UvrD-R284A to unwind DNA and function in DNA repair pathways. It was possible to estimate a $K_m$ for UvrD-R284A from the data in Fig. 5 if we assumed the hydrolytic mechanism was unaffected. The initial slope of a reaction velocity versus substrate concentration graph, such as the one depicted in Fig. 5, is equal to $k_{cat}/K_m\cdot E_c$, where $E_c$ is the total enzyme concentration. If the hydrolytic mechanism of UvrD-R284A were unaffected by the mutation, then $k_{cat}$ should have been equal to that of UvrD. From the slope of the line in Fig. 5B and the UvrD $k_{cat}$ of 162 s$^{-1}$, an estimated $K_m$ for ATP for UvrD-R284A was 143 μM. The plot of initial velocity versus substrate concentration for UvrD-R284A was linear to at least 3 mM ATP. Since there was no deviation from linearity, it was concluded that the actual $K_m$ was significantly higher than 3 μM. Apparent substrate inhibition with wild-type helicase II at high ATP concentrations, coupled with the necessary modifications in reaction conditions, made it impossible to accurately measure hydrolysis at ATP concentrations significantly higher than 3 μM. Therefore, the relationship between the apparent $K_m$ for ATP and the 143 μM value estimated above was not determined. In light of these results, it remains possible that there was a defect in the hydrolytic mechanism of UvrD-R284A in addition to the severe defect in ATP binding.

The helicase activity of the UvrD-R284A mutant was significantly compromised, and the protein failed to function in vivo. Helicase activity is dependent on ATP hydrolysis, and it seemed reasonable to conclude that the lack of unwinding was...
due to the tremendous decrease in ATP hydrolysis that, in turn, was due to a decreased affinity of the mutant enzyme for ATP. However, the possibility that the R284A substitution also directly impaired the unwinding mechanism or the coupling between hydrolysis and unwinding cannot formally be ruled out. Based on the results presented here, the lack of biological function in both DNA repair pathways was probably a result of the ATP binding defect. With a $K_a$ for ATP significantly greater than 3 m,$^\text{m}$ it is unlikely that an intracellular ATP concentration of 3 m,$^\text{m}$ (59) would be sufficient to adequately populate the active helicase II pool and allow the enzyme to function properly in vivo.

To the best of our knowledge, UvrD-R284A represents the first helicase mutant, outside of motifs I and II, whose primary defect is in nucleotide binding. A helicase II mutant in motif III was defective in binding ATP but only in the absence of ssDNA (27). This mutant was also defective in binding ssDNA in the absence of ATP. In the presence of ssDNA, the motif III mutant bound ATP with normal affinity, and it was concluded that the motif III mutant was compromised in the ability to form stable binary complexes with its two ligands.

The ATP binding defect described in this study is consistent with the recently published crystal structure of the PcrA helicase (28). The overall amino acid sequence of the PcrA helicase from 	extit{B. stearothermophilus} is 44% identical to UvrD and 90% identical within the seven conserved helicase motifs. Notably, these seven motifs are clustered together at the base of the enzyme, forming the ATP-binding pocket. In view of the extensive amino acid identity, particularly among the conserved motifs, it is likely that the structure of the nucleotide binding sites of PcrA and UvrD closely resemble one another. From this structure, it seems likely that all seven motifs may be involved in nucleotide binding and/or hydrolysis, and thus, a role for motif IV in ATP binding is not surprising.

The PcrA structure indicates that motif IV forms a bridge connecting the two large domains of the protein at the bottom of the nucleotide binding pocket. This region lies near the adenine base of the bound nucleotide, and the authors suggest that a conserved tyrosine in motif IV makes a stacking interaction with the bound NTP. This tyrosine, which is not conserved in all superfamily I helicases, is immediately followed by the invariant arginine that was altered in this study (see Fig. 1). The apparent absence of additional specific interactions between the adenine base and nearby amino acid residues was suggested as an explanation for the lack of nucleotide specificity in reactions catalyzed by the PcrA helicase. UvrD, on the other hand, exhibits a strong preference for ATP or dATP (60), and it is possible that arginine 284 is involved in a specific interaction with the adenine base on ATP and dATP, whereas its counterpart in PcrA contributes less to specificity. Alternatively, arginine 284 may not directly contact the nucleotide but might mediate a local conformational change that places other residues, such as tyrosine 283, in proper position to interact with the NTP. Yet another possibility is that motif IV, acting as a bridge between the two large domains of the helicase, might mediate a global conformational change in the protein that is required for high affinity NTP binding. A more detailed description of the role of arginine 284 in ATP binding must await a high resolution structure for ATP-bound helicase II.

Acknowledgments—We thank Dr. Thomas Kunkel, Dr. David Porter, and Leah Mechanic for critical reading of the manuscript and Susan Whitfield for preparation of figures. We are especially grateful to Dr. David Porter for assistance with analyzing the steady-state kinetic data.