The effect of *Escherichia coli* Uvr protein binding on the topology of supercoiled DNA

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ABSTRACT

The effects of the binding of the *E. coli* UvrA and UvrB proteins on the linking number (ΔL) of superhelical DNA has been measured. The effects of cofactor ATP structure on UvrAB-nucleoprotein complex formation revealed that nucleotide binding, not hydrolysis, is sufficient to locally unwind the DNA helix of both ultraviolet light-damaged as well as undamaged DNAs. The extent of this unwinding is of the same order of magnitude as the nucleotide distances of the double incision sites generated by the UvrABC endonucleolytic reaction.

INTRODUCTION

The *E. coli uvr* repair system is unique in that its specificity is sufficiently broad to accommodate a wide spectrum of seemingly different kinds of DNA damaging agents. As a consequence, organisms possessing such a system can respond to a plethora of environmental hazards without having to evolve new enzyme systems as new types of agents are introduced into its environment. Parenthetically, the spectrum of agents to which *E. coli uvr* mutants are sensitive is remarkably similar to the damage sensitivity of human repair-deficient skin fibroblasts derived from xeroderma pigmentosum patients (1). These findings suggest that the enzyme mechanisms may be similar and point to the significance of studying the *E. coli uvr* system as a model for a DNA repair deficiency amongst patients predisposed to skin cancer

The incision components of the *E. coli uvr* repair system responsible for the initial recognition of damaged DNA consists of the UvrA protein which in the presence of ATP binds to the damaged DNA, as well as to undamaged DNA duplexes (2). The UvrA protein requires thesimultaneous presence of the UvrB and UvrC proteins to initiate a dual incision event in DNAs containing ultraviolet light induced pyrimidine dimers (3,4) and 6,4-pyrimidine-pyrimidone adducts (3), photoactivated psoralen mono- and diadducts (5), acetylaminofluorine-guanine adducts (5), cis-platinum-guanine adducts (6) and benzo[a]pyrene-guanine adducts (7). In almost all of those experiments carried out with DNA substrates of defined sequences, the dual incision events were shown to involve a 5'-incision event 7 nucleotides to a damaged site and 3-4 nucleotides 3'- to the same site. In spite of the fact that the primary chemical events are dissimilar and the extent to which these agents distort DNA, the sites of incision are

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essentially invariant. It is the implication of these observations that the constancy of breakage sites is probably structurally imposed on DNA by the Uvr proteins.

The subject of this manuscript is to demonstrate the influence of the Uvr proteins on the topological nature of the DNA substrate. The binding of the nucleotide cofactor to the UvrA protein, not its hydrolysis, is required for this structural change.

MATERIALS AND METHODS

Proteins and Enzymes: UvrA and UvrB proteins were purified as previously described (4). The UvrA protein was derived from the ssDNA-cellulose fraction and dialyzed against 50 mM MOPS, pH 7.6, 0.3 M KCl, 2 mM DTT, 1 mM EDTA, 15% glycerol. The UvrB protein used was from the Sephadex G150 fraction. Both were judged greater than 95% pure when analyzed by densitometer scanning of Coomasie stained SDS polyacrylamide gels. Calf thymus topoisomerase I was purchased from BRL. Purified photolyase was a gift from Dr. Aziz Sancar (University of North Carolina).

DNA: The RFI form of fd phage DNA was isolated from infected K37 strains after chloramphenicol amplification (27). After purification through two cesium chloride gradients, the RFI fraction was isolated, the DNA precipitated and then passed through Bio Gel 15M column (0.7 x 25 cm) to remove RNA and salts. DNA concentrations were determined spectrophotometrically using an extinction coefficient of 7400 $cm^{-1} \cdot M^{-1}$. pBR322 DNA was isolated after choroamphenicol amplification following described procedures (8).

Binding Assay: (Figures 1A and 1B). To determine the effect of UvrA binding on the topology of unirradiated covalently closed circular DNA, 0.24 pmol of RFI fd (1 μg) was reacted with UvrA protein for 20 min at 37^0C in a 40 μl reaction volume containing 50 mM MOPS, pH 7.6, 0.1 M KCl, 15 mM $MgCl_2$, 2 mM DTT, 1 mM EDTA and 2.5 mM of indicated nucleotide. When ATP was the nucleotide in the reaction, an ATP regenerating system consisting of 1 unit of pyruvate kinase and 2-4 mM phosphoenolpyruvate was included to limit the accumulation of ADP and depletion of ATP. Five units of calf thymus topoisomerase I were added and incubated for 10 min at 37^0C to allow relaxation of the DNA. Samples were quickly quenched with phenol (warmed to 37^0C) and treated with 5 μl of 5% SDS-0.1M EDTA, pH 8.0. After a second phenol extraction, phenol was removed by ether extraction and the DNA precipitated with isopropanol. The DNA was pelleted, dried and then resuspended in 20 μl of 10 mM Tris, pH 8.0, 1 mM EDTA. Five μl of 25% Ficoll, 5% Sarkosyl, 0.01% bromophenol blue was added and the samples applied to a 0.8% agarose gel containing 19 to 38 μM chloroquine to permit separation of the population of topoisomers (9). Samples were subjected to electrophoresis for 24-30 hrs at 1.5-2 $V \cdot cm^{-1}$ depending on the separation desired. The electrophoresis buffer was recirculated to maintain a relatively constant pH. The gels were stained 6 hrs in 2 μg/ml ethidium bromide (with gentle shaking), destained for 0.5-1 hr in deionized water and then photographed with Polaroid Type 55 negative film. The length of exposure was 6-10 min. The problems of quantitating photographic negatives of ethidium
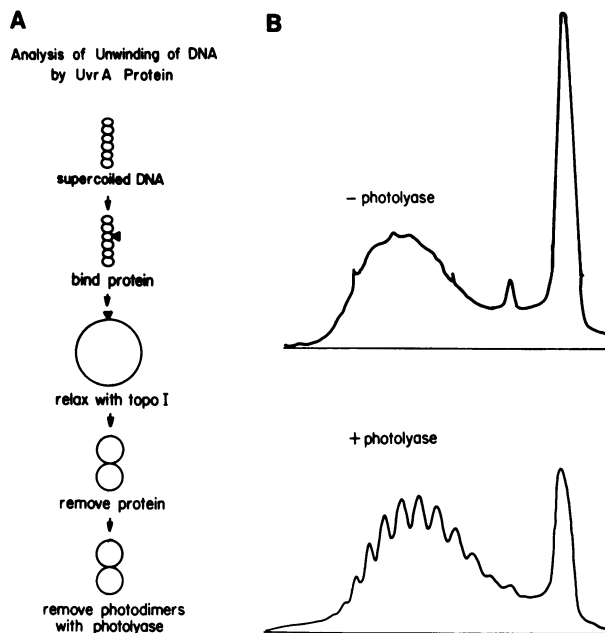
Figure 1A: Assay for DNA Unwinding. UvrA protein is bound to DNA and relaxed with topoisomerase I. The DNA is purified by phenol extraction, precipitated with ethanol and analyzed on chloroquine agarose gels. For UV irradiated DNA, following relaxation and purification, the DNA is treated with photolyase to reverse the cyclobutane dimers.
Figure 1B: Densitometer tracing of UV irradiated RFI fd DNA untreated (top) or treated (bottom) with the *E. coli* photolyase.

bromide stained gels have been described (10). The negatives were scanned using an Helena Laboratories Quick Scan Densitometer and peaks heights and areas determined using a Nelson Analytical 3000 Chromatography Analyzer program. To determine the center of the distribution of topoisomers, the data were analyzed according to the equations developed by Depew and Wang (11).

To determine the effect of UvrA protein binding on the topology of UV irradiated DNA (Figure 1), the protein and DNA were reacted under the same conditions except that the binding phase was 15 min and the time of relaxation by topoisomerase I was increased to 20 min. After quenching the reaction with phenol, samples were ether-extracted and isopropanol-precipitated as before. The dried DNA pellet was resuspended in 20 μl of photolyase buffer consisting of 50 mM Tris, pH 7.4, 50 mM NaCl, 10 mM β-mercaptoethanol, 1 mM EDTA. After addition of photolyase, the samples were irradiated for 1 hr with two 15W F15T8-BLB 'Black lights' at a distance of 15 cm to promote photoreversal. The samples were either quenched with sample buffer or phenol, ether-extracted and precipitated with isopropanol. The samples were analyzed on 0.8% agarose-chloroquine gels.

Binding Reaction at Low Temperature: For reactions executed at lowered temperatures, samples were allowed to incubate at 37^0C for 10 min and then quickly chilled by placing on ice. Five units of topoisomerase I were added and relaxation conducted for 2 hrs at 4^0 (a time determined to be required for complete relaxation).   The samples were quenched with chilled phenol followed by 5 μl of SDS-EDTA solution. The samples were warmed, ether- extracted and isopropanol-precipitated as before. Samples were applied to 0.8% agarose gels in 40 mM Tris, 20 mM acetate, 2 mM EDTA, pH 8.0. Electrophoretic separation of topoisomers was conducted at 2 $V \cdot cm^{-1}$ at room temperature for 30 hrs and analyzed by ethidium bromide staining.

Nucleotides: ATP and ADP were purchased from PL Biochemicals. AMP-PNP and ATP-γ-S were purchased from Boehringer-Mannheim Biochemicals and used without further purification. Both solutions were contaminated with 5% ADP when analyzed by chromatography on PEI cellulose plates. Concentrations of nucleotide solutions were determined spectrophotometrically.

UV Irradiation of DNA: DNA at a concentration of 250 μg/ml in 10 mM Tris, pH 8.0, 1 mM EDTA, was irradiated in 25 μl droplets on a sterile petri dish by a 15 W mercury germicidal lamp at a dose rate of 2 $J.m^{-2} \cdot sec^{-1}$. Under these conditions, cyclobutane photodimers are produced at a rate of 0.068 per sec (12).   In the case of pBR322, as determined by transformation of irradiated plasmid into CSR603 and scoring for tet^r colonies, the rate of photoproduct formation under the same conditions is 0.041 per sec . The rate of photoproduct formation, calculated using Poisson distribution and for pBR322, is in good agreement with published figures (13).

RESULTS

Effect of Nucleotide on Unwinding of Non-UV Irradiated DNA: DNA binding proteins, such as CAP protein, EcoR1 endonuclease , and RNA polymerase can cause unwinding of DNA. The unwinding angle for the CAP protein and Eco RI endonuclease is small, 45 and 25 degrees (^{0}) respectively (14,15). The role of unwinding is uncertain, although it may be a mechanism for recognition of specific sequences (15). For RNA polymerase, the unwinding angle measured has varied between 240^0 (7 bases) to 580^0 (17 bases) and has been suggested to be a part of the mechanism for the formation of the 'open complex' required for initiation of RNA synthesis (16,17). Since UvrA is a DNA binding protein and an ATPase, we have attempted to analyze the effects of UvrA binding, in the presence of various nucleotides, on the structure of DNA. In the presence of ATP and increasing amounts of UvrA protein, very little DNA unwinding is observed at a salt concentration of 100 mM KCl (Figure 2). There is an initial increase in unwinding which quickly plateaus at lower concentrations of UvrA reaching a maximal unwinding, ΔL, of 0.5 turns. Similarly, very little unwinding is observed in the absence of a nucleotide. The interpretation of a lack of unwinding in the presence of ATP is complicated by the possibility that binding of UvrA to DNA may be in a dynamic state accompanied by ATP hydrolysis such that a rapid association-dissociation reaction may be
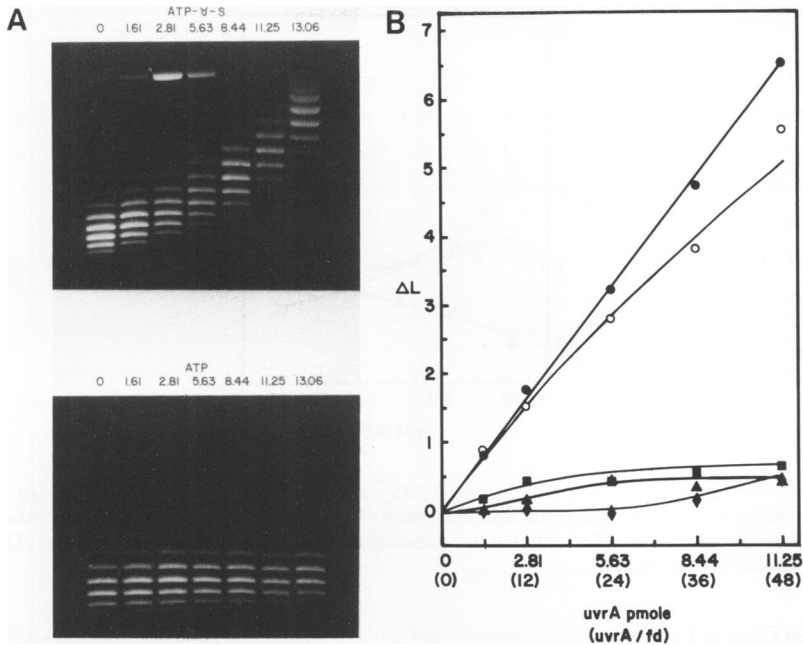
Figure 2: Effect of Nucleotides on the Unwinding by UvrA Protein: All nucleotide concentrations were 2.5 mM. Standard concentrations of KCl were maintained at 100 mM. The binding reaction was for 20 min followed by 10 min of relaxation by topoisomerase I at 37°. A: ethidium bromide stained gel of DNA unwound in presence of ATP-γ-S (upper panel) or ATP (lower panel) with indicated amounts of UvrA protein in pmoles. B: quantitation of unwinding in the presence of nucleotides. Unwinding is represented as ΔL which refers to the change in linking number. a. ATP-γ-S (●), b. AMP-PNP (○); c. ATP (▲); d. ADP (◆); e. - nucleotide (■).

linked to nucleotide hydrolysis. Thus, the life-time of the complexes may not be long enough to be observed in the unwinding assay since the rate of relaxation may be too slow to respond to this association-dissociation driven by conversion of ATP to ADP and orthophosphate. One may expect to see the steady-state condition, that is, the average unwinding seen assuming that a certain fraction of the protein is bound to the DNA at any one time. However, this requires some knowledge of the binding constant for non-damaged DNA. The presence of ADP, an inhibitor of the ATPase and DNA binding activity, results in similar effects to those observed reactions carried out in the absence of nucleotide. However, in the presence of ADP, more UvrA protein is required to observe the small changes in unwinding.

In the presence of ATP-γ-S there is a marked linear increase in unwinding of fd RFI DNA with increasing concentrations of UvrA protein, not attaining saturation even at a ratio of UvrA protein monomer to fd molecule of 64 to 1 (Figure 2). It is not possible to determine the absolute unwinding angle since the nature (size and number) of the binding 'sites' on
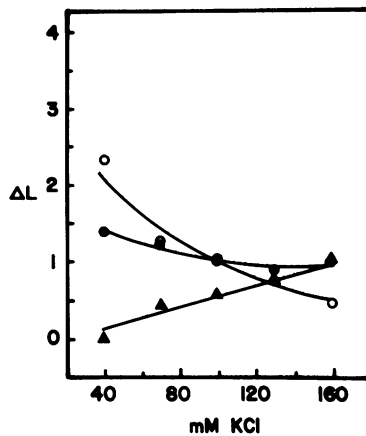
Figure 3: Effect of Salt on DNA Unwinding: 11.25 pmole of UvrA protein was incubated with non-UV irradiated DNA at indicated KCl concentrations either in the absence (●) or presence of 2.5mM ATP (O). The effect of salt on topoisomerase I action in the absence of UvrA protein was also measured (▲).

undamaged DNA and the equilibrium binding constant of UvrA-DNA complex in the presence of ATP-γ-S is not yet known. Assuming that 100% of the UvrA molecules are active and bound to the DNA, an unwinding of 50^0 is calculated for a UvrA monomer. AMP-PNP behaves similarly to ATP-γ-S, in inducing the UvrA protein to unwind DNA by 45^0. However, AMP-PNP consistently induces a nonlinear change in ΔL with increasing concentrations of UvrA protein. Salt concentrations up to 160 mM KCl do not significantly influence unwinding in the presence of either ATP analogs. Recent data indicate that the UvrA protein exists as a dimer in solution under conditions in which it binds to DNA (18). Under such circumstances an unwinding angle approaching 100^0 for a (UvrA)$_2$-nucleoprotein complex should be achieved assuming quantitative binding of the UvrA dimer. If properly extrapolated, such binding represents an unwinding of 3 bases. This is probably an underestimate of the actual value of unwinding in the presence of ATP analogs.

Effect of Salt Concentration on DNA Unwinding: Since salt can affect the interaction of proteins with DNA, the effect of ionic strength on the unwinding of DNA in association with the UvrA protein was measured by adjusting the salt concentration from 40 to 200 mM KCl. The effect of salt concentration on topoisomerase I action in the absence of added protein showed a small linear increase in ΔL when using 40 mM KCl as a reference. This change may be due to a slight inhibition of the topoisomerase I with increasing levels of salt (Figure 3).

In the absence of ATP, UvrA protein unwinds the DNA at low salt concentrations but the effect decreases with increasing levels of salt, approaching zero value at 100 mM KCl. At salt concentrations greater than 160 mM KCl, topoisomerase is inhibited resulting in a
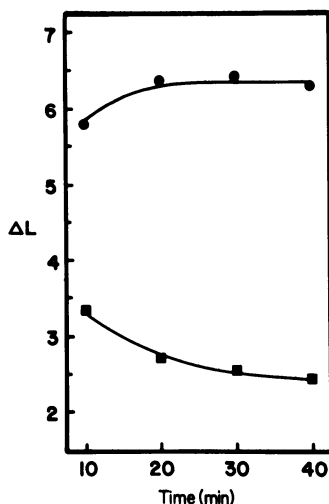
Figure 4: Time course of Binding-Relaxation of fd RFI DNA in the presence of the UvrA Protein: 11.25 pmole of UvrA protein and topoisomerase I were reacted with 1 μg RFI fd DNA for the indicated length of time. The DNA was purified and then analyzed on chloroquine-agarose gels. The UvrA protein in the presence of 2.5 mM ATP-γ-S + non UV irradiated DNA (●). The UvrA protein in presence of 2.5 mM ATP and DNA irradiated to contain 24 dimers per fd molecule (■).

non-Gaussian topoisomer distribution. When ATP is included in the assay, there is slightly more unwinding at low salt concentrations. However, increasing ionic strength decreases unwinding to a level attained in the absence of nucleotide.

Kinetics of Binding and Relaxation: To obtain an accurate determination of ΔL both the binding of the protein and the relaxation of the DNA should be at or near equilibrium. If binding of UvrA has not reached equilibrium, then the value of ΔL will be an underestimate since the fraction of protein bound will be less than at equilibrium. Conversely, if the relaxation is incomplete or the binding of protein inhibits the relaxation, this will lead to an overestimate of the value of ΔL. To determine the optimal times of binding and relaxation, protein and topoisomerase I were added simultaneously and incubated for various times (Figure 4). In the presence of UvrA protein and ATP-γ-S, the level of ΔL increases from 5.5 to a plateau value of 6.3 between 10 and 20 min of incubation. The increase suggests that maximal binding of protein to DNA occurs before 20 min. Relaxation of supercoiled DNA by topoisomerase I is complete by 10 min in the absence of UvrA protein (data not shown). Previous estimates, therefore, of unwinding were obtained under equilibrium conditions.

Unwinding of UV Irradiated DNA: When the unwinding of UV irradiated DNA was studied, the basic assay was modified to allow for the resolution of damaged topoisomers by treating the DNA with the *E. coli* photolyase to photoreverse the cyclobutane dimers (Fig 1). One of the
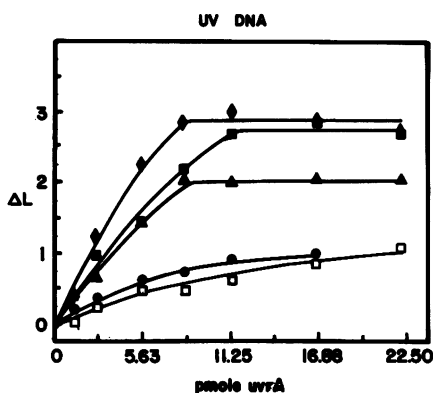
UV DNA

Figure 5: Unwinding of UV Irradiated DNA by the UvrA Protein in the Presence of ATP: fd RFI DNA was reacted under described conditions in the presence of indicated amounts of UvrA protein. An ATP-regenerating system was included so as not to deplete ATP a; non UV irradiated DNA + ATP (●); b, UV irradiated DNA with 12 dimers per fd - ATP (□); c, UV irradiated DNA 6 dimers per fd + ATP (▲); d, 12 dimers per fd + ATP (■); e, 18 dimers per fd + ATP (◆).

main drawbacks encountered in the analysis of topoisomers of UV irradiated DNA is the loss of resolution of topoisomer bands containing pyrimidine photoproducts. The lack of resolution of topoisomers made it difficult to apply the method of Depew and Wang (11) which requires that the center of the topoisomer distributions be determined. The assumption that the level of resolution between topoisomers is constant, allows analyses of ΔL by measuring the Rm value of the peak of the smear. However, such measurements could not be made since the mobility and separation distance between topoisomers varied when UV irradiated DNA was treated with UvrA protein and toposiomerase. The loss of resolution has been observed with DNA modified by other bulky adducts such as cis-platinum and psoralen (19,20). Pyrimidine cyclobutane dimers however, are unique damage products in that they can be enzymatically photoreversed using photolyase. Hence, to overcome the loss of resolution with UV irradiated DNA after its relaxation with topoisomerase I, the UV irradiated DNA was treated with *E. coli* photolyase to remove the cyclobutane dimers. The topological effects of the UvrA protein binding should be retained with the topoisomer bands being more clearly resolved (Figure 1B). Slight losses of resolution are still observed since photoproducts such as 6-4 pyrimidine-pyrimidones are not substrates for the photolyase.

In an analysis of the kinetics of binding-relaxation reactions of UvrA protein with UV irradiated DNA in the presence of ATP, there is a gradual decrease in ΔL with incubation time with fd RF1 DNA irradiated to contain on the average 24 dimers per molecule (Figure 4). Since filter binding experiments show maximal binding of the UvrA protein to DNA occurs well within the 15 min incubation period, the relaxation rate of UV irradiated DNA by topoisomerase I defines the time required to reach equilibrium. Therefore, relaxation of the
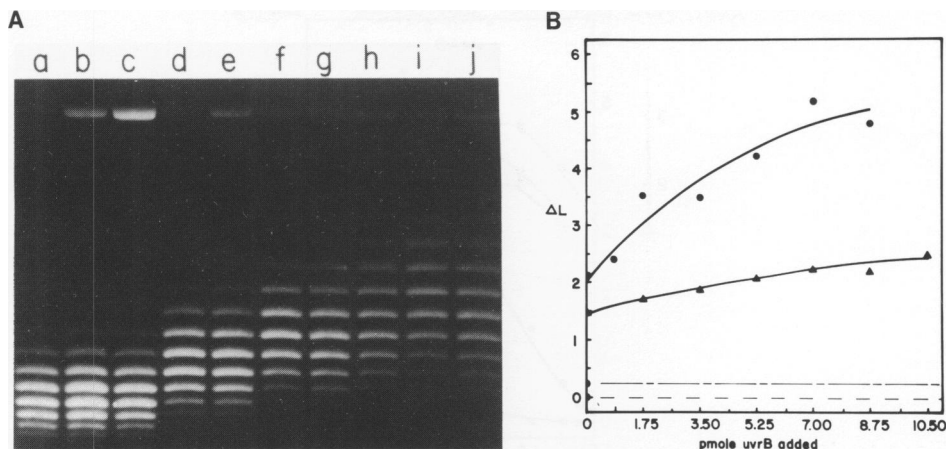
Figure 6A: Influence of the UvrB Protein on the DNA Unwinding by the UvrA Protein: ethidium bromide stained chloroquine-agarose gel containing a, no protein ; b, 7.0 pmole UvrB + ATP-γ-S, c, 2.81 pmole UvrA + 7.0 pmole UvrB - ATP-γ-S, d, 2.81pmole UvrA + ATP-γ-S, e-j, 2.81 pmole UvrA + ATP-γ-S + 0.88, 1.75, 3.50, 5.25, 7.00, and 8.75 pmole of UvrB protein, respectively.

Figure 6B: Quantitation of Unwinding in the Presence of Fixed UvrA and Increasing Amounts of UvrB Protein: a, non UV irradiated DNA in the presence of 2.5 mM ATP-γ-S (●); b, UV irradiated DNA containing 6 dimers per fd in the presence of 2.5 mM ATP and ATP regenerating system (▲); c, 7.00 pmole Uvr B protein in 2.5 mM ATP-γ-S (—•——); d, 2.81 pmole UvrA and 7.00 pmole UvrB protein minus nucleotide (————).

UV treated DNA was allowed to proceed for 20 min at 37^0C. It has been reported that UV irradiation inhibits the action of topoisomerase I (21).

Unirradiated DNA is not a substrate for the UvrABC endonuclease, hence, it is not unexpected that little change in ΔL is observed in the presence of ATP since there are no specific sites available for interaction. With UV irradiated DNA, however, specific binding sites are present and the ΔL should be readily observed in the presence of ATP and UvrA protein. The final value of ΔL should be dependent on the average number of dimers per fd and should reach finite values directly related to the limiting number of specific sites. fd RFI DNA was irradiated with various doses of UV light and reacted with UvrA protein in the presence of ATP. For a DNA irradiated to contain 6 dimers per fd, there is an initial rise in ΔL which then plateaus at ΔL of 1.9 (Figure 5). The amount of UvrA protein necessary to bind all sites in an fd DNA molecule containing 6 dimers per fd should be approximately 2.8 pmole if the protein is bound as a monomer while 5.6 pmole of UvrA should be required if the active binding species is a dimer, assuming quantitative binding by the active species. Experimentally, the amount of UvrA protein required to reach a plateau value is approximately 8.5 pmole, suggesting that the active unit is a dimer of UvrA as mentioned previously. With increasing doses of UV irradiation, further unwinding is observed but the increase in unwinding does not
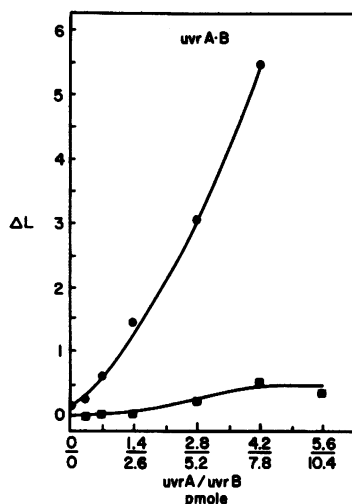
Figure 7: DNA Unwinding by UvrAB Protein Complex: unirradiated DNA was incubated with indicated concentrations of UvrA and UvrB proteins at a molar ratio of 1:2 either in the presence of 2.5 mM ATP-γ-S (●) or 2.5 mM ATP (■).

correlate to the increase in UV dose (i.e. pyrimidine cyclobutane dimer content). Unirradiated DNA undergoes a gradual unwinding but the level is substantially less than for DNA containing dimers. The level of unwinding of a DNA containing 12 dimers, incubated in the absence of ATP, is identical to that observed for unirradiated DNA. The nonlinearity of the final values of ΔL with UV dose may suggest either nonlinearity of photoproduct formation with UV dose or a limit on loading of UvrA protein on DNA. It is also unclear why UV damaged DNA do not show parallel increase in unwinding due to nonspecific interaction (undamaged DNA) after attaining saturation of UvrA protein on damaged sites.

A calculation of ΔL at point of saturation for a DNA substrate with 6 dimers per fd suggests approximately a 80^{0} unwinding per photodimer site after subtracting for non UV-dependent unwinding. This is similar to the change in ΔL seen for unirradiated DNA and ATP-γ-S if a dimer of UvrA is assumed to bind to a damaged site. The value of ΔL derived from data using ATP-γ-S may not represent a true value since the UvrA protein may interact differently with ATP analogs compared to ATP generating differing values of DNA unwinding.

One of the difficulties encountered in this analysis was the increase in production of nicked circular DNA. This may imply that DNA conformations created by UV irradiation may inhibit the reclosure step in the topoisomerase I-catalyzed reaction since UvrA protein or photolyase did not seem to significantly nick the DNA by themselves. If this is the case, then there may be a bias toward a lower ΔL as the fraction of DNA with a higher content of UV photoproduct is preferentially nicked versus a fraction containing a lesser number of photoproducts.

Effect of UvrB Protein on Unwinding of DNA: The presence of the UvrB protein vastly alters the retentive properties of UvrA-nucleoprotein complexes on nitrocellulose filters, causing a decrease in the nonspecific binding and increasing stability of the complex bound to a damaged site (12). Similarly UvrB protein can activate the UvrA-associated ATPase activity 5-10 fold in a DNA dependent manner (18,22). The UvrB protein itself does not bind to DNA nor does it have any demonstrable ATPase activity. To determine the effect of UvrB protein on the unwinding of unirradiated DNA by UvrA protein, UvrA protein levels were kept constant and increasing amounts of UvrB protein added (Figure 6). In the presence of ATP-γ-S, there is a doubling of ΔL. The UvrB protein in the presence of ATP-γ-S has no DNA unwinding activity itself and demonstrable changes in ΔL induced by UvrA-UvrB protein absolutely requires the presence of a nucleotide analog. The change in ΔL is nonlinear, suggesting site-saturation for UvrB binding which seems to plateau at a UvrA:UvrB ratio between 1:2 and 1:3. A titration curve using a constant 1:2 ratio of UvrA to UvrB protein leads to a somewhat nonlinear increase in ΔL with an unwinding angle of 180-220^0 per UvrA dimer, nearly twice that seen for UvrA alone (Figure 7). The increase in ΔL may be due to an increase in the fraction of UvrA protein bound in the presence of UvrB protein or due to an alteration in the mode of interaction with DNA for UvrA protein already bound to DNA. The latter implies further alteration in DNA conformation at the site bound by UvrA protein through interaction with UvrB protein.

If UvrB protein is affecting binding in a qualitative sense, then it may be detected by analyzing the unwinding of UV irradiated DNA in the presence of ATP. If the pyrimidine cyclobutane dimer sites can be saturated with UvrA protein, then the addition of UvrB should cause further unwinding if it affects the mode of UvrA binding but should not affect unwinding if the presence of UvrB affects the fraction of UvrA bound to the dimer site, assuming the UvrAB complex does not bind or recognize non-damaged sites. Analyses of the unwinding of unirradiated DNA by the UvrA and UvrB proteins in the presence of ATP reveal very little if any unwinding the extent of which is similar to that seen for UvrA unwinding in the absence of cofactor (Figure 7). To measure the unwinding effects of damage, fd RFI was irradiated to give on the average 6 dimers per fd. UvrA protein was added at 5.7 x 10^{-8} M to saturate all dimers sites. The addition of increasing amounts of UvrB protein induced a gradual increase in the unwinding angle suggesting UvrB affects the mode of binding of UvrA protein to DNA (Figure 7). Further support for this explanation comes from recent analyses of the size of DNA protected by UvrA protein from nuclease digestion. These data show an alteration in the length of DNA protected by the UvrA protein when in the presence of UvrB protein (18).

DISCUSSION

We have sought to analyze the mode of binding of the UvrA protein to DNA by examining the unwinding of covalently closed circular DNA. Demonstrable unwinding is measurable under
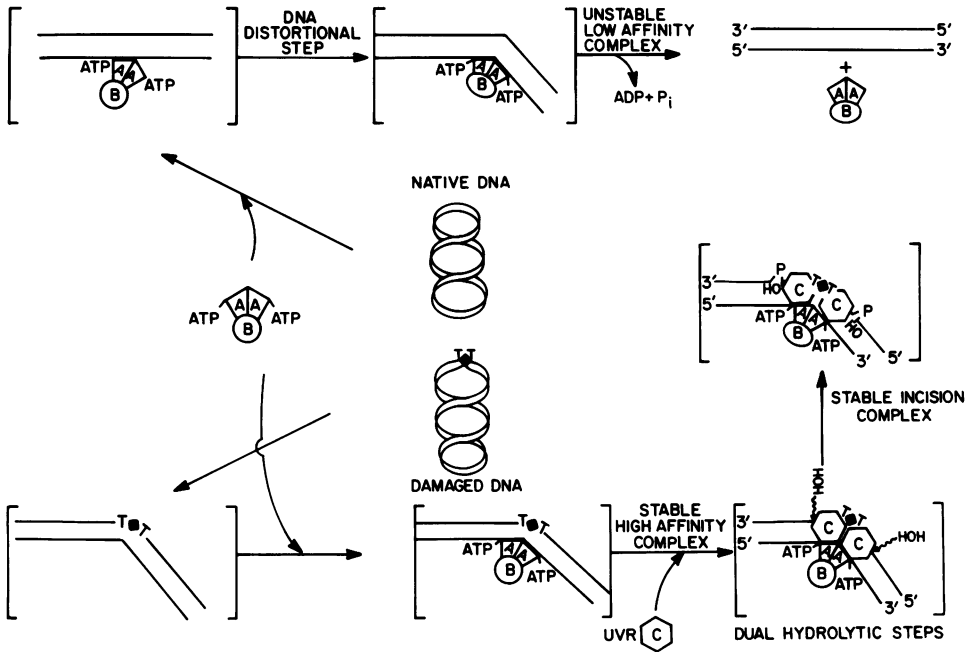
Figure 8: A Model for the Role of UvrA and UvrB Proteins in Recognition of DNA Damaged by Bulky Adducts: in the interaction of the UvrA and UvrB proteins with undamaged DNA the active unit of UvrA protein exists as a dimer. The active unit of UvrB protein is either a monomer or dimer. The interaction of the UvrA protein is assumed to be asymmetric since only one of the subunits interacts readily with DNA. The interaction of the second subunit of UvrA with DNA requires the UvrB protein for the bending-unwinding of the DNA. The interaction is unstable and readily dissociates due to the DNA structure. The interaction of other DNA binding sites with DNA induced by UvrB protein serves to explain the increase in the UvrA-associated ATPase activity influenced by its interaction with the UvrB protein. It is not known whether the increase in ATPase activity is due to the increased length of time spent in contact with DNA or whether the interaction at specific sites with DNA causes an increase in the ATPase activity.

In the case of damaged DNA, the structure of the DNA at damaged sites, indicated by the bent nature of the DNA, allows interaction of the DNA with both subunits of the UvrA dimer complex. Some degree of allostery mediated by UvrB protein between the UvrA subunits causes an induced fit of the DNA so as to complement the geometry of the damaged DNA sites. The complex associated with the DNA contains a bound nucleotide(s), is stable and recognized by the UvrC protein . The presence of all three proteins and the nucleotide cofactor allows DNA to be locked into a specific conformation which is conducive for the incision events at precise distances from the damage.

conditions of low salt. However, under those salt conditions optimized for catalysis, where little unwinding is observed, the substitution of ATP with poorly hydrolyzable analogs leads to clearly demonstrable DNA unwinding. It is not clear whether the residual DNA unwinding observable in low salt and in the absence of added nucleotide represents a different mode of

DNA interaction than the unwinding observed in the presence of the required cofactor. There is also detectable unwinding of UV damaged DNA in the presence of ATP.

The interaction of the UvrB protein with the UvrA nucleoprotein complex results in a doubling of DNA unwinding. This observation is consistent with several additional effects of the UvrB protein on UvrA protein-associated activities such as activation of the ATPase activity of UvrA, increased stability of the UvrAB nucleoprotein complex at damaged sites, a decrease in UvrA non-specific binding, and an alteration in the length of DNA protected from nuclease digestion.

The significant unwinding of supercoiled DNA by UvrA protein is apparently associated with nucleotide binding and not with its hydrolysis since the ATP analogs are the most effective in inducing stable unwinding of undamaged DNA. The requirement for the binding of nucleotide, independent of hydrolysis, is also associated with the allosteric effects of ATP on the conformational changes of the UvrA protein. A component of this allostery leads to the dimerization of the UvrA protein (18). Non-hydrolyzable ATP analogs have similar effects on the recA protein (22) and also on DNA gyrase (23).

If it is assumed that the binding of the nucleotide analog accurately reflects the binding of ATP, then the initial steps in the mechanism of recognition of damage sites involves conformational changes of the structure of UvrA protein which imposes the observed structural effects on DNA binding sites. This ATP-induced binding of the UvrA protein to DNA leads to a deformation of DNA at the bound site as reflected in DNA unwinding (Fig.8). Other types of deformation such as bending and kinking may occur (24), however, this assay does not specifically detect such conformations. Enhanced topological changes then occur upon the association of the UvrB protein to the UvrA-nucleoprotein complex. A mechanism can be postulated in which deformation of DNA reflects the role of the Uvr proteins in imposing a conformationally appropriate site to act as a substrate site for catalysis. The UvrAB protein complex on binding serves to further deform the DNA to provide a uniformly consistent structure for the double incision events driven by the binding energy of ATP.

In the absence of DNA damage the degree of rigidity in the DNA conformation may not fit optimally into the UvrAB-induced conformation required for productive binding and, hence, would lead to an unstable complex which rapidly dissociates upon ATP hydrolysis. However, the peculiar and varied nature of a DNA containing 'bulky adducts', many of which have been suggested to kink and denature DNA, may more readily interact with the UvrAB protein complex and not require substantial structural perturbations at the substrate site. The geometry of a damaged site may be such that the binding sites on both subunits of the UvrA protein can interact readily with DNA whereas in nondamaged DNA only a limited subset of DNA binding sites are available with any significant affinity. The former complex, in which both subunits of UvrA protein through allosteric effects induced by UvrB protein are stably complexed to DNA, should provide the proper architecture with the UvrC protein binding leading to the cleavage reactions. The artificial conditions under which the UvrC protein can

associate with the UvrAB-nucleoprotein complex bound to nondamaged sites is currently under investigation. In any case, damaged DNA must possess some common structural element recognizable by the UvrABC proteins which allows formation of a cofactor-protein-DNA complex competent to undergo incision events. It is interesting to note that a prominent feature of the UvrABC endonuclease is the asymmetry of the incision sites relative to site of damage. Futhermore, the 5' cleavage is precise irrespective of the nature of damage while there is some ambiguity in the 3' cleavage sites. This suggests a possible asymmetry in the nature of the damage site and in the mode of interaction of UvrABC proteins with the damaged site. A 'looseness' or 'wobble' in the 3' interaction may explain the slight variation in 3' cleavage sites and in part helps to explain the ability to accommodate to a variety of seemingly unrelated DNA damage products. It may also serve to explain an observation made in this laboratory that the incision events in dimer-containing DNA are sequential in which the 5' site is cleaved prior to the 3' site (25).

If the role of ATP in the UvrABC endonucleolytic reactions is to modify the structure of both the enzyme complex and its DNA substrate for optimal dual incision events, it serves to explain a peculiarity of the incision event itself. The nucleotide excision system controlled by the *E. coli uvr* system acts in a virtually non-specific manner in that it is induced to act on DNA's damaged by a wide variety of structurally unrelated damage, yet in each case the asymmetric dual incision sites are essentially invariant whether the damage is a pyrimidine dimer (3,4), an acetylaminofluorine-guanine adduct (5), a cis-platinum guanine intra-strand dimer (6) or a psoralen-thymine monoadduct (5). That these damaging agents, all producing different structural perturbations are acted upon in virtually the same manner strongly suggests that it is the UvrABC protein complex which is imposing a structure on the DNA molecule to satisfy its own substrate requirements. The role of damaged sites, in all likelihood, serve to reduce the Km for UvrAB binding, the extent of which may be inversely related with the structural characteristics of distortion imposed by the damaging agents on DNA.

Recent experiments show that binding of nucleotide and not its hydrolysis is required to initiate the endonucleolytic event (25). Such a bound nucleotide can act as an effector to 'lock' the protein DNA complex in a productive conformation. It is of further interest to note that the distances of the potentially excisable fragment are essentially a single helical turn in all cases. This is the approximate nucleotide length unwound by the UvrAB nucleoprotein complex.

One observation with intriguing possibilities in allowing an analysis of the recognition mechanism is the activation of UvrABC endonuclease by *E. coli* photolyase (26). Unlike the UvrD protein which allows turnover of the UvrC protein and therefore affects the extent but not the rate of incision (28,29), photolyase accelerates both the rate and extent of incision. It has not been determined whether photolyase affects turnover of the UvrABC complex or influences recognition of damaged sites by the UvrABC endonuclease. Both possibilities should be easily testable. In accelerating the rate of incision, the binding of photolyase to a

pyrimidine cyclobutane dimer may alter the structure of DNA near the damaged site such that it is more readily recognized by the UvrABC protein complex. It has been suggested that pyrimidine cyclobutane dimers are poor substrates when compared to other damaged products such as psoralen, acetylaminofluorine, and cis-platinum. The photolyase may alter the photodimer site, lowering the Km for UvrABC binding to one resembling DNA modified by these other agents.

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