Both ATPase Sites of Escherichia coli UvrA Have Functional Roles in Nucleotide Excision Repair

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The roles of the two tandemly arranged putative ATP binding sites of Escherichia coli UvrA in UvrABC endonuclease-mediated excision repair were analyzed by site-directed mutagenesis and biochemical characterization of the representative mutant proteins. Evidence is presented that UvrA has two functional ATPase sites which coincide with the putative ATP binding motifs predicted from its amino acid sequence. The individual ATPase sites can independently hydrolyze ATP. The C-terminal ATPase site has a higher affinity for ATP than the N-terminal site. The invariable lysine residues at the ends of the glycine-rich loops of the consensus Walker type "A" motifs are indispensable for ATP hydrolysis. However, the mutations at these lysine residues do not significantly affect ATP binding. UvrA, with bound ATP, forms the most favored conformation for DNA binding. The initial binding of UvrA to DNA is chiefly at the undamaged sites. In contrast to the wild type UvrA, the ATPase site mutants bind equally to damaged and undamaged sites. Dissociation of tightly bound nucleoprotein complexes from the undamaged sites requires hydrolysis of ATP by the C-terminal ATPase site of UvrA. Thus, both ATP binding and hydrolysis are required for the damage recognition step enabling UvrA to discriminate between damaged and undamaged sites on DNA.

The surveillance of the integrity of genetic material by organisms is monitored by DNA repair enzymes. One of the best characterized repair processes in Escherichia coli is nucleotide excision repair. The nucleotide excision repair enzyme UvrABC endonuclease is unique in its dual incision pattern that is seven nucleotides 5' and three to four nucleotides 3' to a UV-damaged site (Yeung et al., 1983; Sancar and Rupp, 1983). This enzyme complex exhibits broad specificity by recognizing lesions that may cause a common secondary structure, formed in the major or minor groove such as bulky nucleotide adducts, UV-mimetic lesions, or intra- or interstrand cross-links (Grossman et al., 1988; Pu et al., 1986; Van Houten, 1990).

The UvrA protein is suggested to be the damage recognition subunit of the endonuclease. UvrA possesses a DNA-independent ATPase/GTPase (Seeberg and Steinum, 1982; Oh et al., 1986). Its binding affinity to DNA is enhanced by ATP and DNA damage (Seeberg and Steinum, 1982; Yeung et al., 1986a). A monomer-dimer equilibrium of UvrA protein is established in the presence of ATP (Oh et al., 1989; Orren and Sancar, 1989, Mazur and Grossman, 1991), and it is shifted toward dimer formation in the presence of ATPγS or ADP (Oh et al., 1989). The UvrAB protein complex manifests an enhanced ATPase activity dependent on DNA (Oh et al., 1989), which drives a helicase activity (Oh and Grossman, 1987, 1989). The helicase activity of the UvrABC complex is limited to a short stretch of about a 22-base-paired region that requires either ATP or dATP hydrolysis for its activity (Oh and Grossman, 1987, 1989). The endonuclease activity of the UvrABC complex stably bound damaged DNA is driven by ATP binding and not its hydrolysis (Caron and Grossman, 1988).

The predicted amino acid sequence of UvrA and UvrB proteins, two of the subunits of the UvrABC endonuclease, have sequence motifs common to many ATP binding proteins and ATPases (Husain et al., 1986; Doolittle et al., 1986; Arikan et al., 1986; Backendorf et al., 1986; Walker et al., 1982; Fry et al., 1986; Higgins et al., 1988). Most of these ATPases contain two distinct motifs, an "A" type consensus of a hydrophobic stretch of β-strand-GXXGXG (a flexible glycine-rich loop)-KS/T (α-helix) (Fig. 1) and a "B" type consensus of hydrophobic stretch of β-strand-DE/D (Gorbulevna and Koonin, 1990). It is suggested that the central flexible loop of the A motif binds the phospholyl moiety of ATP possibly by forming a giant anion "hole" (Schulz, 1987). It is thought that the conserved aspartate residue of the B motif chelates Mg2+ or MgNTP (Fry et al., 1986). The UvrA protein is one of the few proteins to have two ATP binding motifs tandemly arranged in a linear sequence (Fig. 1). It is generally believed that internal duplication or exon shuffling during evolution may generate such tandem motifs (Doolittle et al., 1986; Dorit et al., 1990).

In this study, we have investigated the role(s) of the two nucleoside triphosphate binding motifs of UvrA in UvrABC-mediated excision repair. It is our goal to understand whether the two motifs are functional and what roles they play in excision repair. This would serve as a useful prototype model to study proteins with two tandemly arranged ATP binding motifs. This question is approached by generating site-directed mutations at each motif as well as both motifs. The invariable lysine residue at the end of the glycine-rich loop in the A motif was mutated to an alanine, a polar glutamine, or a conservative arginine residue (Fig. 2). Mutagenesis of the invariable lysine residue in proteins containing a single ATP binding motif in two of the repair proteins, namely UvrB and...
RAD3, and other proteins such as adenylate kinase, led to the loss of their functionality (Seeley and Grossman, 1989, 1990; Sung et al., 1988; Reinstein et al., 1988). The present study suggests that both of the ATP binding motifs of UvrA are indispensable for its functioning and localizes the roles of ATP binding and hydrolysis in the pre-incision partial repair reaction.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains—**E. coli strains JM109 (recA1, endA1, hsdR17, relA1, SupE44, X-, Δlac-proAB), [F', trdD63, proAB, lacY162ΔM15]) and CJ236 (delu, ungI, thiI, relA1/pCJ105(Cm')] were used as the uracil incorporated DNA repair proficient and deficient strains, respectively, in the site-directed mutagenesis experiments. The uraA deletion mutant strain HI1.1A (araD39, Δ[araL]7697, ΔluxC74, galU, galK, Strα, ΔourA) was kindly provided by Dr. J. A. Brandsma (Leiden University, The Netherlands).

**Molecular Cloning—**The uraA gene was excised from plAC1, a derivative of pATY1103 (Cassen and Grossman, 1991; Casseen et al., 1991). The largest BamHI/HindIII fragment of pLAC1 carrying the uraA gene was cloned into pTZ18R (Pharmacia LBK Biotechnology Inc.) at the HindIII/BamHI site in the multiple cloning region to generate pSS1. The F10 gene of pSS1 derived from the pTZ18R enabled this plasmid to be used in generating single-stranded uraA gene containing template for mutagenesis experiments. pSS10 is a derivative of pSS1 with the uraA gene under control of the P1 promoter, which is in turn regulated by the cI857 repressor on the same construct. pSS1 was digested with ScaI for linearization. pHE6 (Milman, 1987) was digested with HaelI/XmnI, and the largest fragment carrying the P1 promoter and cI857 region was isolated. The isolated fragment of pSS10 linearized by ScaI and the HaelI/XmnI fragment of pH6E6 were first treated with mung bean nuclease (Pharmacia). The fragments were isolated again and digested with BamHI. The BamHI/mung bean nuclease-treated fragments derived from pSS1 and pHE6 were ligated to generate pSS10. Mutant uraA gene generated using the pSS1 construct were recloned into the same construct. pSS1 was digested with SacI for linearization. pHE6 was digested with Mung bean nuclease (Pharmacia). The fragments were isolated again and digested with BamHI. The BamHI/mung bean nuclease-treated fragments derived from pSS10 and pHE6 were ligated to generate uraA gene containing fragments and ligating it into the BamHI/XmnI fragment of pSS10 deleted of uraA wild type gene but containing the P1 promoter and cI857 repressor gene.

**Preparation of Single-stranded DNA—**The CJ236 strain of E. coli transformed with pSS1 was used to generate a uracil-incorporated single-stranded DNA (ssDNA) template. Cells from a minimal plate were grown at 30°C 30-60 min and heat-inactivated at 70°C for 10 min and allowed to cool to the room temperature. Second strand synthesis was continued in a 50-ml reaction mixture of 20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 2 mM DTT, 4% (w/v) glycerol, 600 µM dNTPs (150 µM each), 500 µM ATP, 1.5 µM/ml SSB, and 2 µg of DNA poly II holohexyrase (gift from Dr. R. Stephens and Dr. R. M. Vazquez). The reaction was carried out at 30°C for 30-60 min and heat-inactivated at 70°C for 10 min. The ligation reaction was continued overnight at 15°C by the addition of 2 units of T4 DNA ligase (New England Biolabs). The ligation mixture was transformed into competent E. coli JM109 cells, and plasmids isolated from randomly picked colonies were sequenced using T7 DNA polymerase (Pharmacia) by the Sanger dideoxy method (Sanger et al., 1977; Torhorst and Richardson, 1979).

**UV Survival—**UV survivability of the UvrA mutant and wild type strains was determined by streak tests and the sensitivity range obtained by this procedure was used to perform UV survival experiments. The mutant and wild type uraA gene carrying pSS1 or pSS10 and the control pTZ18R plasmids in an E. coli HI1.1A background were used in these experiments.

The streak tests were performed as follows. The overnight cultures were streaked across the 50 µg/ml carbenicillin containing 2 x YT plates. Shortwave (254 nm) UV lamp in the dark room was set at 12 microwatts/cm² to generate an exposure of 7.5 J m⁻² min⁻¹ at a distance of 41 cm. The UV exposure was increased from left to right perpendicular to the bacterial streaks by moving an opaque shield across the plate from right to left. The exposures were as indicated in Fig. 34. At the end, the plates were immediately transferred to an incubator set at 30°C and protected from laboratory light exposure in order to avoid photoactivation.

The UV survival curves were executed as follows. The bacterial strains were grown up to midlog phase (A₆₀₅ of 0.7-0.8); the cells were centrifuged, resuspended in M9 salts lacking a carbon source, and permitted to grow with shaking for another 1.5 h. The cells were diluted in M9 salts lacking a carbon source to yield approximately 3000 cells/ml and one tenth was plated onto YT plates containing 50 µg/ml carbenicillin and further diluted for mock irradiation. The UV exposed cells at the exposures indicated in Fig. 3B were incubated in the dark at 30°C. The colonies were counted after an overnight incubation.

**Protein Purification—**The mutant proteins K37A, K646A, and K37A K646A as well as wild type protein were purified from pSS10 and pSS1 constructs transformed into HI1.1A strains by a modified procedure of Yeung et al. (1986b). Cells carrying the appropriate plasmid were grown at 30°C in 2 x YT media containing 50 µg/ml carbenicillin until it reached an A₆₀₅ of 0.7-0.8. The temperature was shifted to 42°C to start induction of protein expression, and the cells were grown for another 1.5 h. The cells were immediately chilled and centrifuged at 10000 x g and pellets were resuspended in 0.1 M Tris-HCl, pH 7.5, and stored at ~80°C. Twenty grams of cell pellet was used in each preparation. Cells were thawed in a buffer of 0.1 M Tris-HCl, pH 7.5, 12 mM EDTA, 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 1.5 mg/ml lysozyme at 4°C. After 1.5-2 h of incubation, 5 mM NaCl was added to a final concentration of 0.3 M and sonicated to further disrupt the cells. The crude extract was obtained after centrifugation and used in the subsequent steps of purification.

**Site-directed Mutagenesis—**Oligonucleotide primers 23 nucleotides long were synthesized on an Applied Biosystems 380A automated synthesizer by Scott Morrow (Dept. of Biochemistry, The Johns Hopkins University) or 3 units of T4 DNA polymerase (Pharmacia) by the Sanger dideoxy method (Sanger et al., 1977) is used at 30°C. The present study essentially consisted of Affi-Gel blue (Bio-Rad) and Phenyl SePhorose (Pharmacia) chromatography. The samples were applied onto the column and washed with a buffer of 0.1 M KPO₄, pH 7.5, 1 mM EDTA, 2 mM DTT, 15% (v/v) glycerol, and 0.1 M KCl. The column was washed again with a buffer of 50 mM K⁺-MOPS, pH 7.5, 1 mM EDTA, and 2 mM DTT (buffer C) and 0.1 M KCl to remove the phosphbate. Buffer C, containing 0.1 M KCl and 5 mM ATP, was used to wash the column again to remove any contaminating proteins which bind ATP but not DNA. Washing with buffer C and 0.1 M KCl was followed to remove excess ATP. The wild type UvrA protein was eluted with buffer C and 0.3 M KCl. The column material with bound mutant UvrA proteins was first washed with buffer C and 0.3 M KCl and eluted with a linear gradient of 0.3 to 0.6 M KCl in buffer C.

The salt concentration was lowered, and proteins were concentrated simultaneously by a two-step centrifugation in Centriprep 30 and Amicon 30 microcentrator cartridges, respectively. The purified mutant and wild type proteins were judged to be greater than 90% pure by SDS-PAGE (Laemmli, 1970) on 10% polyacrylamide gels after staining with Coomassie Blue (Diesel et al., 1972).

**UV Irradiated DNA—**UV-irradiated DNA was obtained by exposure to 720 J m⁻² generating a distance of 41 cm. The UV exposure was increased from left to right perpendicular to the bacterial streaks by moving an opaque shield across the plate from right to left. The exposures were as indicated in Fig. 3A. At the end, the plates were immediately transferred to an incubator set at 30°C and protected from laboratory light exposure in order to avoid photoactivation.

**ATPase Sites of UvrA**
approximately six pyrimidine dimers per kilobase pair of random DNA sequences. Reactions were initiated by adding ATP. At various time points, 0.5-µl aliquots of the reaction mixture were spotted onto polyethyleneimine-cellulose TLC plates (Brinkman) prespotted with 0.5 µl ATP/ADP (100 mM) markers. The TLC plates were developed with 1 N formic acid, 0.5 M LiCl. The dried spots corresponding to ATP and ADP were visualized using shortwave ultraviolet light (254 nm) and excised to determine the level of radioactivity in scintillation fluid (Bio-Safe NA, Research Products International). The initial rates were determined by linear regression analyses, and the kinetic parameters were calculated from the slope of the regression line.

**ATP Binding Assay**—The binding of ATP to the UvrA proteins was assayed by retention of ATP/γS employing a modification of the procedure described by Gonsky et al. (1990). The binding assay (75 µl) contained 10 mM K+-MOPS, pH 7.6, 100 mM KCl, 15 mM MgCl₂, 50 µg/ml bovine serum albumin, 10% (v/v) glycerol, 150 ng [³²P]HPE6 phosphodi (Milman, 1987; Yeung et al., 1986b) equivalent to 57-fmol circles. The reaction was incubated at 37°C for 30 min. 20-µl portions of the reaction mixture were filtered through 4.5-µm nitrocellulose filters (HAWP025, Millipore) pre-equilibrated in 0.1% SDS. The dried spots were developed with 1 M formic acid, 0.5 M LiCl. The dried spots were counted in a scintillation fluid. The radioactivity bound to the filters was calculated from the subtraction of the background radioactivity without protein.

*DNA Binding Assay*—Nuclease protein complexes were assembled in a 100-µl reaction volume consisting of 50 mM K+-MOPS, pH 7.6, 100 mM KCl, 15 mM MgCl₂, 1 mM DTT, 50 µg/ml bovine serum albumin, 10% (v/v) glycerol, 150 ng [³²P]HPE6 phosphodi (Milman, 1987; Yeung et al., 1986b) equivalent to 57-fmol circles (specific activity = 8.0 x 10⁶ cpm/µg) and 2 mM ATP, ADP, or ATP/γS. Whenever the reaction mixture contained ATP, an ATP regenerating system (4 mM phosphoenolpyruvate and 500 units/ml pyruvate kinase (Boehringer) was included in the reaction mixture for 10 min prior to the binding assay. The radioactivity in the filter buffer was determined by counting in scintillation fluid. The level of radioactivity recovered by the UvrA proteins was corrected by subtraction of the background radioactivity without protein.

**Half-life Measurement of 2³³P**—The binding assay mixture was filtered in a similar manner except for the inclusion of UvrA protein. The reactions were incubated at 37°C for 30 min. 20-µl portions of the reaction mixture were filtered through 4.5-µm nitrocellulose filters (HAWP025, Millipore) pre-equilibrated in the filter buffer (10 mM K+-MOPS, pH 7.6, 100 mM KCl, 15 mM MgCl₂, and 1 mM EDTA). The filter was washed once with 100 µl of filter buffer and dried for 10 min, and the level of ATP/γS adsorbed onto the filters was determined by counting in scintillation fluid. The level of radioactivity retained by the UvrA proteins was corrected for the background radioactivity.

The described mutagenesis technique involved a 10 to 80% yield of mutants in a plasmid construct pSST1 which was easily manipulated for further cloning and eventual expression of the respective mutant proteins (Fig. 4). Further, there was no evidence that the mutations (ATP binding sites) as the mutant(s) reverted back to wild type when the mutant segment of the DNA was replaced with the corresponding wild type segment of DNA in the plasmid constructs (data not shown).

The yield of ssDNA template for the mutagenesis experiments using the M13K07 superinfection method was very poor when pSST10, an 8.1-kb plasmid with the phase origin was used. However, this was overcome by using the 6.6-kb plasmid pSST11. It is possible that the phase capsid

| Protein | Site | Sequence | Residues |
|---------|------|----------|----------|
| Ec UvrA | ATPI | L I V V T G L S G S G R S L S L | 26-40 |
|         | ATPI | F T C I G V S G S K S L T L | 635-659 |
| Mi UvrA | ATPI | M V V F T G L S G S G S L S L | 57-71 |
|         | ATPI | L A T V G T S G S K S L T L | 687-701 |
| Factor Y | ATPI | A W L L A G Y T G S K T E V | 119-133 |
|         | ATPI | Y T O V A A R A G K A G Q E | 578-593 |
| RbsA    | ATPI | V M A L S G E N A G K S T M | 32-46 |
|         | ATPI | I L G V S L G M A T E L | 280-294 |
| PfdmR   | ATPI | T Y A F V G E S G C G C S T | 408-422 |
|         | ATPI | T T A I V E G S C G C S T | 1156-1170 |
| STE6    | ATPI | T F I I V G X S G S C G C S T | 387-401 |
|         | ATPI | T I T I V G X S G S C G C S T | 1086-1096 |
| mmdrI   | ATPI | T V A L V N S G C G C S S T | 421-435 |
|         | ATPI | T L A V G S S G S S C S T | 1063-1077 |
| mmdrII  | ATPI | T V A L V N S G C G C S S T | 421-435 |
|         | ATPI | T L A V G S S G S S C S T | 1061-1075 |
| CTR     | ATPI | T V A L V N S G C G C S S T | 422-436 |
|         | ATPI | T L A V G S S G S S C S T | 1065-1075 |
| SrCTR   | ATPI | L L A V G S T C A G C T S L | 453-467 |
|         | ATPI | R V G L L E R G S T C S T | 1239-1253 |

*Fig. 1. Proteins with two tandemly arranged putative ATP binding motifs.* EcUvrA is the UvrA subunit of *E. coli* UvrABC endonuclease (Husain et al., 1986; Doolittle et al., 1986); MiUvrA is the UvrA analog of *Micrococcus luteus* (Shiota and Nakayama, 1989); Factor Y(?) is a component of the replication-priming apparatus of *E. coli* and x174 (Nurse et al., 1986; Lee et al., 1986); RbsA is a ribose transport protein in *E. coli* (Bell et al., 1988); PfdmR is the multiple drug resistance gene in *Plasmodium falciparum* (Fonte et al., 1989); STE6 is the gene for the α-factor pheromone export system of *Saccharomyces cerevisiae* (McGrath and Varshavsky, 1988); mmdrI and mmdrII are members of a mammalian multiple drug resistance gene family (Gros et al., 1988; 1989); kdrI is a human multiple drug resistance gene (Chen et al., 1986); CFTR is the human cystic fibrosis gene (Riordan et al., 1989).
imposes a limitation on the size of the ssDNA which can be packaged.

Effect of the Mutations on UV Survival—Wild type and mutant uvra gene containing plasmid (pSST1 or pSST10) in the Δuvra background was studied by streak tests and UV survival curves (Fig. 3, A and B). K37R was the only mutant able to complement the chromosomal defect in uvra to generate a UV-resistant phenotype. The levels of resistance conferred by the other mutants were as follows: K37Q > K37A > K646A > K646Q, K646R, K37A K646A, K37Q K646Q, and K37R K646R. The estimated $D_{10}$ values (the UV dose sufficient to produce a single inactivating hit, $\ln N/N_0$ of 1 = 0.37) for wild type uvra gene complementation was 16 J m$^{-2}$ while it was 1.3 J m$^{-2}$ for the host strain MH1ΔA with a chromosomal deletion of the gene. The $D_{10}$ values for complementation of the host defect by the ATPase site mutations were as follows: K37A, 2.7 J m$^{-2}$; K37Q, 4.6 J m$^{-2}$; K37R, 16.3 J m$^{-2}$; K646A, 1.4 J m$^{-2}$; K646Q, K646R, K37A K646A, K37Q K646Q, and K37R K646R. Both types of plasmids (pSST1 and pSST10) carrying the mutant and wild type genes of uvra yielded similar results despite the fact that the gene is expressed at slightly higher levels in pSST10 due to "leakiness" of the temperature-sensitive repressor (cI857) regulated P$_I$ promoter. The levels of UvrA protein in all of the strains were found to be comparable (data not shown). From such observations it is suggested that the presence of both invariant lysine residues at the ends of the glycine-rich loops of the A consensus Walker type motif of NTP binding proteins present in the UvrA protein enables this subunit to confer full activity of the excision repair enzyme UvrABC endonuclease.

FIG. 2. DNA sequences of the wild type and mutant UvrA proteins at their putative ATP binding motifs. The wild-type (WT) protein has lysine residues at both first (ATP-I) and second (ATP-II) motifs with a corresponding DNA sequence of AAA. K37A, K37Q, and K37R are first site mutants, K646A, K646Q, and K646R are second site mutants, and K37A K646A, K37Q K646Q, and K37R K646R are double mutants. The DNA sequence changes corresponding to the amino acid changes lysine (K) to alanine (A), lysine (K) to glutamine (Q), and lysine (K) to arginine (R), respectively, are as follows: K to A, AAA to CGC; K to Q, AAA to CAG; K to R, AAA to CCG. Details of mutagenesis are described under "Experimental Procedures."

FIG. 3. UV survival of wild type and mutant UvrA plasmid carrying strains in a ΔUvrA background. A, streak test results: UV exposure and the streaks corresponding to different uvra plasmid-carrying strains are indicated in the figure. B, survival curves of wild type and mutant uvra gene carrying plasmid in MH1ΔA strain: WT, ○; MH1ΔA, ●; K37A, △; K37Q, □; K37R, ▽; K646A, ■; K646Q, ●; K646R, ▲; K37A K646A, ◧; K37Q K646Q, ○; K37R K646R, □. The notations for the different mutants are described in the legend for Fig. 2. See "Experimental Procedures" for methodology.
UvrA proteins were purified for further characterization (Fig. 5). The levels of UV resistance, sites of the mutations, and the amino acid change were considered in choosing the proper mutant protein. The K37A and K646A single mutants and the K37A K646A double mutant proteins were chosen to study since they represent an increasing level of UV sensitivity. Further, mutations were localized at each of the single site and both sites, and all had a similar lysine to alanine change in the ATP binding motif.

Expression of the Wild Type and Mutant UvrA Proteins—The analysis of the soluble fractions of the mutant and wild type uvrA gene carrying pSS710/MH1 ΔA strains suggest that the majority of the UvrA protein in each of the fractions does not form inclusion bodies due to overexpression (Fig. 4). The presence of higher levels of UvrA protein in pSS710/MH1 ΔA than pSS71/MH1 ΔA and MH1 ΔA (data not shown) even at 30 °C (before induction) suggests that the \( pL \) promoter is not tightly repressed (“leaky”) by cI857.

Purification of Proteins—The mutant proteins were purified by essentially the same protocol as the wild type UvrA (Fig. 5). However, unlike the wild type protein, proteins were eluted from the ssDNA column using a higher salt gradient. This suggested that the mutant proteins are stably bound to ssDNA. Similarly, the mutant proteins were surprisingly more stably bound to double-stranded DNA (Fig. 7, Table II). Western blotting of the purified wild type and mutant UvrA proteins using anti-UvrA and anti-UvrB antibodies confirmed that the mutant proteins could cross-react with the anti-UvrA raised against the wild type protein, and the protein preparations have no contaminating UvrB (data not shown).

ATPase Activity—Both of the single site mutants retained ATPase activity while the double mutant was unable to hydrolyze ATP (Table I). These observations suggest a crucial role of the lysine residue in the mechanism of ATP hydrolysis. Further, it is assumed that the ATPase activities exhibited by the single mutants are derived from the nonmutated ATP-ase site(s). This assumption relies on the absence of any gross conformational changes in the mutant UvrA protein(s) which could affect the ATPase activity derived from the nonmutated site(s). The ability of the mutant proteins to bind ATP at levels similar to the wild type protein indicate that the conformation of the ATP binding pocket is not significantly altered by these ATPase site mutations (Fig. 6). Furthermore, while the double mutant completely lost its ability to hydrolyze ATP, the single mutants retained ATPase activities with different kinetic parameters (Table I). The ATPase activity of the wild type protein has an apparent \( K_m \) of 149 \( \mu \)M, intermediate between the apparent \( K_m \) values of the C-terminal site (69 \( \mu \)M) and the N-terminal site (312 \( \mu \)M). The apparent second order rate constant (\( K_{cat}/K_m \)) for the association of the enzyme and substrate of the C-terminal site (derived from K37A) is only slightly decreased while that of the N-terminal site (derived from K646A) is about 10-fold lower than the wild type ATPase. Thus, the C-terminal site apparently has a higher affinity for ATP binding than the N-terminal site. In addition, a substantial decrease in the \( K_{cat} \) values of the individual sites compared to the wild type protein suggests cooperativity between the sites in the catalysis of ATP hydrolysis.
ATPase Sites of UvrA

The initial rates of ATPase activity were measured using polyethyleneimine-cellulose TLC based assay as described under "Experimental Procedures." The assays contained 24 nM UvrA. The DNA used in these assays was pPYC3 plasmid DNA. UV exposure was at 720 J m⁻². The K646A ATPase activity in the presence of DNA was measured using 20 to 650 μM ATP (labeled with [³²P]ATP) while 20 to 350 μM ATP was used in all other measurements. WT, wild-type; NT, N-terminal ATPase; CT, C-terminal ATPase; ND, not detectable (the initial rates of ATP hydrolysis at all the concentrations tested were less than 0.1 nmol/min).

Table I: Kinetic parameters of wild-type and mutant ATPase activity

| Effective ATPase | Kₐ | Vₐₐₜ | Kₐₐ | Kₐ/Kₐₐ | Relative Kₐ/Kₐₐ |
|------------------|----|------|-----|--------|----------------|
| No effectors     |    |      |     |        |                |
| WT               | NT and CT | 149 | 4.1 | 171 | 1.15 | 1.00 |
| K37A             | CT  | 60  | 1.3 | 53  | 0.89 | 0.77 |
| K646A            | NT  | 312 | 0.8 | 34  | 0.11 | 0.09 |
| K37A K646A       | None | ND  |   | ND  | ND  | ND  |
| DNA              |    |      |     |        |                |
| WT               | NT and CT | 24 | 1.0 | 40  | 1.67 | 1.00 |
| K37A             | CT  | 308 | 0.7 | 31  | 0.10 | 0.06 |
| K646A            | NT  | 355 | 1.3 | 55  | 0.10 | 0.06 |
| K37A K646A       | None | ND  |   | ND  | ND  | ND  |
| uvDNA            |    |      |     |        |                |
| WT               | NT and CT | 85 | 1.4 | 56  | 0.66 | 1.00 |
| K37A             | CT  | 238 | 0.8 | 32  | 0.14 | 0.20 |
| K646A            | NT  | 270 | 1.3 | 55  | 0.20 | 0.30 |
| K37A K646A       | None | ND  |   | ND  | ND  | ND  |

UV-damaged as well as native DNA modulate wild type UvrA-associated ATPase activities exhibited by both of the individual sites in the mutant proteins as well as the wild type protein. The apparent Kₐ values of the individual ATPase sites are 3- to 23-fold higher and the apparent second order rate constants are 3- to 17-fold lower than the wild type protein with both functional ATPase sites operating. These results further suggest allosteric interactions between the sites in both ATP binding and hydrolysis when in the presence of DNA. The significant levels of inhibition of ATPase activity of UvrA protein in the presence of either UV-damaged or native DNA is in agreement with the notion that ATP-bound UvrA protein has an enhanced affinity for DNA binding while hydrolysis of the bound ATP favors its dissociation from DNA.

Effect of ATPase Site Mutations on Binding to ATP—Mutagenesis of the lysine at the end of the glycine-rich loop of the consensus NTP binding motifs did not significantly affect these mutant UvrA proteins to bind ATP (Fig. 6). The wild type and the mutant UvrA proteins showed essentially similar levels of ATP binding. These findings further suggest that the inability of the double mutant UvrA protein to hydrolyze ATP is not due to a defect in ATP binding (Table I). The retention of ATP binding ability by these ATPase site mutants provides a system with great potential for an examination of the role(s) of binding and hydrolysis of ATP by the UvrABC subunit of the UvrABC endonuclease.

Effect of Nucleotide Cofactors on DNA Binding—The requirement of nucleotide cofactor binding and/or hydrolysis in support of UvrA-DNA binding was analyzed. ATP has been previously shown to be the preferred nucleotide cofactor for UvrA-DNA complex formation (Seeberg and Steinum, 1982; Yeung et al., 1986a; Seeley and Grossman, 1989). However, these studies could not distinguish between the role(s) of ATP binding and the role of ATP hydrolysis in nucleoprotein complex formation. Since the single and double mutations in the putative ATP binding sites led to the loss of ATPase activity and retention of ATP binding ability of the mutated site(s) (Table I and Fig. 6), it provided a useful system by which to separate these roles in nucleoprotein complex formation.

The mutant proteins as well as the wild type protein bind DNA in the absence of nucleotide (Fig. 7A). However, in the presence of ATP, the double mutant (K37A K646A) resulted in the highest level of DNA binding while the binding of the single mutants (K37A, K646A) were intermediate between wild type and double mutant (Fig. 7C). The introduction of ADP in the binding reaction reduced the level of nucleoprotein complex formation by the mutants as well as the wild type protein (Fig. 7B). These observations suggest that it is the binding of ATP which has a greater influence on nucleoprotein formation, whereas the hydrolysis of ATP seems to favor nucleoprotein complex dissociation. Interestingly, the wild type protein exhibited the lowest level of nucleoprotein complexed compared to the mutants in the presence of ATP, suggesting that the wild type UvrA is in an equilibrium between the bound form driven by nucleotide binding and the unbound form driven by the hydrolysis of ATP.

Substitution of ATPγS for ATP in the Binding Reaction—ATPγS as a poorly hydrolyzable analog of ATP is expected to result in binding of the wild type protein to a similar level of binding exhibited by the double mutant (Fig. 7D). The variability in the level of binding in the presence of ATPγS compared to ATP may be due to the difference in the inter-
actions of ATP and ATPγS to the UvrA protein.

**Binding of Mutant and Wild Type Proteins to uvDNA—**

The binding of wild type UvrA protein to DNA was enhanced as a consequence of DNA damage (Fig. 8A). In contrast, the binding of the mutant proteins shows no differential binding between native DNA versus uvDNA. In some cases, there is a reduction in binding to the uvDNA by mutant UvrA proteins (Fig. 8, B–D). There appears to be a loss of differential recognition of damaged sites on DNA by the UvrA mutations. This is consistent with further analyses of whether hydrolysis of ATP by either one ATPase site or both sites may be important in locating a damaged site by the UvrA protein. It was approached by examining the chelator-resistant residence times of the mutant and wild type proteins on DNA (Table II).

**2 x SSC-resistant Nucleoprotein Complex Formation—**

The half-lives of chelator-resistant UvrA-DNA complexes were found to be less than 5 s. However, in the presence of UvrB and damaged DNA, the half-life was increased to an hour (Yeung et al., 1986a). The increase in the residence time of these nucleoprotein complexes reflects binding of the UvrAB complex to damaged sites. In the present study, we find that the residence times of the nucleoprotein complex on both undamaged and damaged DNAs are dramatically increased in the C-terminal ATPase site (ATPII) mutant protein (K646A) and in the double mutant (K37A K646A), while the N-terminal ATPase site (ATP1) mutant (K37A) protein and the wild type protein bound to the undamaged DNA formed very unstable complexes (Table II). Further, there is a noticeable increase in the half-life of wild type protein bound to the damaged DNA compared to the undamaged DNA. The substitution of ATPγS for ATP in the binding reaction of wild type UvrA protein to either damaged or undamaged DNA increases its half-life in 2 x SSC from <3–4.5 s to 30–40 s and 25% of the nucleoprotein complexes remain stable with a half-life of more than 3 h. These observations suggest that the initial binding of UvrA protein is largely nonspecific, and hydrolysis of ATP by the C-terminal ATPase site leads to dissociation of the protein enabling it to repeat the binding-release cycle until it finds a damaged site. This diffusion-controlled repetition of random binding and dissociation cycles may be a component of the discrimination required for damage recognition by the UvrABC system. However, the UvrA protein seems to be the damage recognition subunit irrespective of its independent action.

**FIG. 7.** Binding of DNA to wild type and mutant UvrA proteins. The titles of the figures B–D indicate the type of nucleotide cofactor included in the binding assay. Figure A is a control lacking nucleotide cofactor in the reaction. WT, O; K37A, Δ; K646A, V; K37A K646A, -O. The description of the DNA binding assay is given under "Experimental Procedures."

**FIG. 8.** Binding of native DNA versus uvDNA to wild type and mutant proteins. The titles of the figures indicate the proteins used in the binding assay. A, WT:DNA (O), uvDNA (●); B, K37A-DNA (Δ), uvDNA (▲); C, K646A-DNA (V), uvDNA (●); D, K37A K646A-DNA (O), uvDNA (●). Please refer to "Experimental Procedures" for the assay conditions.

**DISCUSSION**

The roles of the two ATPase sites present in the UvrA protein have been analyzed by undertaking site-directed mutagenesis of the gene and biochemical characterization of the representative mutant proteins. The results of these experiments indicate that both of these UvrA-associated ATPase sites are functional in the overall nucleotide excision repair in E. coli mediated by the UvrABC endonuclease (Fig. 3, A and B). The biochemical characterization of the respective mutants revealed that these two sites play different roles as well as function together or influence each other by allosteric interactions between the sites. The C-terminal site has a higher affinity for ATP binding than the N-terminal site. The two ATPase sites can hydrolize ATP independently of each other. ATPase activities exhibited by both of the sites are modulated by undamaged as well as UV-damaged DNA (Table I). The ability to bind ATP is unaffected by these mutations at the ATPase sites (Fig. 6). The complete loss of ATPase activity in the double mutant protein with retention of its ability to bind ATP identifies that the conserved lysine residue at the end of the glycine-rich loop of the consensus A type ATP binding motif as assuming a key role in the mechanism of ATP hydrolysis.

In the presence of ATPγS, the level of wild type UvrA nucleoprotein complex formation reaches the level observed with the double mutant protein (K37A K646A) that is completely defective in ATP hydrolysis (Fig. 7D). Similar conditions lead to a 10-fold increase in the half-life of chelator-resistant wild type UvrA-DNA complex. Furthermore, the single mutants defective in ATP hydrolysis at only one of the ATP binding sites show an increased ability to bind DNA compared to wild type protein (Fig. 7C). These findings suggest that ATP binding, but not hydrolysis at both of the ATP binding sites of UvrA, favors nucleoprotein complex formation. Similarly, it is ATP binding to UvrA that favours its dimerization to form (UvrA)_2. In further support of this, ATPγS shifts the monomer-dimer equilibrium toward (UvrA)_2 formation (Oh et al., 1989). (UvrA)_2 exhibits a greater increase in binding UV-damaged DNA than undamaged DNA (Fig. 8A). The moderately increased residence time in the presence of chelators of wild type UvrA bound to UV-damaged DNA relative to undamaged DNA (Table II) provides additional support for the notion that the UvrA protein has an intrinsic ability to discriminate between damaged and undamaged sites on DNA. Analyses of the residence times of these proteins on damaged and native DNA in the presence of
nucleation for the subsequent steps of nucleotide excision repair. Because of the low level of discrimination between damaged and undamaged sites, it is unlikely that (UvrA)₂ could find a single damaged site in 10⁶ nucleotides in a diffusion-controlled reaction. An alternate mechanism for damage recognition by the UvrABC system is becoming increasingly evident from the recent observation that UvrAB acts as a helicase and tracks on DNA in search of damage (Oh and Grossman, 1987, 1989; Koo et al., 1991). Damage recognition by the UvrABC endonuclease system, however requires the hydrolysis of ATP presumably localized at the C-terminal ATPase site of UvrA. This binding/hydrolysis step seems to provide a sensing step localizing the complex at damaged sites to initiate repair processes.

It is apparent from UV sensitivity and survival experiments of the uvr mutants that productive nucleoprotein complex formation is critical to repair (Fig. 3, A and B). The extreme sensitivity of the C-terminal ATP binding site mutants and the double mutants is due to essential irreversible binding of those mutant UvrA proteins to nonspecific sites, thus blocking their ability to locate UV adducts on DNA. However, reduced UV sensitivity of the N-terminal site mutants suggests that binding of ATP but not hydrolysis is required at this site by the UvrA protein during the damage recognition step. The wild type level of survival exhibited by the K37R mutant further supports this hypothesis and suggests ATP binding is not greatly impaired by the conservative change of lysine to arginine in this region of UvrA.

The biochemical characterization of the site-directed mutant proteins of UvrA has not only allowed for an understanding of the different roles of ATP in the initial steps of damage recognition by UvrA, but also provides direct evidence that UvrB exhibits ATPase activity in the presence of UvrA and DNA. This conclusion is reached from the observation that the ability of the double mutant protein (K37A K646A) to manifest ATPase activity depends on the presence of UvrB protein and DNA. The requirement for ATP binding and hydrolysis at either ATP binding site by the UvrA subunit for the function of the UvrAB complex and the UvrABC system are under current investigation.

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