ATPase activity of the UvrA and UvrAB protein complexes of the *Escherichia coli* UvrABC endonuclease

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ABSTRACT

We have analyzed the ATPase activity exhibited by the UvrABC DNA repair complex. The UvrA protein is an ATPase whose lack of DNA dependence may be related to the ATP induced monomer-dimer transitions. ATP induced dimerization may be responsible for the enhanced DNA binding activity observed in the presence of ATP. Although the UvrA ATPase is not stimulated by dsDNA, such DNA can modulate the UvrA ATPase activity by decreases in K_m and V_m and alterations in the K_i for ADP and ATP-γ-S. The induction of such changes upon binding to DNA may be necessary for cooperative interactions of UvrA with UvrB that result in a DNA stimulated ATPase for the UvrAB protein complex. The UvrAB ATPase displays unique kinetic profiles that are dependent on the structure of the DNA effector. These kinetic changes correlate with changes in footprinting patterns, the stabilization of protein complexes on DNA damage and with the expression of helicase activity.

INTRODUCTION

In E. coli., the incision events initiating repair of 'bulky adduct' type DNA damage requires ATP and the UvrA, UvrB, and UvrC proteins (1). The Uvr endonuclease acts on a diverse set of dissimilar DNA damage that include pyrimidine-pyrimidine cyclobutane dimers, 6−4 pyrimidine-pyrimidones, photoactivated psoralen mono and diadducts, N-acetoxy-N-acetylaminofluorene, benzo[a]pyrene, and cis-platinum, but does not act on base mismatches, DNA hairpins, or apyrimidinic/apurinic sites (2−9). Unlike the case for other DNA repair proteins such as photolyase and the glycosylases, the Uvr system does not recognize the modified base itself. Therefore, common DNA structural perturbations such as kinks and bends induced by the DNA damaging agents have been suggested to be determinants recognized by the repair complex (10).

Biochemical analysis identified ATP hydrolysis as a requirement for effective incision near DNA damage (1,2,3). The UvrA protein displays an ATPase activity and its amino acid sequence contains putative nucleotide binding sites (11). The UvrB protein also contains a nucleotide binding site (based on sequence analysis) and exhibits a cryptic ATPase activity (12,13). In association with the ATPase activities, ATP can enhance the DNA binding activity of UvrA and can stabilize the binding of UvrAB protein complexes on DNA damage sites (16). ATP-γ-S can induce UvrA to unwind supercoiled DNA (31). In addition, the UvrAB protein complex can unwind short DNA duplexes in a manner that is dependent on ATP hydrolysis (28). Some studies of the ATPase activities have been conducted but have been limited in scope because of the use of only a single DNA effector or the lack of description of the relevant kinetic constants (13,22). To obtain an understanding of the various effects of nucleotide, we have analyzed the ATPase activities of the UvrA and

Table 1. Kinetic Parameters for the UvrA ATPase Activity

Protein + DNA effector	$K_{m(app)}$ μM	Vm $\mu M min^{-1}$	TON mole min^{-1}
UvrA	150	2.98	125
UvrA + 30 μM dsDNA	102	0.85	36
UvrA + 300 μM dsDNA	67	1.98	82
UvrA + 300 μM UV dsDNA	31	2.03	84
UvrA + 300 μM ssDNA	5	1.08	45

The initial rates of the ATPase activity were measured using the TLC based assay. The assays contained 0.44 pmole UvrA. Double stranded DNA was pRLM24 and ssDNA was from fd phage. UV irradiation of double stranded DNA was for 720 J m^{-2}. TON is the turnover number per mole UvrA.

UvrAB protein complexes in more detail. We observe unique changes in the kinetics of the ATPase activities depending on the nature of the DNA effectors. The importance of these changes in affecting or reflecting the biochemical properties of the various intermediates leading to the incision of damaged DNA are discussed.

MATERIALS AND METHODS

Proteins. The UvrA, UvrB and UvrC proteins were purified according to previously described procedures (14). The UvrA employed in these experiments represents the ssDNA-Sepharose column fraction and was dialyzed against 50 mM MOPS, pH 7.6, 0.3 M KCl, 30% glycerol, 2 mM DTT, 1 mM EDTA. The UvrB, obtained as the Sephadex G-150 fraction, was concentrated by dialysis against 50 mM MOPS, pH 7.6, 300 mM KCl, 50% glycerol, 2 mM β-mercaptoethanol, 1 mM EDTA. The UvrB used in this study does not contain the proteolyzed form of the protein (13). UvrC was from the ssDNA-Sepharose

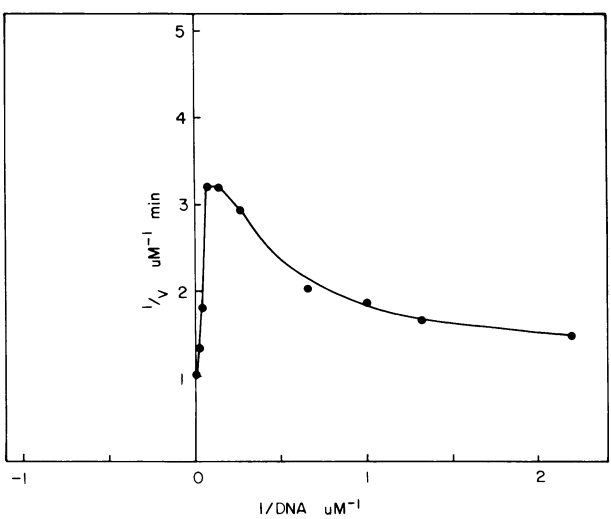


Figure 1. Modulation of the UvrA ATPase activity by double stranded DNA. Initial rates of the ATPase activities were measured at an ATP concentration of 150 μM in the presence of various concentrations of pRLM24 DNA. The TLC based assay was used with 0.44 pmole UvrA in each reaction.

fraction. Catalase and aldolase, purchased as ammonium sulfate suspensions (Cooper Biomedicals), were prepared by resuspending the precipitate in 50 mM MOPS, pH 7.6, 0.1 M KCl, 15% glycerol, 2 mM DTT and dialyzed against the same buffer before use. β-galactosidase was purchased from Sigma and bovine serum albumin (BSA) was from Miles.

DNA. Replicative form I (RFI) DNA of fd phage was prepared from phage infected E. coli K37 cells by standard procedures using CsCl-ethidium bromide gradients (15). A [^{3}H]-thymidine labeled RFI fd DNA was prepared as described (16). Supercoiled pRLM24 DNA, a replication defective runaway plasmid, was prepared from E. coli. C600 cells harboring the plasmid. Single stranded fd DNA was prepared from purified phage by phenol and phenol/chloroform extractions followed by ethanol precipitation (17).

ATPase assays. ATPase activities were either measured by the charcoal adsorption assay or by thin layer chromatography on polyethyleneimine (PEI) cellulose plates. For the charcoal adsorption assay, the reactions contained 50 mM MOPS, pH 7.6, 100 mM KCl, 15 mM $MgCl_2$, 2 mM DTT in a total volume of 100 ml. ATP concentrations were 0.2 to 2 mM ATP and contained 1 to 2 μCi of γ-[^{32}P]-ATP (2500 Ci/mmol; New England Nuclear). Mixtures were warmed to 37°C and then proteins added to initiate the reaction. Each reaction contained 2.2 pmole UvrA and 4.4 pmole UvrB unless indicated. At various times, 10 μl aliquots were transferred to 200 μl of 20% Norit A charcoal (w/v, acid washed) in 50 mM KPO_4, pH 5.5, placed on ice several minutes, and then followed with 200 μl of 50 mM KPO_4. Samples were vortexed, centrifuged, and 200 μl of the supernatant counted in 4 ml of scintillation fluid (Baker). The percent recovery of released phosphate was determined with either [^{32}P]orthophosphate or [^{3}H]-glycine. The concentrations of DNA used in the assay has no effect on the adsorption of nucleotide to charcoal. An ATP regenerating system consisted of 4 mM phosphoenolpyruvate and 5 units of pyruvate kinase (Boehringer-Mannheim).

ATPase assays based on thin layer chromatography on polyethyleneimine (PEI) cellulose plates were conducted in a volume of 20 μl and contained 50 mM MOPS, pH 7.6, 100 mM KCl. 15 mM $MgCl_2$, 2 mM DTT, 50 μg/ml BSA, 0.44 pmole UvrA and 1.11 pmole UvrB. Reactions were initiated by the addition of [^{3}H]-ATP (Amersham). The final specific activities ranged from 0.25 Ci/mmole to 20 Ci/mmole. The initial rates were analyzed by taking 0.5 μl or 1 μl aliquots and spotting on PEI cellulose plates pre-spotted with 0.5 μl of 0.1 M ATP and 0.1 M ADP. The plates were developed with 0.5 M formic acid/1.0 M lithium chloride and visualized by short wave UV light. Regions corresponding to ADP and ATP were excised and counted in scintillation fluid. Under most circumstances, initial rates were obtained before 10% of the substrate had been hydrolyzed. Lineweaver-Burke plots of the initial rates were used to determine the kinetic constants. All nucleotides were purchased from P.L. Biochemicals.

Preparation of Radiolabeled UvrA protein. Microgram quantities of [^{35}S]-methionine labeled UvrA were obtained using the plasmid, pHE6-uvrA (29). The plasmid encodes the C_I857 repressor and contains the uvrA gene whose expression is under control of the λP_L promoter. Upon induction of expression at 42°C, UvrA accumulates to the level of 30% of the total cellular protein. Approximately 50% of the radiolabeled protein is UvrA.

The cells (C600) were grown overnight at 30°C in M9 media containing 100 μg/ml ampicillin. The cells were pelleted by centrifugation and resuspended (final OD_{550} between 0.05−0.1) in 100 ml of M9 minimal salts media containing 50 μg/ml of each of the amino acids except methionine: cysteine was present at 0.1 mg/ml. Cells were then grown at

Table 2. Kinetic Parameters for the UvrAB ATPase Activity

Protein + DNA Effector	$K_{m(app)}$ μM	Vm $\mu M min^{-1}$	TON mole min^{-1}
UvrA + B	70	0.68	28
UvrA + B + 30 μM dsDNA	527	5.73	228
UvrA + B + 300 μM dsDNA	400	12.89	536
UvrA + B + 30 μM UV dsDNA	103	8.98	373
UvrA + B + 300 μM UV dsDNA	72	11.73	488
UvrA + B + 30 μM ssDNA #1	65	11.45	476
# 2	844	44.65	1858

Double stranded DNA is pRLM24 and ssDNA is from fd phage. UV irradiation was for 720 J m^{-2}. The TLC based assay was used. Each assay contained 0.44 pmole UvrA and 1.11 pmole UvrB. TON is the turnover number per mole UvrA.

30°C to OD_{550} of 0.4. [^{35}S]-methionine (0.5 mCi; New England Nuclear) was added after shifting the temperature to 42°C and at every 30 minutes afterwards for 3.5 hrs. Labeling was halted by the addition of unlabeled methionine to a final concentration of 50 μg/ml. The cells were immediately chilled on ice, pelleted by centrifugation, and placed at −70°C overnight or used directly for purification. A crude extract was prepared by sucrose plasmolysis (1) except that the final salt concentration was 0.3 M KCl. Approximately 60% of the UvrA is solubilized. All subsequent purification steps used a buffer consisting of 50 mM MOPS, pH 7.6, 15% glycerol, 2 mM DTT, 1 mM EDTA. The crude extract was applied onto a 4 ml Affigel Blue column (Bio Rad) equilibrated with 0.1 M KCl. The column was washed successively with 0.1 M KCl and 0.4 M KCl. The labeled UvrA was eluted with 2−3 column volumes of 0.8 M KCl. The eluent was dialyzed overnight

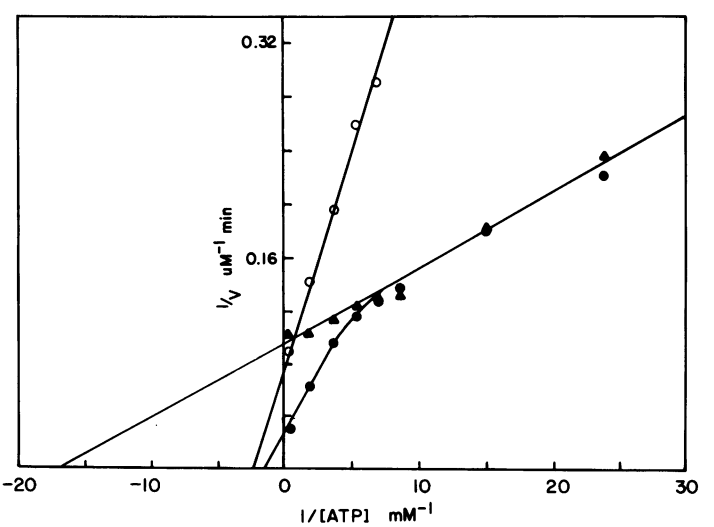


Figure 2. Comparison of the double reciprocal plots of UvrAB ATPase activity with dsDNA, UV irradiated dsDNA and ssDNA effectors: UvrAB + 300 μM dsDNA (open circles); UvrAB + 300 μM UV irradiated dsDNA (triangles); UvrAB + 30 μM ssDNA (closed circles). UV irradiation was for 720 Jm^{-2}. Kinetic constants are listed in Table 2.

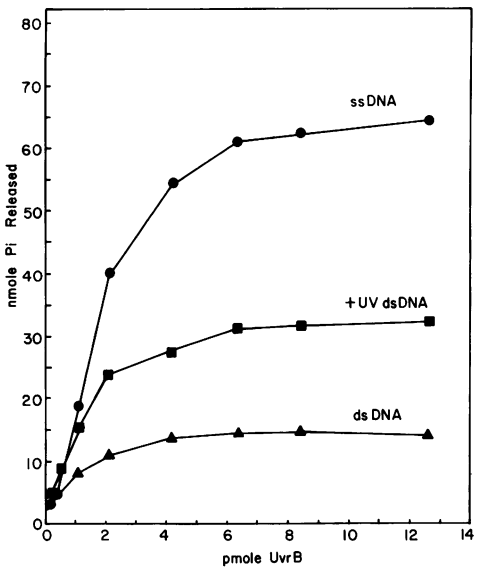


Figure 3. Titration of UvrB protein at a fixed UvrA concentration in the presence of various DNA effectors. UvrA was held constant [2.2×10^{-8}M (250 ng)] and the ATP concentration was 2 mM. Assays used the charcoal assay and reactions were for 20 min at 37°C. The ATP hydrolysis rate is linear up to 20 min for assays conducted at 2 mM ATP. All DNA concentrations were 30 μM. Double stranded DNA was supercoiled pRLM24 while ssDNA was from fd phage. UV irradiation was for 720 Jm^{-2}; ssDNA (circles); UV irradiated dsDNA (squares); dsDNA (triangles).

against 0.09 M KCl and then applied on a 3 ml phosphocellulose (P-11; Whatman) column. After washing the column with 0.1 M KCl, the UvrA was eluted with 2 column volumes of 0.3 M KCl. The salt concentration was lowered by dilution or dialysis to 0.1 M KCl. The sample was applied onto a 5 ml ssDNA-Sepharose column and the column washed with 0.1 M KCl. The UvrA was eluted with 0.3 M KCl and the protein concentrated by centrifugation in an Amicon 30 Microconcentrator cartridge. The resulting UvrA is 80–85% pure by densitometer scanning of Coomasie Blue stained SDS-polyacrylamide gels and is radiochemically greater than 90% pure. The specific activity was approximately 6.9×10^4 cpm/μg. The protein was used within a month of its purification. Protein concentrations were determined by the Bradford assay (18) using bovine serum albumin as a standard.

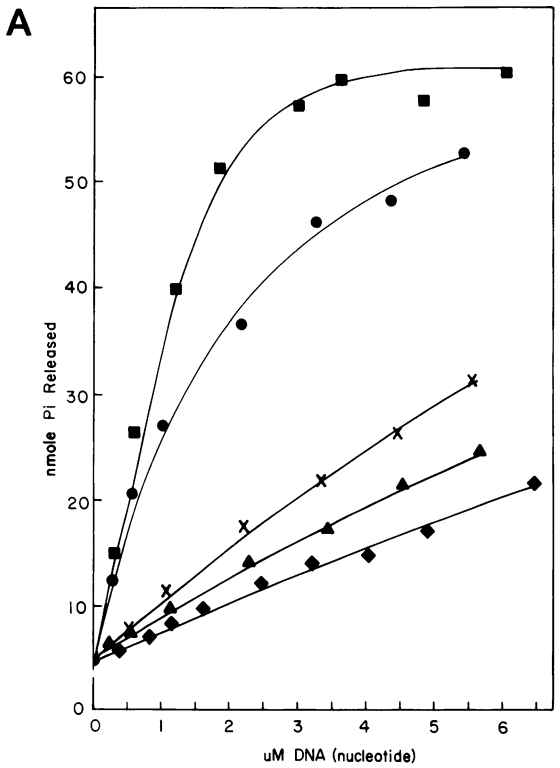
Table 3. Inhibition by ADP and ATP-γ-S

	K_i	
	ADP (μM)	ATP-γ-S (μM)
UvrA	20	50[a]
UvrA + dsDNA	160[b]	0.7
UvrAB + dsDNA	110[b]	1.8

Assays were conducted as described in Tables 1 and 2. Double stranded DNA concentrations were 300 μM (in nucleotides).

[a] –value from reference 1.

[b] –estimated from the linear region of double reciprocal plots on the assumption that inhibition is pure competitive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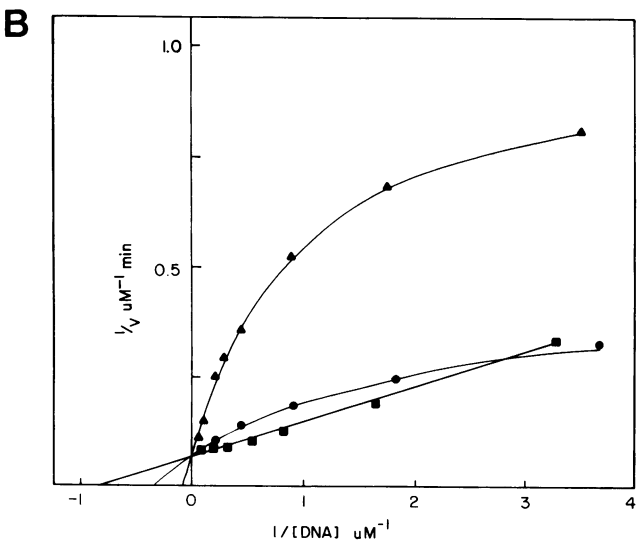
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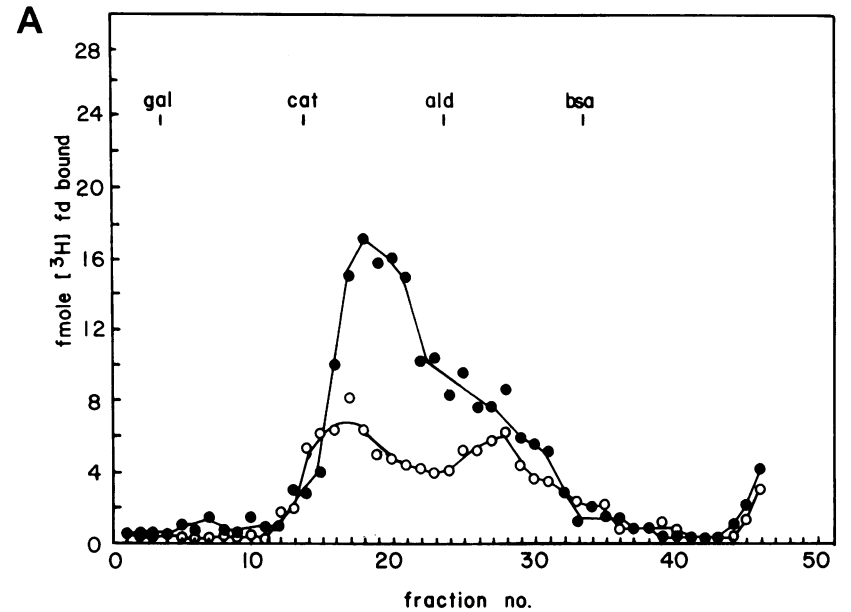
Glycerol gradient sedimentation. Sedimentation analyses were conducted in 15−35% glycerol gradients in 50 mM MOPS, pH 7.6, 100 mM KCl. 2 mM DTT, 1 mM EDTA and the indicated concentration of nucleotide. Centrifugation was for 24 hrs at 4°C at 45000 rpm in a Beckman SW 50.1 rotor. Five drop fractions were collected. [^{35}S]-methionine labeled samples were counted in 4 ml of scintillation fluid. Non-radiolabeled UvrA was assayed by analyzing for UvrABC endonuclease activity (16). Molecular weight standards consisted of catalase (20 μg; MW 247500), β-galactosidase (20 μg; MW 515000), aldolase (40 μg; MW 149100) and bovine serum albumin (40 μg; MW 66200). Protein standards were identified by assaying for activity or by SDS-polyacrylamide gel electrophoresis. *Equilibrium Sedimentation in an airfuge.* Sedimentation equilibrium analysis using a Beckman airfuge was conducted as described (19,20). The sample volume was 150 μl and centrifugation was for 28 hrs at 4°C. The running temperature of the rotor under these conditions is 8°C. The radial speed, determined using a stroboscope, was approximately 28000 rpm. Following centrifugation, 10 μl aliquots were removed from the meniscus for analyses. Concentrations of aldolase, used as the gradient stabilizer and as the molecular weight standard, were determined by the Bradford assay. [^{35}S]-Methionine labeled UvrA was placed in 200 μl of water and counted in scintillation fluid.

RESULTS.

UvrA ATPase activity. Several laboratories have shown that purified UvrA is an ATPase (1,21,22). Although purified UvrB and UvrC do not significantly hydrolyze ATP (1,21,22), ATPase activity in a proteolyzed form of UvrB (UvrB*) has recently been shown (13). The ATPase activity of UvrA used in this study displays a K_m for ATP of 150 μM and a V_m of 2.98 μM/min (Table I). The calculated turnover number is 125 mol ATP/min/mol UvrA which is twice as high as has been previously reported (1,22). The UvrA ATPase also displays substrate inhibition beyond 1 μM ATP (unpublished observations).

Although the UvrA ATPase is not activated by the presence of DNA, the ATPase activity is modulated by changes in the DNA concentration (Figure 1). An analysis of the initial rates at 150 μM ATP, which is the K_m for the UvrA ATPase, shows a decrease in the rate at low DNA concentrations. Further increases in the DNA concentrations result in a gradual return to the rates similar to that seen in the absence of DNA (Figure 1). Determination of the kinetic constants show decreases in the K_m and V_m that are dependent on the DNA concentration and on the type of DNA effector. For instance, the presence of ssDNA results in the lowest K_m value of 5 μM. Situations that increase the concentration of single stranded-like regions, such as high DNA concentrations (300 uM dsDNA) or UV irradiation of DNA, also result in decreases in K_m. The modulatory effects of DNA on the ATPase are also indicated by the changes in the K_i for competitive inhibitors ADP and ATP-γ-S (Table 3). When DNA is present in the reaction, the K_i of ADP increases eight fold from a value of 20 μM. On the other hand, the K_i for ATP-γ-S

Figure 4. Dependence of activation of the UvrAB ATPase on the length of oligonucleotide effector. **A.** Polynucleotides (pdA_n) of various lengths were used as effectors and compared to fd ssDNA for their ability to stimulate the UvrAB ATPase activity. Reaction mixtures contained 2 mM ATP, 2.2×10^{-8}M UvrA and 4.4×10^{-8}M UvrB. Reactions were conducted for 20 min at 37°C and the amount of ATP hydrolyzed determined by the charcoal adsorption assay. The amount of phosphate released is calculated for a 100 μl reaction volume: pdA_6 (diamonds); pdA_{10}, (crosses); pdA_{20}, (triangles), pdA_{40-60} (circles); fd ssDNA (squares). **B.**Double reciprocal plot of the UvrAB ATPase activity as a function of DNA concentration (in nucleotides).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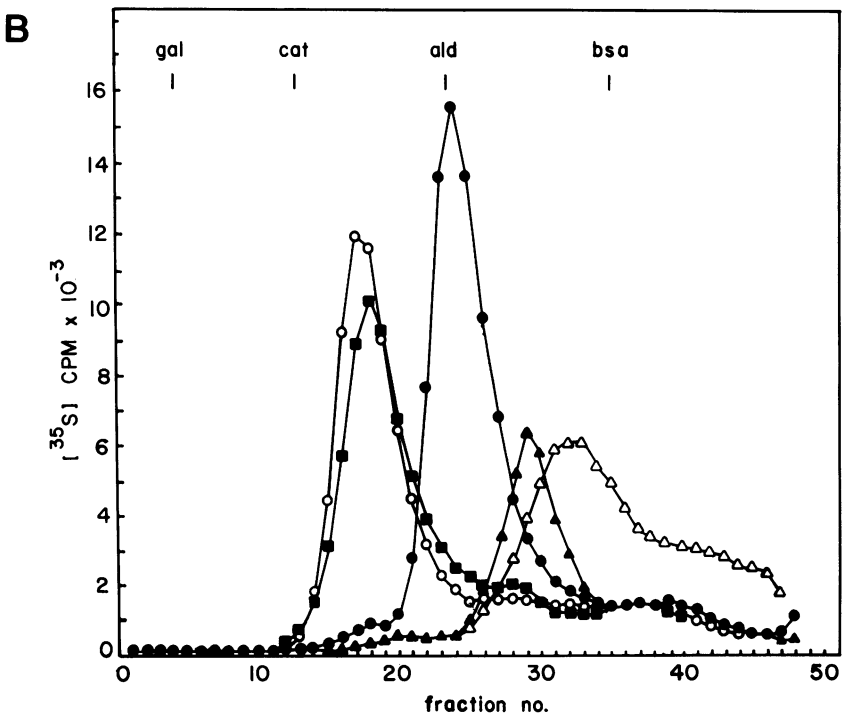
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decreases seventy one fold from the reported value of 50 μM. The increase in the affinity of the UvrA-DNA complex for ATP-γ-S agrees with the increase in affinity for ATP (seen as decreases in K_m). In addition, inhibition by ADP results in non-linear Lineweaver-Burke plots that indicate the presence of cooperativity (23).

The absence of an undamaged dsDNA-activated ATPase activity for UvrA is not due to the presence of contaminating ssDNA. UvrA purified by chromatography on a heparin-Sepharose column displays identical kinetic behavior to protein purified on an ssDNA-Sepharose column. Secondly, if contaminating ssDNA or dsDNA is present in the protein preparations, the addition of UvrB should result in a noticeable increase in the V_m (Table 2). The predicted change is not observed. Instead, the V_m of the ATPase decreases when UvrB is added to UvrA. We conclude that the protein preparations are not contaminated with ssDNA and that the lack of dsDNA dependence is an inherent property.

Sedimentation analysis of the UvrA Protein. The binding of a ligand such as a nucleotide can affect the quaternary structure of protein complexes. For example, the T4 phage gene41 protein is a helicase involved in the replication of phage DNA (30). Although the protein is a monomer, GTP binding induces formation of the active dimer. Interestingly, the T4 gene41 protein displays some DNA independent ATPase activity that may be, in part, caused by the monomer-dimer transitions. Since the UvrA ATPase also displays DNA independence, we analyzed the effect of nucleotides on the UvrA quaternary structure. UvrA was sedimented through 15−35% glycerol gradients and assayed for UvrABC endonuclease activity (Figure 5a). In the absence of ATP in the gradient, two peaks of equal activity are observed with transport properties indicative of dimers and monomers. The presence of ATP in the gradient results in a broad peak of activity that migrates between the monomer and dimer species. Moreover, the proportion of the species migrating near the dimer form is greatly increased relative to the monomer fraction and the recovery of total UvrA activity is several fold more than that obtained in the absence of ATP.

To further analyze the effect of nucleotides, we sedimented [^{35}S]-methionine labeled protein through glycerol gradients (Figure 5b). In the absence of Mg^{2+} and ATP, the labeled protein migrates to a position corresponding to a monomer: the recovery of radiolabeled protein is only 20−30%. Although the presence of 15 mM $MgCl_2$ shifts the peak slightly toward the bottom of the gradient, the labeled protein still migrates with the characteristics of a monomer. In the presence of ATP-γ-S or ADP, the recovery of protein increases to about 70% and the labeled protein migrates like a dimer. This increase in recovery is the same as the increase in the recovery of endonuclease activity previously seen in the sedimentation analysis of the non-radiolabeled protein. With ATP in the gradient, we found a single peak migrating between the dimer and monomer forms. Unlike the broad

Figure 5. Glycerol gradient sedimentation analysis of purified UvrA protein. **A.** UvrA protein (3.3 μg) was treated with 2 μM ATP or no ATP at 37°C for 10 min. Samples were loaded onto 15−35% glycerol gradients containing 4 μM ATP (closed circles) or lacking nucleotide (open circles), and centrifuged as described in 'Material and Methods'. After fractionation, 50 μl of each sample was removed and treated with 5.6 μM phosphoenolpyruvate and 5 units of pyruvate kinase for 10 min at 37°C to convert ADP to ATP. The samples were then analyzed for UvrABC endonuclease activity by incubation of fractions with UvrB and UvrC in the presence of UV irradiated [^{3}H]-fd DNA (containing approximately 6 photoproducts per fd DNA) for 20 min at 37°C. The DNA was denatured in alkali, renatured, and passed through nitrocellulose filters. The filters were dried and counted in scintillation fluid. The assay detects the conversion of covalently closed circular DNA to nicked DNA. **B.** Glycerol gradient sedimentation analyses of [^{35} S]-methionine labeled UvrA is shown. Approximately 1 μg of radiolabeled protein was sedimented as described for unlabeled protein: no $MgCl_2$ (open triangles); 15 μM $MgCl_2$ (closed triangles); 2 μM ATP (closed circles); 2 μM ADP (open circles); 2 μM ATP-γ-S (closed squares).

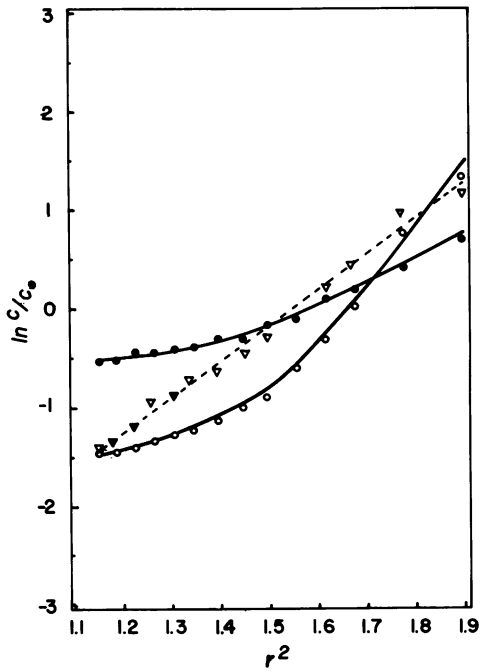

Figure 6. Sedimentation equilibrium in a airfuge. Samples were prepared in a volume of 200 μl and contained 50 mM MOPS, 100 mM KCl, 2 mM DTT, pH 7.6 and either $MgCl_2$ or ATP as indicated. Aldolase and [^{35}S]-methionine labeled UvrA was at 777 ng and 1.2 μg, respectively. Volume used for sedimentation is 150 μl.: 15 mM $MgCl_2$ (closed circles); 2 mM ATP + 15 mM $MgCl_2$ (open circles); aldolase (triangles).

distribution displayed by the non-radiolabeled protein, the radiolabeled protein shows a well defined peak. This difference is probably due to the higher protein concentration used for the sedimentation analysis of non-radiolabeled protein.

The low recovery of protein in the sedimentations conducted in the absence of nucleotide is not due to aggregation of UvrA. Recoveries that are identical to those from glycerol gradients are obtained when UvrA is chromatographed through a Bio-Gel 0.5M gel filtration column (unpublished observations). We observe no peak corresponding to an aggregated form in the void volume when ATP is absent from the elution buffer. Furthermore, equilibrium sedimentation analysis (see below) clearly shows that UvrA is not aggregated in the absence of nucleotide. The binding of nucleotide alters the properties of the protein such that nonspecific adsorption is reduced.

The changes observed by glycerol gradient sedimentation either can be explained by alterations in the subunit composition or by changes in the hydrodynamic properties of the UvrA monomer. To distinguish between these possibilities, the radiolabeled protein was subjected to equilibrium sedimentation analysis in an air driven centrifuge (Figure 6). The equilibrium sedimentation analysis should be affected only by the molecular weight and not by the hydrodynamic properties of the protein. When the protein was sedimented in the absence of nucleotide, the ln c/c^o vs r^2 plot is slightly curvilinear indicating some heterogeneity. However, the slope of the ln c/c^o vs r^2 plot for the entire gradient is lower than the aldolase (MW 149100) standard demonstrating that UvrA exists substantially as

a monomer. Equilibrium sedimentation in the presence of ATP results in a ln c/c^o vs r^2 profile with increased curvilinearity and a slope at the bottom half of the gradient that is significantly greater than that of aldolase (i.e. a molecular weight greater than aldolase): similar profiles are observed with ADP and ATP-γ-S. Although we have not quantitated the amount of ATP hydrolyzed during the centrifugation, the equilibrium sedimentation data clearly demonstrates that changes observed by glycerol gradient sedimentation are due to the formation of UvrA dimers and not due to changes in the hydrodynamic properties of the UvrA monomer.

UvrAB ATPase activity. It is known that UvrA and UvrB in the presence of ATP can form a stable complex on UV damaged DNA (16,27). Such complexes are resistant to dissociation by EDTA and can undergo endonuclease cleavage events upon addition of UvrC. Furthermore, the UvrAB complex also displays a helicase activity that acts on short DNA duplexes (28). The interaction of UvrA with UvrB however, does not significantly alter the affinity of the protein complex for DNA damage when compared to UvrA by itself (25). The ATPase resulting from interaction of UvrA and UvrB shows lower K_m and V_m when compared to UvrA (Table 2). The decrease in K_m and V_m mimic those seen for UvrA in the presence of DNA. The addition of DNA results in an activation of the UvrAB ATPase. The activation is specific to DNA since polyribonucleotides of all forms do not stimulate the ATPase (23). Furthermore, stimulation is greater with UV irradiation than with unirradiated DNA (23, Figure 3). An analysis of the kinetic constants for the DNA stimulated UvrAB ATPase shows similar V_m in presence of dsDNA and UV dsDNA (Figure 2, Table 2). However, there is a five to six fold reduction in the K_m for ATP when UV irradiated dsDNA is used as the effector. A ten fold lower concentration of DNA still results in five fold difference in the K_m. This demonstrates that the apparently higher rate of ATPase activity observed with UV irradiated DNA versus undamaged DNA is due to differences in the K_m and not due to differences in the V_m. Analyses of competitive inhibitors ADP and ATP-γ-S results in K_i values similar to those seen for UvrA-DNA complexes (Table 3). Non-linear Lineweaver-Burke plots are also observed with inhibitor ADP (23).

When single stranded DNA is used as an effector, a biphasic double reciprocal plot is obtained. One phase, with a K_m of 65 μM is similar to that obtained with UV-irradiated dsDNA while the second phase with a K_m of 844 μM is similar to that obtained with undamaged DNA. Although V_{m1} is similar to the V_m observed for UV irradiated dsDNA, V_{m2} in nearly 3.5 times greater than the V_m obtained for the dsDNA stimulated ATPase. The nonlinear double reciprocal plots obtained with ssDNA is indicative of either negative cooperativity or the presence of two catalytic entities with $K_{m1} V_{m1} << K_{m2} V_{m2}$. To insure that the differences in the kinetic parameters were not due to the interaction of DNA with protein complexes containing different ratios of UvrA and UvrB, a titration with UvrB was conducted while keeping UvrA and DNA concentrations constant (Figure 3). With the various DNAs, all the reactions displayed half maximal activation at the same concentration of UvrB suggesting that the protein complexes are the same in all cases.

To examine the influence of DNA length on the ATPase activity, oligonucleotides (pdA) of various lengths were tested (Figure 4a,4b). Since activation by single stranded DNA exhibits multiple K_ms with the highest near 0.8 mM, assays were conducted near a V_m condition of 2 μM ATP. Although oligonucleotides of 6, 10, and 20 bases in length were capable of activation, they were required at higher concentrations to attain the same level of stimulation caused by fd ssDNA. A 40 to 60 base long oligonucleotide elicited a kinetic

profile similar to that of fd ssDNA: a slight concavity in the double reciprocal plot is observed with the pdA_{40-60} (Figure 4b). Thus, interaction with DNAs of 40−60 bases are required to fully activate the ATPase activity. The absence of significant differences between the ATPase rates induced by circular fd ssDNA and pdA_{40-60} suggests that translocation on single stranded DNA, at least over long distances, is not taking place. Such differences are observed for the T4 gene41 helicase (30). The oligonucleotide length of 40−60 nucleotides approximates the length of DNA that is protected by the UvrA protein (24).

DISCUSSION

In this study, we analyzed the ATPase activities of the UvrA and UvrAB protein complexes. The ATPase activities reveal several important features about the intermediates leading to incision of damaged DNA. We infer from the sedimentation data that the first step in the pathway to incision is a nucleotide induced dimerization of UvrA. It is the dimer which constitutes the active DNA binding unit (25). The enhancement of dimerization may result in the enhancement of the general DNA binding activity seen in the presence of ATP. Upon hydrolysis of ATP, the dimer reverts to the monomer. This transition accounts for the peak migrating between monomers and dimers in glycerol gradients containing ATP. The peak represents a time averaged value due to the rapid equilibrium between monomers and dimers as opposed to the strictly dimeric state induced by ADP or ATP-γ-S. These monomer-dimer transitions can account for the DNA independent ATPase activity of UvrA.

Interactions between UvrA and DNA leads to changes in the interaction of UvrA with nucleotide cofactors. This is evident from the ability of DNAs to modulate the K_m and V_m of the ATPase and to alter the K_i for ADP and ATP-γ-S. There is a general increase in the affinity for ATP-γ-S and ATP by the UvrA-DNA complex. These changes in the ATPase properties of UvrA, when bound to DNA, may be necessary for the cooperative interactions with UvrB that result in a DNA stimulated ATPase. It may also limit monomerization and dissociation from DNA upon ATP hydrolysis. From careful analyses, we conclude that UvrA by itself is not a DNA activated ATPase. This is similar to the MuA protein involved in transposition of Mu phage DNA (26).

Formation of the UvrAB complex results in a DNA-stimulated ATPase. The kinetic constants of this ATPase change depending on the nature of the DNA effector. The interaction with UV damaged DNA results in a 6 fold decrease in K_m as compared to interactions with undamaged DNA. This alteration in the kinetic profile reflects changes in the biochemical characteristics of the UvrAB-DNA complex. For instance, UvrAB bound to a DNA damage site is resistant to dissociation by EDTA while binding to undamaged DNA is EDTA sensitive (16) . In addition, the binding to a damage site results in a change in the length of the DNA 'footprint' as compared to that observed for the UvrA alone (24). Both of the changes are dependent on the presence of ATP.

Previous work has suggested that a prerequisite for incision is the alteration of the DNA damage site through the helicase activity of the UvrAB complex (28). Interestingly, ssDNA stimulates an ATPase with biphasic kinetic behavior similar to that elicited by a combination of UV dsDNA and dsDNA. The kinetic profile of the ATPase activity of UvrAB-UV DNA complexes may reflect interactions with unwound single stranded regions near the damage site. The induction of two kinetic phases in the ssDNA stimulated ATPase can result from

ssDNA interacting simultaneously with DNA binding sites responsible for nonspecific binding and specific binding to DNA damage.

The UvrAB ATPase differs on several points from the UvrB ATPase activity reported previously (13). The UvrB ATPase activity is observed for UvrB*, the proteolyzed UvrB protein, while the intact UvrB protein has barely detectable ATPase activity. The UvrB* ATPase is activated by ssDNA and chaotropic agents, but not activated by dsDNA. Thus, the activation is a general one resulting from global changes in protein structure. The activation of the UvrAB ATPase activity, on the other hand, is specific for DNA since polyribonucleotides of all forms fail to activate the ATPase. This specificity is indicated by several other properties. (1) The UvrAB ATPase is easily saturated by DNA (both dsDNA and ssDNA) while the UvrB* ATPase is not saturated even at a hundred fold higher ssDNA concentration than that used in this study. (2) The UvrB* ATPase displays simple Michaelis-Menton kinetics and does not display the biphasic kinetics observed with the UvrAB-ssDNA complex. (3) The shift in the K_m observed for the UvrAB ATPase when the DNA effector is UV irradiated dsDNA is not seen with the UvrB* ATPase. (4) A UvrAB* complex is incapable of forming an endonuclease that incises damaged DNA. The same complex does not display the DNA stimulated ATPase activity that is readily observed with the intact UvrAB complex.

We have not determined which of the subunits in the UvrAB protein complex is responsible for the various ATPase activities. The variations in the K_m and V_m of the UvrA and the UvrAB ATPases does not allow a specific assignment of the responsible subunit by a comparison of the kinetic values displayed by the individual proteins (i.e. UvrA and UvrB*). A previous study suggested that UvrB might be the DNA activated ATPase with UvrA being an anchor that juxtaposes UvrB and DNA (13). This conclusion was based on the observation that the UvrA ATPase can hydrolyze GTP while the UvrB* and UvrAB ATPases are specific for ATP or dATP. If UvrB is the only DNA-activated ATPase, it must be differentially activated by dsDNA and UV damaged DNA as implied by the changes in K_m of the UvrAB ATPase.

It is possible that the 'DNA activation' may occur through UvrA-UvrB protein contacts rather that UvrB-DNA contacts. If this were the case, the only requirement for unmasking of the UvrB ATPase should be the interaction of UvrB with UvrA protein bound to DNA. Since UvrA-DNA complexes are readily formed in the presence of GTP or GTP-γ-S, UvrAB-DNA complexes would display a DNA stimulated ATPase in spite of the addition of GTP-γ-S to inhibit the UvrA ATPase (13). The ability of UvrA to distinguish between damaged and undamaged DNA would provide a basis for explaining the changes in the K_m of the UvrAB ATPase.

In any case, if recognition of damaged DNA results in a conformational change in UvrA which is translated to UvrB by means of protein-protein interactions, the UvrB ATPase activity may function to modulate the interaction of the dimeric UvrA protein with DNA. The results of such changes may be the formation of stable, EDTA resistant protein-DNA complexes on DNA damage and elaboration of the helicase activity. However, it should be noted that UvrA contains two putative nucleotide binding sites either of which may be activated under various conditions and display different nucleotide specificities. The similar K_i for ADP and ATP-γ-S of the UvrA and UvrAB ATPases (irrespective of the DNA effector) implies that the UvrA ATPase is operative in the UvrAB-DNA complex.

Furthermore, both ATPase activities of UvrA and UvrB are required for endonuclease activity (13). A clear delineation of the function of the UvrA and UvrB ATPase activities will come from mutagenesis of the various nucleotide binding regions. The differences in the kinetic properties of the UvrA and UvrAB ATPases described in this report should aid in the study of the mutated proteins.

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ABBREVIATIONS

MOPS	(N-morpholino)propanesulfonic acid
ssDNA	single stranded DNA
dsDNA	double stranded DNA
ATP-γ-S	adenosine-5′-O-(3-thiotriphosphate)
TLC	thin layer chromatography

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