THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 4, pp. 2184–2194, January 27, 2006

Base Flipping in Nucleotide Excision Repair*

Received for publication, August 12, 2005, and in revised form, October 28, 2005 Published, JBC Papers in Press, November 9, 2005, DOI 10.1074/jbc.M508901200

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UvrB, the ultimate damage-binding protein in bacterial nucleotide excision repair is capable of binding a vast array of structurally unrelated lesions. A β-hairpin structure in the protein plays an important role in damage-specific binding. In this paper we have monitored DNA conformational alterations in the UvrB-DNA complex, using the fluorescent adenine analogue 2-aminopurine. We show that binding of UvrB to a DNA fragment with cholesterol damage moves the base adjacent to the lesion at the 3′ side into an extrahelical position. This extrahelical base is not accessible for acrylamide quenching, suggesting that it inserts into a pocket of the UvrB protein. Also the base opposite this flipped base is extruded from the DNA helix. The degree of solvent exposure of both residues varies with the type of cofactor (ADP/ATP) bound by UvrB. Fluorescence of the base adjacent to the damage is higher when UvrB is in the ADP-bound configuration, but concomitantly this UvrB-DNA complex is less stable. In the ATP-bound form the UvrB-DNA complex is very stable and in this configuration the base in the non-damaged strand is more exposed. Hairpin residue Tyr-95 is specifically involved in base flipping in the non-damaged strand. We present evidence that this conformational change in the non-damaged strand is important for 3′ incision by UvrC.

DNA is constantly being threatened by various damaging agents, which can be either exogenous (chemicals or irradiation) or endogenous (reactive metabolites). If left unrepaired the damage could lead to mutations or cell death and therefore several repair mechanisms have evolved to avoid these effects. One of these mechanisms, nucleotide excision repair (NER)[2] is characterized by the unique feature that it is able to recognize and repair a large variety of structural different damages (1). In bacteria, NER is initiated by three different proteins: UvrA, UvrB, and UvrC (reviewed in Refs. 2 and 3). First UvrA and UvrB form a complex in solution, which is able to search the DNA for possible damage (4). Once damage has been found, UvrA leaves the complex resulting in a UvrB-DNA preincision complex. Atomic force microscopy (5) and bandshift analysis (23) of this complex have shown that it contains two UvrB molecules, with one monomer bound to the damaged site and the second monomer more loosely associated. The role of this second UvrB subunit in the repair reaction, however, still needs to be determined. UvrC subsequently binds to the preincision complex, thereby releasing the loosely associated UvrB subunit (5, 23). Next UvrC will make the incisions in the DNA. First an incision is made at the 4th or 5th phosphodiester bond 3′ to the damage (6, 7), which is then followed by an incision at the 8th phosphodiester bond 5′ to the damage (8). The 5′ incision is often followed by an extra incision seven nucleotides 5′ to the original 5′ incision, which has been shown to be the result of recognition of the 5′ nick by UvrB (9). The resulting oligonucleotide is removed by UvrD after which PolI fills in the gap and ligase closes the nick in the DNA. In some bacterial species like *Escherichia coli* a second NER-specific nuclease is present (Cho) that can induce a 3′ incision at the 9th phosphodiester bond 3′ to the damage and is believed to serve as a back-up system for UvrC (10).

During the NER reaction ATP binding and hydrolysis by UvrA and UvrB play multiple roles. Each of the two UvrA monomers contains two ATPase sites. The precise role of these ATPase sites in damage detection is still unknown, but it has been shown that ATP hydrolysis by UvrA is essential for dissociation from non-damaged sites (11), thereby playing an important role in the initial screening for potential damage. The UvrB protein contains one ATPase site and the ATP hydrolysis in this site becomes activated by UvrA and (damaged) DNA (12). AFM studies revealed that ATP binding by UvrB promotes wrapping of the DNA around one of the UvrB monomers, both in $UvrA_2B_2$ complexes in search of a damage and in $UvrB_2$ complexes bound to a damaged site (5, 13). The ATPase activity of UvrB has been shown to be associated with strand destabilization activity (14–16), which is most likely used to distort the DNA at a lesion to facilitate damage-specific binding. After the ATPase mediated loading of UvrB on the damaged site a new ATP molecule needs to be bound to form a productive preincision complex that can subsequently be processed by UvrC (17).

The crystal structures of UvrB from *Baccillus caldotenax* (18) and *Thermus thermophilus* (19, 20) reveal a prominent feature of UvrB: a flexible β-hairpin containing a number of highly conserved aromatic residues at its base and tip. Removing the tip of the hairpin produces a protein that is unable to form a stable preincision complex (21). A "padlock model" for DNA binding by UvrB was proposed, where the β-hairpin inserts itself between the two strands of the DNA, clamping one of the strands between the β-hairpin and domain 1b of the protein (18).

In addition to many different types of base alterations, UvrB has also been shown to efficiently recognize a one-nucleotide gap in the DNA (22). From this observation a model for damage recognition was proposed where UvrB probes the DNA for the presence of damage by trying to flip bases out of the DNA helix. Lesion-induced alterations in base stacking would facilitate this base flipping and a one-nucleotide gap would mimic such a flipped configuration.

Substitution of residues Tyr-92 and Tyr-93 at the base of the β-hairpin with alanine leads to UvrB proteins that are lethal to the cell because of stable binding to non-damaged sites (22, 23). This could also be explained by the base flipping model for damage recognition. In the absence of damage, flipping is prevented because the non-damaged base is held in place by stacking interactions with its neighbors. This subsequently results in a sterical clashing with Tyr-92 and Tyr-93 and dissociation of UvrB from the DNA. When damage is present the damaged

* This work was supported in part by CERC3 Grant 700.52.706 from the Netherlands Organization for Scientific Research (NWO). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[2] The abbreviations used are: NER, nucleotide excision repair; 2-AP, 2-aminopurine; CK, creatine kinase; CP, creatine phosphate; ATPγS, adenosine 5′-*O*-(thiotriphosphate); wt, wild type; AU, arbitrary units.

base can be flipped out of the DNA helix and residues Tyr-92 and Tyr-93 occupy the vacated space (22).

Base flipping is not uncommon in DNA repair, because co-crystal structures of uracil-DNA glycosylase (24), alkyladenine glycosylase (25), 8-oxoguanine-DNA glycosylase (26), and photolyase (27) have shown that the damaged base exists in an extrahelical position. Flipping, however, is not solely confined to the damaged nucleotide, because in the co-crystal structure of T4 endonuclease V it was shown that it is not the cyclobutane pyrimidine dimer lesion itself, but a adenine opposing the cyclobutane pyrimidine dimer that becomes extrahelical (28).

In this study we have probed changes in the helical structure induced by the UvrB protein using the fluorescent adenine analogue 2-aminopurine (2-AP). 2-Aminopurine can be incorporated in the DNA using automated synthesis and gives only minimal distortion to the DNA if paired with a thymine with which it can form a Watson-Crick-type base pair (29). Excitation of 2-AP with light at a wavelength around 310 nm results in an emission of about 370 nm. In a double-stranded DNA fragment this fluorescence is significantly quenched by base stacking interactions and therefore 2-AP can be used as a spectroscopic probe for base flipping (30). Examples of studies where 2-AP has been used to demonstrate base flipping can be found for DNA photolyase (31), methyltransferases (30, 32), T4 endonuclease V (33), and T7 RNA polymerase (34).

We show that binding of UvrB to a DNA fragment with a cholesterol lesion forces the base adjacent to the lesion at the 3′ side in an extrahelical position. Also the nucleotide opposite this flipped base is extruded from the DNA helix in the UvrB-DNA complex. UvrB mutants with substitutions in the β-hairpin revealed that residues Tyr-92 and Tyr-93 are not needed for the observed base flipping. Residue Tyr-95 appears to be specifically involved in flipping the base in the non-damaged strand and this base flipping seems to be required for the 3′ incision by UvrC.

MATERIALS AND METHODS

Proteins and DNA Fragments—The plasmid expressing the UvrB mutant (Y92A/Y93A) has been described (22). The plasmids expressing UvrB(K45A), UvrB(Y95A), and UvrB(Y96A) were constructed in a similar way using site-directed mutagenesis. All plasmid constructs were verified by DNA sequencing. Mutant UvrB630 lacks the 43 C-terminal amino acids (37). The UvrA (35), (mutant) UvrB (22), UvrC (35), and Cho (10) proteins were purified as described. The 50-bp DNA substrates used in this study were obtained commercially (Eurogentec) and are shown in Fig. 1. Creatine kinase (CK) was obtained from Roche Diagnostics.

Chemicals—Creatine Phosphate (CP), ADP (containing <1% ATP), ATP (containing <0.5% ADP), and ATPγS (containing <10% ADP) were obtained from Roche.

Incision Assay—The DNA fragments were labeled at the 5′ side of the top strand using polynucleotide kinase as described (35). The DNA substrates (0.2 nM) were incubated with 2.5 nM UvrA, 100 nM UvrB, and 25 nM UvrC or Cho in 20 μl of Uvr-endo buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM KCl, 0.1 μg/μl bovine serum albumin, and 1 mM ATP). After incubation at 37 °C for 30 min the reactions were terminated by using 3 μl of EDTA/SDS (0.33 M EDTA, 3.3% SDS) and 2.4 μl of glycogen (4 μg/μl) followed by ethanol precipitation. The incision products were visualized on a 15% denaturing polyacrylamide gel.

Gel Retardation Assay—The 5′ terminally labeled DNA substrates (0.2 nM) were incubated with 1.25 nM UvrA and 100 nM UvrB in 10 μl of Uvr-endo buffer for 10 min at 37 °C. The mixtures were analyzed on a cooled 3.5% native polyacrylamide gel containing 1 mM ATP and 10 mM $MgCl_2$ in 1× Tris borate/EDTA as described (35). To determine UvrB binding under conditions used for the 2-aminopurine measurement the same amount of 5′ terminally labeled DNA substrate (tracer) was mixed with 0.5 μM unlabeled DNA substrate. The proteins (0.45 μM UvrA, 3.75 μM UvrB, and 0.17 μg/μl CK) were incubated with the DNA in 10 μl of Uvr-endo buffer (without bovine serum albumin) for 10 min at 37 °C in the presence of 10 or 20 mM CP, after which they were loaded on a 3.5% native gel as described above. To follow UvrB complex formation in time, the same incubation conditions were used and the reaction was terminated at different time points by putting the incubations on ice.

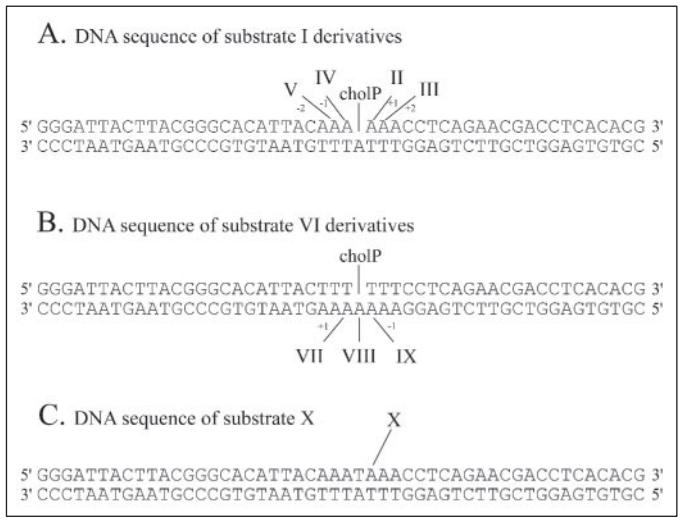

FIGURE 1. **DNA substrates used in this study**. *A*, nucleotide sequence of substrate I. The cholesterol lesion (*cholP*) in the center of the *top strand* and the positions of the 2-AP substitutions in substrates II–V are indicated. *B*, nucleotide sequence of substrate VI. The positions of the 2-AP substitutions in substrates VII–IX are indicated. *C*, nucleotide sequence of substrate X without a cholesterol lesion and 2-AP at position 28 in the *top strand*.

2-Aminopurine Fluorescence Measurements—60-μl samples containing 0.5 μM DNA, 0.45 μM UvrA, 3.75 μM UvrB, and 0.17 μg/μl CK were incubated in Uvr-endo buffer (without bovine serum albumin) for 10 min at 37 °C in the presence of 10 or 20 mM CP as indicated. Where indicated 300 mM acrylamide was added to the sample after the incubation. The sample was transferred to a 3 × 3-mm quartz cuvette and placed in the cuvette holder of the fluorimeter. Fluorescence emission spectra were obtained using a PerkinElmer LS 50B fluorimeter, which was connected to a temperature variable water bath to maintain a temperature inside the cuvette of 37 °C. The excitation wavelength was set at 310 nm, and spectra were obtained by scanning from 325 to 475-nm emission wavelengths. The excitation and emission slit widths were 5 and 10 nm, respectively. Spectra were corrected for background fluorescence by subtracting the spectrum obtained from the buffer. The free 2-aminopurine signal was obtained by measuring 0.5 μM 2-aminopurine riboside-3′,5′-cyclic monophosphate (BioLog) in 60 μl of Uvr-endo buffer at 37 °C. For the time-dependent fluorescence measurements, proteins and DNA were mixed and immediately put in the cuvette after which emission at 370 nm was recorded over a 1-h period. For testing the influence of the cofactor on fluorescence, 3 mM ADP, ATP, or ATPγS were added to the mixture (which initially contained 1 mM ATP) at different time points as indicated.

RESULTS

2-AP Does Not Influence UvrB Binding—For the fluorescence experiments, 2-AP was incorporated at specific positions in the top and bottom strands of a 50-bp substrate containing a cholesterol lesion. Because the 2-AP base pairs with dT, two different DNA sequences

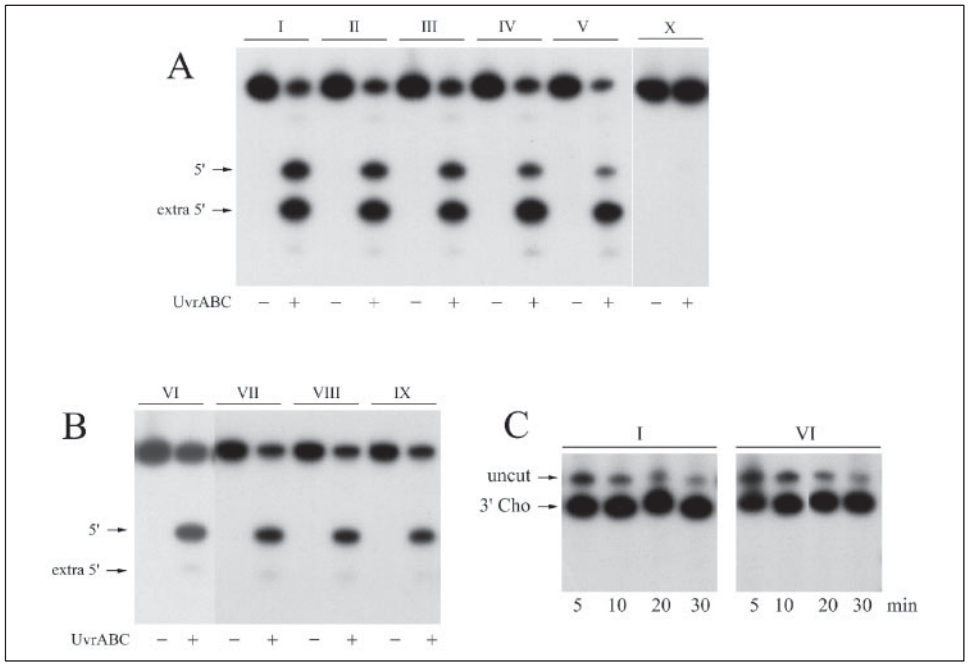

FIGURE 2. **UvrABC and UvrABCho incision of substrates I–X.** The 5′ terminally labeled DNA fragments were incubated with 2.5 nM UvrA, 100 nM UvrB, and 25 nM UvrC (*A*, *B*) or 25 nM Cho (*C*) at 37 °C for 30 min (*A* and *B*) or for the indicated times (*C*). The incision products were analyzed on 15% denaturing polyacrylamide gels. The substrates used are indicated *above* the gels. The incision products are indicated with *arrows*. The proteins added are indicated *below* each lane (*A* and *B*). The different time points after which the reaction was terminated are indicated *below* the gels (*C*).

were used (I and VI, see Fig. 1). Four DNAs were prepared (substrates II-V) in which the individual four adenines flanking the cholesterol lesion in substrate I were substituted with 2-AP (Fig. 1*A*). The three other DNA substrates (VII–IX) have three individual adenines opposite the lesion in substrate VI replaced with 2-AP (Fig. 1*B*). As a control we also made a DNA fragment (substrate X) with a 2-AP without the cholesterol (Fig. 1*C*).

First we tested whether the presence of the 2-AP base influences UvrABC incision of the DNA fragments. The 2-AP base itself is not recognized as damaged by the Uvr proteins, because substrate X with only the 2-AP does not show significant incision (Fig. 2*A*). The incisions of substrate I (without 2-AP) and the same DNA fragments with the 2-AP substitutions (II–V) are very similar (Fig. 2*A*). Likewise the incisions of substrate VI (without 2-AP) and the derivatives containing 2-AP (VII–IX) are also comparable (Fig. 2*B*). The overall incisions of the substrate VI derivatives, however, are significantly lower (50%) than those of the substrate I derivatives (>90%). Because of this lower incision the extra 5′ incision is hardly observed with the substrate VI derivatives. It has been shown before that the sequence context of the lesion can influence the efficiency by which UvrC incises the DNA (36). To confirm also that in this case the difference in incision is caused by UvrC and not by a difference in UvrB binding, we repeated the incisions of substrates I and VI using the Cho nuclease instead of UvrC (Fig. 2*C*). Indeed now both DNA sequences are very efficiently incised, indicating also that UvrB binding is very efficient.

To test UvrB binding more directly we also performed a bandshift analysis on the different substrates with or without 2-AP. Fig. 3, *A* and *B*, shows that the amount of UvrB-DNA complex is similar for all substrates, indicating that 2-AP does not significantly alter formation or stability of these complexes. As expected no UvrB-DNA complexes are formed on substrate X with only the 2-AP and no damage (Fig. 3*A*).

Setting Up the Conditions for 2-AP Fluorescence Measurement—To obtain a significant fluorescence signal of 2-AP, a DNA concentration of 0.5–1 μM is needed (30–34). This is significantly higher than the DNA concentrations used in the incision and DNA binding assays described above. Therefore we first needed to determine the optimal protein concentration for formation of the UvrB-DNA complex at these high DNA concentrations. The concentrations of UvrA (from 0.045 to 0.45 μM) and UvrB (0.95 to 7.5 μM) were varied, but for both substrates I and VI the amount of UvrB-DNA complexes obtained with 0.5 μM DNA remained significantly lower compared with the complexes formed with 0.2 nM DNA (not shown).

Searching for and binding to DNA damage involves ATP hydrolysis by UvrA and UvrB. Therefore at high protein and DNA concentrations the amount of ATP might become limiting and the ADP formed might "poison" the proteins. To prevent this we included an ATP-regenerating system in our incubation mixture consisting of CK and CP. With this ATP-regenerating system incubation of 0.5 μM DNA with 0.45 μM UvrA and 3.75 μM UvrB leads to a substantial amount of UvrB-DNA complexes (Fig. 3*C*). Note that these complexes migrate at the position of the UvrB dimer. It has been shown before (23) that the second UvrB subunit, which is more loosely associated with the complex, dissociates during electrophoresis at low UvrB concentration (resulting in monomer complexes in Fig. 3, *A* and *B*) but is stabilized at high UvrB concentration (resulting in dimer complexes in Fig. 3*C*). All 2-AP containing substrates gave similar UvrB-DNA complexes with these same incubation conditions (not shown).

Base Flipping at the 3′ Side of the Damage—The fluorescence spectrum of free 2-AP shows a very strong peak at 370 nm (Fig. 4*A*). When incorporated in double-stranded DNA without a lesion (substrate X) the fluorescence is almost completely quenched (Fig. 4*B*). Quenching is less when the 2-AP containing DNA is in the single-stranded form (Fig. 4*B*), but fluorescence is still much lower than the free 2-AP.

For each of the 2-AP containing damaged substrates four different emission spectra were made: (*a*) the double-stranded DNA without proteins; (*b*) the DNA with UvrA alone; (*c*) the DNA with UvrA + UvrB; and (*d*) the corresponding 2-AP containing single-stranded DNA. All spectra were recorded at least twice and typically deviated from their mean peak value by no more than 5%. Measurements of the proteins in the absence of DNA did not result in any significant fluorescence signal (results not shown).

First we will consider the effect of the lesion on the fluorescence of the individual 2-APs in the double-stranded DNA of substrate I derivatives (2-AP in the damaged strand). For substrates III and V, fluorescence is

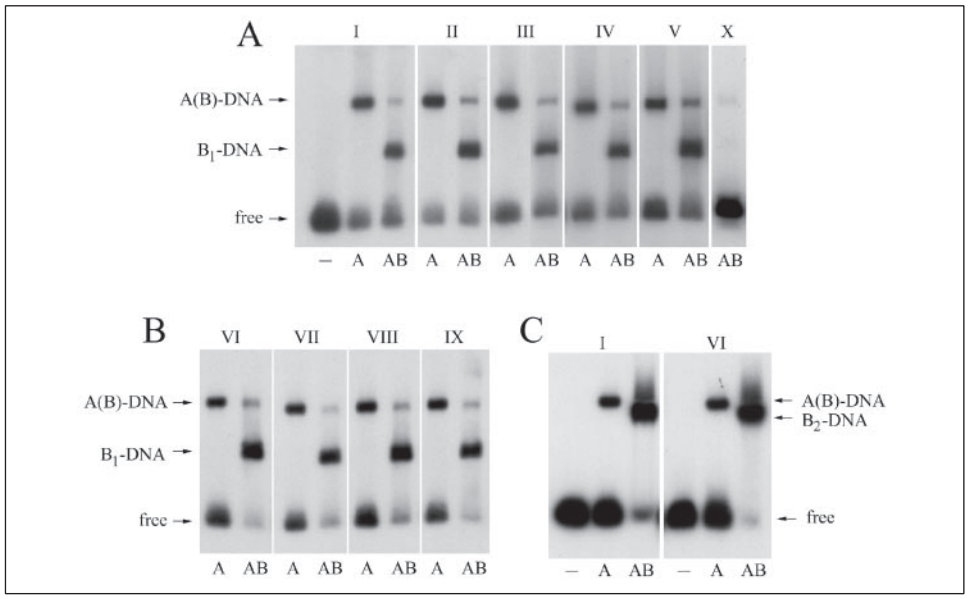

FIGURE 3. **Protein-DNA complexes formed by UvrAB on substrates I–X**. *A* and *B*, the 5′ terminally labeled DNA substrates were incubated with 1.25 nM UvrA and 100 nM UvrB for 10 min at 37 °C. *C*, The 5′ terminally labeled DNA substrates (tracer) were mixed with 0.5 μM unlabeled DNA and incubated with 0.45 μM UvrA and 3.75 μM UvrB for 10 min at 37 °C in the presence of the CP/CK system. The mixtures were analyzed on 3.5% native polyacrylamide gels containing 1 mM ATP. The proteins used are indicated *below* each lane. The substrates used are indicated *above* the gels. The positions of the different protein-DNA complexes are indicated with *arrows*. The UvrA-DNA and UvrAB-DNA complexes migrate at the same position in the gel. The position of the $UvrB_1$-DNA complex corresponds to that of a monomer of UvrB bound to the DNA. The $UvrB_2$-DNA complex contains a dimer of UvrB bound to the DNA.

comparably low as for substrate X without damage (Fig. 4, compare *D*, *F* with *B*). Apparently the cholesterol lesion does not significantly alter the conformation of bases at positions −2 and +2 with respect to the damage. For the 2-APs at position −1 (substrate IV) and +1 (substrate II) the damage does influence the conformation. In particular, the 2-AP immediately 5′ to the damage shows a significant fluorescence (Fig. 4*E*), indicating a perturbation in base stacking. The effect on the 2-AP 3′ to the damage is also detectable, but less pronounced (Fig. 4*C*).

Addition of UvrA alone to DNAs II–V does not result in significant change in fluorescence of any of these substrates (Fig. 4, *C-F*). In particular, for positions +1 and +2 it is clear that 2-AP fluorescence is higher in the free single-stranded DNA than in the UvrA-bound double-stranded fragment (Fig. 4, *C* and *D*). This might indicate that damage binding by UvrA does not cause significant base pair disruption at the 3′ side of the damage. On the other hand the amount of damage-specific UvrA-DNA complexes might be too low (see Fig. 3) to be able to detect any UvrA-induced conformational changes.

When UvrA + UvrB are included in the incubation, allowing formation of the UvrB-DNA complex, a considerable change in 2-AP fluorescence at the 3′ side of the damage is observed. For the 2-AP at position +1 (substrate II) UvrB binding results in a fluorescence level that is a factor of three higher than the level of the corresponding single-stranded DNA (Fig. 4*C*). The signal of this 2-AP in the UvrB-DNA complex is about 50% of the signal of a comparable concentration of the free 2-AP base (Fig. 4*A*). This very high fluorescence can only be explained by UvrB-induced base flipping, thereby removing the stacking interactions between 2-AP and its neighbors. The 2-AP at position +2 (substrate III) also shows a significant increase in fluorescence upon binding of UvrB (Fig. 4*D*). In this case the signal is a factor of two higher than the signal of the corresponding single-stranded DNA again indicating that quenching is reduced by destacking of the 2-AP base. This can be caused by a direct effect of the UvrB protein on the base at the +2 position. Alternatively the enhanced fluorescence of substrate III might merely reflect the base flipping at position +1, because base flipping at +1 is expected to reduce quenching at position +2.

At the 5′ side of the damage, no significant change in fluorescence upon UvrB binding is observed. Fluorescence of substrate V remains very low, showing that in the UvrB-DNA complex the 2-AP at position −2 is fully stacked (Fig. 4*F*). Also the fluorescence of 2-AP at position −1 does not change upon UvrB binding (substrate IV, Fig. 4*E*). Because fluorescence of this residue was already higher in the free DNA, as a result of destacking by the damage, this might indicate that the position of the damage does not change in the UvrB-DNA complex.

Base Flipping in the Non-damaged Strand—Fluorescence of the three individual 2-APs opposite the damage is significantly increased compared with the non-damaged substrate, indicating a perturbation of all three residues by the damage itself (Fig. 4, *G–I*). The effect is most significant in substrates VIII and IX, because the fluorescence signals of these double-stranded fragments are higher than the corresponding single-stranded DNAs (Fig. 4, *H* and *I*). Apparently the bases directly opposite the lesion and at −1 are partly pushed out of the helix by the cholesterol moiety.

Binding of UvrA again does not give a significant change in fluorescence of the different 2-APs. Upon binding of UvrB the signal in substrate IX does increase considerably (Fig. 4*I*). It reaches a level that is a factor of three higher than that of the corresponding single-stranded DNA and about 35% of the free 2-AP base. Apparently concomitant with the base 3′ to the lesion, the opposite partner is also pushed in an extrahelical position by UvrB. The level of fluorescence of this 2-AP in the non-damaged strand is less than that of the 2-AP adjacent to the damage. Still the amount of UvrB-DNA complexes formed on both substrates (II and IX) is the same (Fig. 3, *A* and *B*). This means that the fluorescence of the 2-AP in the bottom strand is still partially quenched, either because it is only partially extruded from the helix or by stacking of residues of the UvrB protein.

Fluorescence of 2-AP directly opposite the lesion (substrate VIII) or at position +1 (substrate VII), which are already enhanced in the free DNA through influence of the damage, do not change significantly upon UvrB binding (Fig. *4*, *G* and *H*). Again this could indicate that UvrB binding does not alter the position of the cholesterol moiety in the DNA helix. It can, however, not be excluded that in the UvrB-DNA complexes the bases opposite the lesion and at +1 are no longer destacked by the lesion itself, but that now the UvrB protein displaces these 2-AP residues leading to a similar level of fluorescence.

The Base in the Damaged Strand Is Flipped into a Pocket of the UvrB Protein—A closer examination of the fluorescence signal of substrate II in complex with UvrB reveals that the λ_{max} value (366.5 nm) is shifted

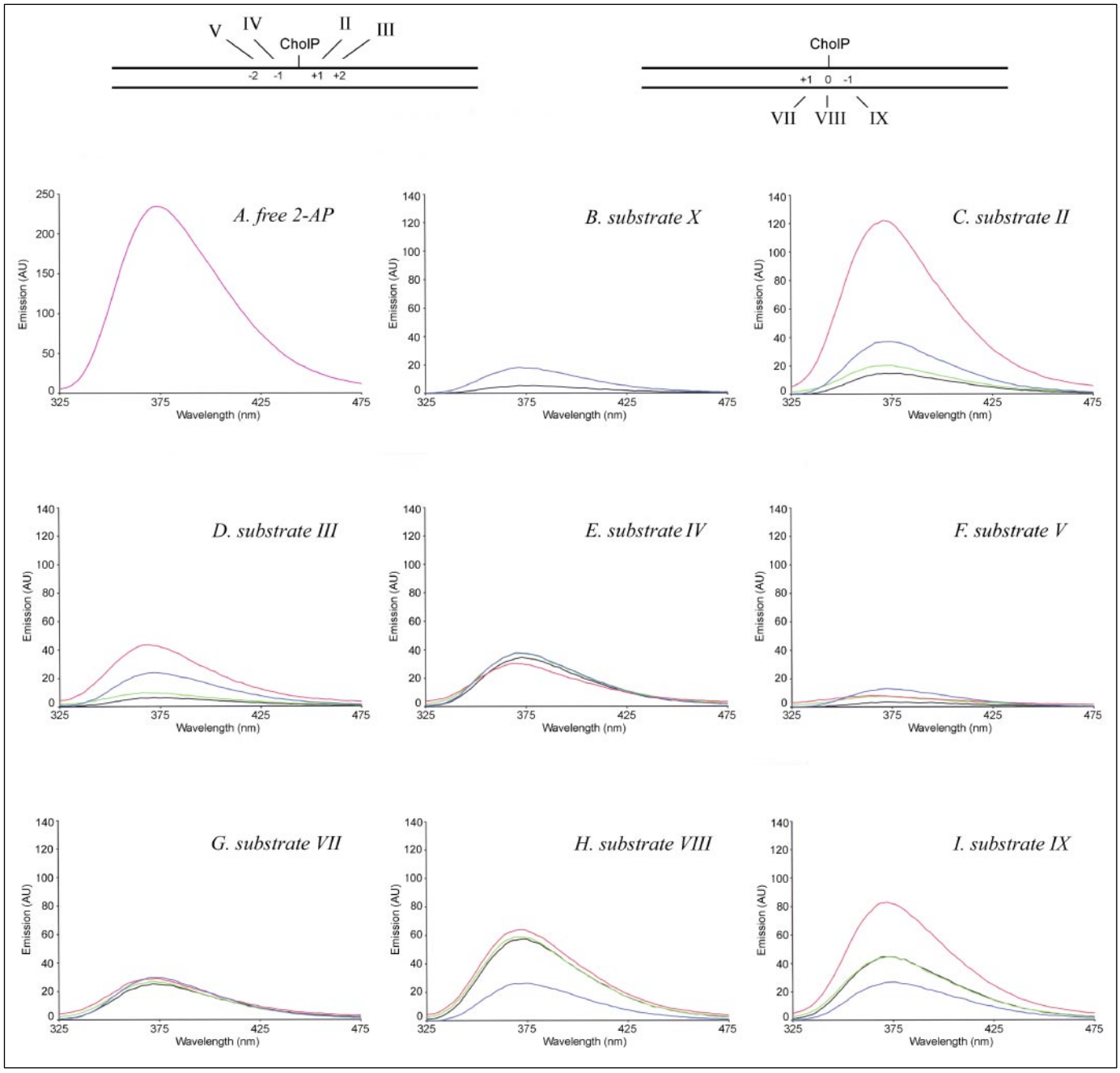

FIGURE 4. **Fluorescence emission spectra.** *A*, fluorescence emission spectrum of free 2-AP (0.5 μM). *B*, emission spectrum of substrate X (without cholP). *C–I*, fluorescence emission spectra of the different 2-AP containing DNA fragments (substrates II–V with 2-AP in the damaged strand and substrates VII–IX with 2-AP in the non-damaged strand) in the presence or absence of UvrAB. The DNA (0.5 μM) was incubated for 10 min at 37 °C with or without 0.45 μM UvrA, 3.75 μM UvrB, and the CP/CK system using 10 mM CP. After incubation the sample was transferred to a cuvette and emission spectra were recorded at 37 °C (excitation at 310 nm). For each DNA substrate separate measurements were done for the 2-AP containing single-stranded DNA without proteins (*blue*), the double-stranded DNA without proteins (*black*), the double-stranded DNA with UvrA (*green*), and the double-stranded DNA with UvrA and UvrB (*red*). The positions of the 2-AP residues are schematically shown *above* the spectra.

compared with the λ_{max} value of the free 2-AP base (370 nm). Such a blue shift indicates that the extrahelical 2-AP residue in the substrate is positioned in an environment with a lower dielectric constant (41). To further assess the environment of the extrahelical 2-AP, quenching studies were performed using acrylamide. Addition of 300 mM acrylamide to the free 2-AP base resulted in a reduction of the fluorescence signal from ~240 to ~17 arbitrary units (AU). Addition of the same amount of acrylamide to the UvrB-DNA complex formed on substrate II did not give any significant reduction of fluorescence, showing that the extrahelical 2-AP base is not accessible to the molecule. This strongly suggests that the base adjacent to the damage is flipped into a pocket of the UvrB protein, which is too tight to allow access of acrylamide.

We also performed quenching studies with the UvrB-DNA complex formed on substrate IX. Addition of 300 mM acrylamide resulted in a 50% reduction of the fluorescence signal from ~90 to ~45 AU. With a gel retardation assay we confirmed that the acrylamide did not in any way affect the stability of the UvrB-DNA complex (data not shown). Therefore the decreased fluorescence is because of acrylamide quenching of the signal. This indicates that the base in the non-damaged strand is positioned extrahelically and that it is not completely shielded by residues of the UvrB protein.

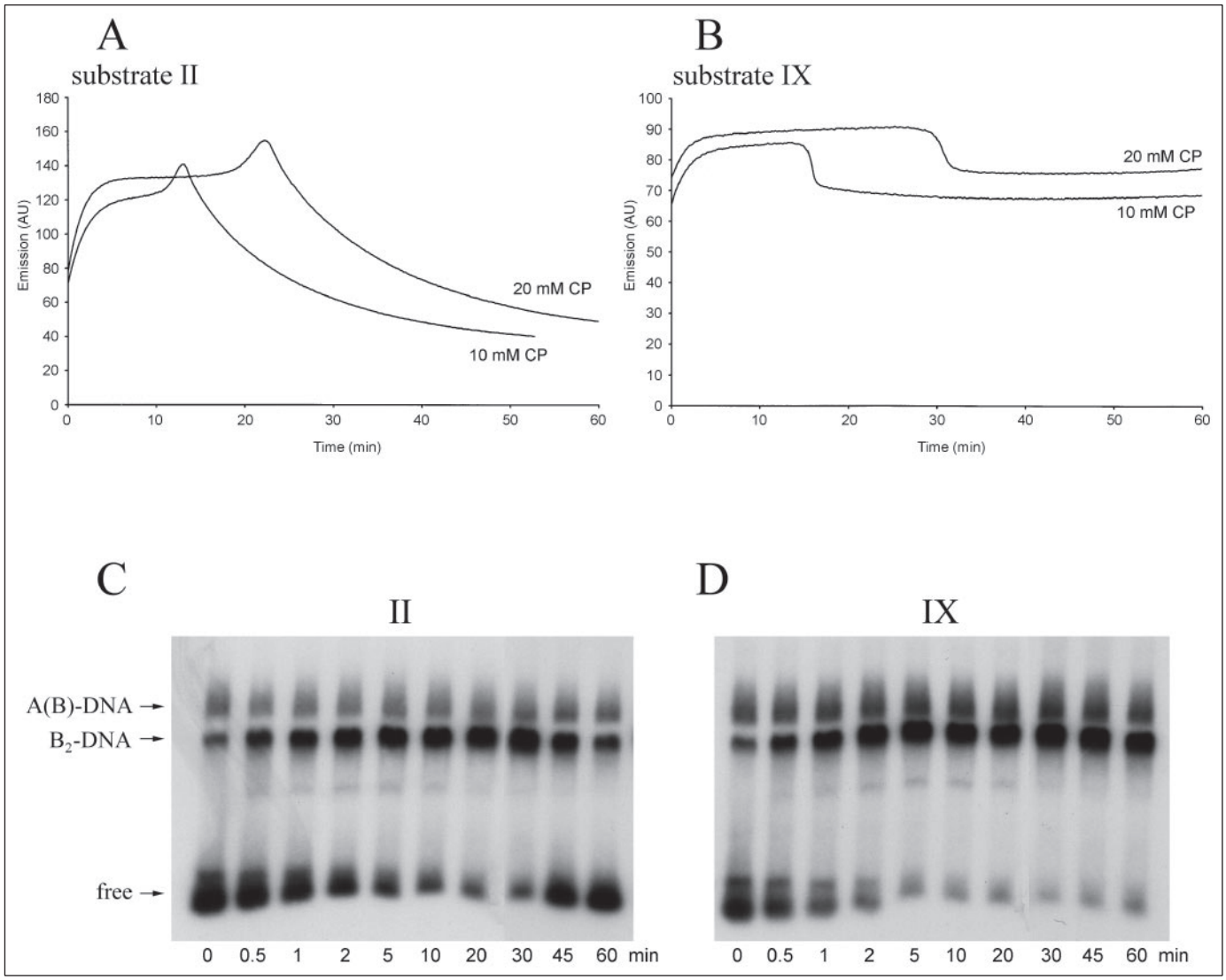

FIGURE 5. **Fluorescence of substrates II and IX measured in time.** DNA (0.5 μM) of substrate II (*A*) or substrate IX (*B*) was mixed with 0.45 μM UvrA and 3.75 μM UvrB in the presence of the CP/CK system using 10 or 20 mM CP and immediately put in the cuvette after which emission at 370 nm was recorded over a 1-h period at 37 °C. UvrB complex formation of substrates II (*C*) and IX (*D*) was monitored in time in a gel retardation assay by incubating the 5′ terminally labeled DNA substrates (tracer) and 0.5 μM unlabeled DNA with 0.45 μM UvrA and 3.75 μM UvrB in the presence of the CP/CK system, using 20 mM CP. At different time points the reaction was stopped by putting the samples on ice. The protein-DNA complexes were analyzed on a 3.5% native gel containing 1 mM ATP. The UvrB-DNA complex migrates at the position corresponding to the dimeric $UvrB_2$-DNA complex.

Base Flipping Is Dependent on Cofactor Binding by UvrB—The enhanced fluorescence signals in the UvrB-DNA complexes formed on substrates II and IX allow us to monitor this complex in time. Therefore the emission at 370 nm was recorded during a 1-h period, starting immediately after mixing protein and DNA as described under "Materials and Methods." First these experiments were done with substrate II in the presence of the ATP-regenerating system, using 10 mM CP, as described above (Fig. 5*A*). After mixing the components a sharp rise in fluorescence was observed during the first minutes until it reaches a plateau level after about 5 min. Next after ~10 min a second rise in fluorescence was observed that was then followed by a gradual decrease. To test whether this change of fluorescence in time might be related to a change in the ADP/ATP ratio, we repeated the experiment in the presence of a higher amount of CP (20 mM). Again initially fluorescence rises sharply, reaching a plateau after 5 min, but now this level remains constant up to 20 min, after which again it is followed by a further increase of the signal and a subsequent gradual decrease toward the level of that of free DNA. Apparently, the presence of additional CP, which allows the regeneration of ATP over a longer time, delays the second part of the fluorescence pattern. Therefore the observed peak and subsequent decrease of fluorescence must be caused by a shortage of ATP and therefore probably reflect the UvrB in the ADP-bound form. The level of fluorescence after 5 min is higher with 20 mM CP than with 10 mM CP, suggesting that an increase in the ATP/ADP ratio results in more UvrB-DNA complexes.

Also for substrate IX, a time-dependent change in fluorescence was observed, which was related to the ADP/ATP ratio in the mixture (Fig. 5*B*). For both CP concentrations, fluorescence reaches a plateau level after about 5 min that is followed by a decrease of the signal after about 15 (10 mM CP) or 30 min (20 mM CP). The decrease of the signal with substrate IX after depletion of the CP, however, was not gradual, as for substrate I, but it reaches a second plateau value that is still significantly above that of the free DNA (~45 AU, see Fig. 4*I*).

To test whether the observed fluorescence pattern is related to formation and dissociation of the UvrB-DNA complex we also monitored formation and stability of these complexes in time during a gel retardation assay (Fig. 5, *C* and *D*). Substrates II and IX were incubated with UvrA and UvrB and the ATP-regenerating system using 20 mM CP. At

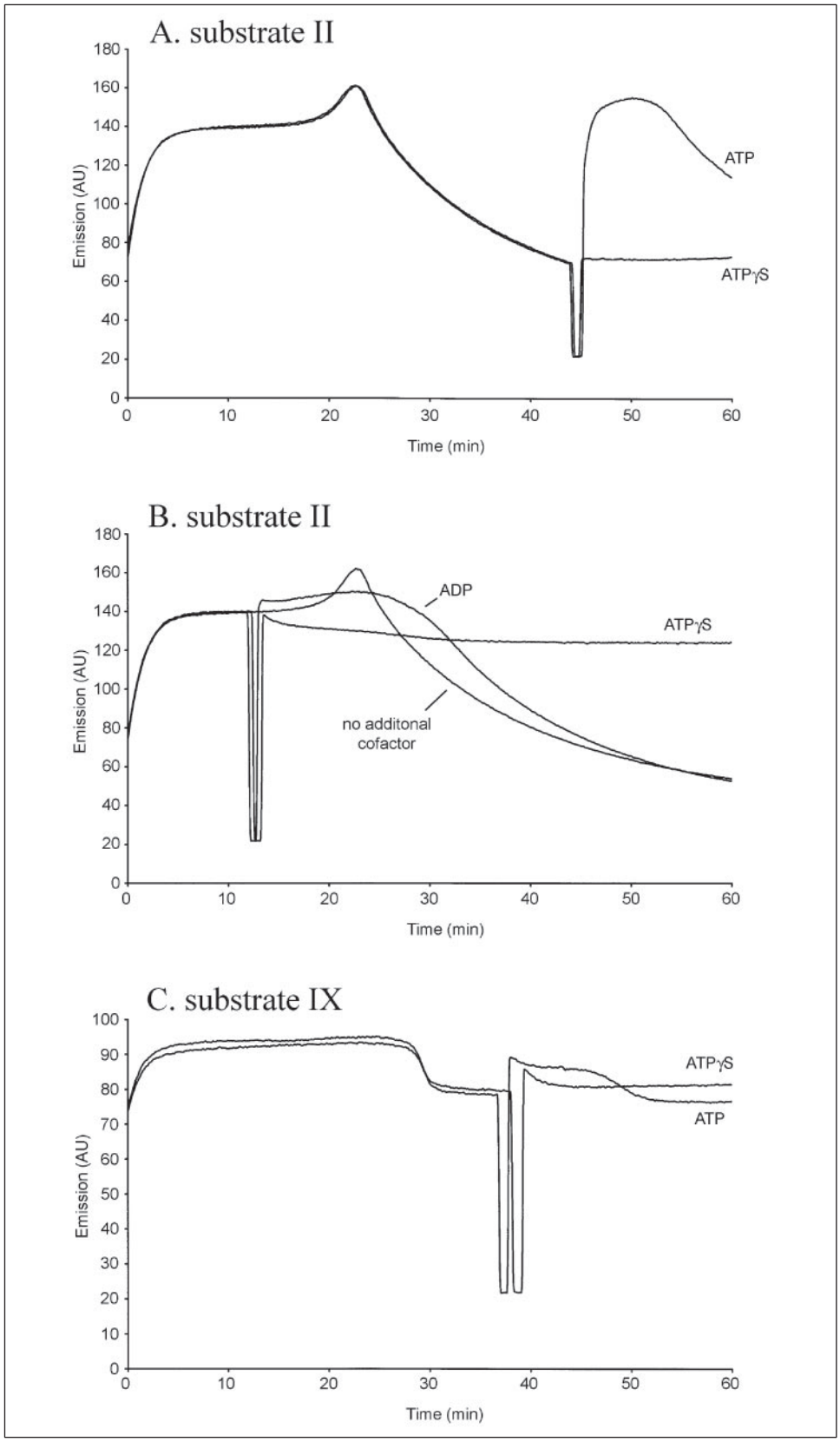

FIGURE 6. **The effect of additional cofactor on the time-dependent fluorescence of substrates II and IX.** Incubation conditions were as described in the legend to Fig. 5, using 20 mM CP. *A*, fluorescence pattern of substrate II without additional cofactor and the same substrate after addition of 3 mM ATP or ATPγS, around 45 min after the start of the incubation. *B*, addition of 3 mM ATPγS or 10 mM ADP to substrate II, around 12–13 min after the start of the incubation. *C*, fluorescence pattern of substrate IX after addition of 3 mM ATP or ATPγS, around 37–38 min after the start of the incubation.

different time points the reaction was stopped by putting the samples on ice. On both substrates already at $t = 0$, a small amount of B-DNA complexes can be observed. Probably these complexes are formed on ice or during loading of the gel. Already after 0.5 min the amount of complexes increases significantly to reach a maximum around 5–10 min on both substrates. This corresponds very well to the increase in fluorescence signals of substrates II and IX that reach their respective plateau levels around the same time. This confirms that destacking the 2-AP

residues in both DNA strands is related to formation of the UvrB-DNA complex. For substrate IX the amount of UvrB-DNA complexes remains the same up to 60 min of incubation (Fig. 5*D*). This means that the drop in fluorescence is not because of dissociation of the complex, but rather to a difference in the conformation of the 2-AP residue depending upon whether the UvrB contains ATP (first plateau level) or ADP (second plateau level). For substrate II, however, a different stability of the UvrB-DNA complex was observed (Fig. 5*C*). Like for substrate IX the complex remains stable during the first 30 min, when it is expected to be in the ATP-bound form, but it dissociates gradually later in time, most likely because it is now in the ADP-bound form. Dissociation of the UvrB complexes as monitored in the gel retardation assay seems to be at a later time point than in the fluorescence experiment. This can be explained by the assay method. Because both gel and gel buffer contain ATP, UvrB that has dissociated from the DNA can bind again as soon as the mixture is loaded on the gel. Therefore reduction of the UvrB-DNA complexes in the gel can only be detected when most of the UvrB is in the ADP-bound form. The difference in stability of the ADP-containing complexes on substrates II and IX must be explained by the sequence context of the damage, *i.e.* a stretch of purines in top *versus* bottom strand, which might give a different contact of UvrB with the DNA surrounding the lesion.

To further test the influence of the cofactor on the 2-AP conformation in the two DNA substrates, we repeated the time-dependent fluorescence measurements with the same amount of ATP (1 mM) and 20 mM CP and added extra ATP, ATPγS, or ADP (3 mM) at different time points after the start of the incubation (Fig. 6). After addition of ATPγS to substrate II, 45 min after onset of the reaction the signal remained constant (Fig. 6*A*). Apparently the exchange of ADP with the non-hydrolyzable ATPγS prevents further dissociation of the remaining complexes. The addition of ATP at the same time point results in a sharp increase of the signal, which was indicative of reloading UvrB on the DNA. Because after 45 min the concentration of CP is expected to be very low, ATP will be quickly consumed and therefore the fluorescence goes back to the level of the ADP-bound UvrB. The subsequent reduction of fluorescence can again be ascribed to dissociation of the complex. When ATPγS was added after 10 min, neither the peak nor the subsequent decrease in fluorescence were observed (Fig. 6*B*), confirming that it is indeed the ADP that is responsible for these alterations of the fluorescence signal. The level of fluorescence remains constant up to 1 h after addition of ATPγS, indicating that in the ATP-bound form the UvrB-DNA complex is very stable. Moreover this experiment shows that, once the UvrB-DNA complex has been formed, ATP hydrolysis is no longer required for the observed base flipping.

To try to demonstrate more directly that the peak in the fluorescence pattern of substrate II is indeed because of the ADP-bound form of UvrB, we also added ADP to the mixture after 10 min (Fig. 6*B*). Because at this early time point there is still enough CP left in the mixture that will allow conversion of the ADP to ATP, we added a large excess of ADP (10 mM). As a result the sample becomes more diluted (10%), which will affect the height of the signal. Indeed fluorescence goes up after addition of ADP and this rise is significant considering the 10% dilution factor. Because there is still ATP left in the mixture and part of the ADP will also be converted to ATP, the signal that is obtained is probably an average of the ADP- and ATP-bound complexes. When after 30 min all the ATP is consumed again the complexes dissociate and fluorescence drops again (Fig. 6*B*).

Similar experiments were done with substrate IX (Fig. 6*C*). ATP or ATPγS were added 40 min after onset of the reaction, when all the ATP is expected to be hydrolyzed to ADP. Each of the cofactors results in an increase of the fluorescence signal. The increase with ATP is somewhat higher than with ATPγS, which might be caused by the presence of a small amount of ADP in the ATPγS preparation. With ATPγS the level of fluorescence remains constant, but with ATP after about 10 min the level drops again below that of ATPγS and reaches a similar level of fluorescence as before addition of the ATP. This confirms that indeed the two plateau levels of the fluorescence pattern of substrate IX reflect the ATP- and ADP-bound forms of UvrB, respectively.

TABLE 1
Effect of UvrB mutant proteins on the fluorescence of substrates II and IX

The DNA substrates (0.5 μM) were incubated for 10 min at 37 °C with 0.45 μM UvrA and 3.75 μM (mutant) UvrB in the presence of the CP/CK system using 20 mM CP. The excitation was at 310 nm, and emission was recorded at 370 nm. Each fluorescence value is the average of at least two experiments.

Protein(s)	Substrate II	Substrate IX
UvrA	33 ± 1	52 ± 3
UvrA + wtUvrB	137 ± 4	91 ± 4
UvrA + UvrB(K45A)	41 ± 1	53 ± 1
UvrA + UvrB630	151 ± 2	85 ± 4
UvrA + UvrB(Y92A/Y93A)	162 ± 2	83 ± 6
UvrA + UvrB(Y95A)	140 ± 2	56 ± 4
UvrA + UvrB(Y96A)	44 ± 3	57 ± 2

Residue Tyr-95 Is Involved in Base Flipping in the Non-damaged Strand—We also tested the effect of different UvrB mutants on base flipping in substrates II and IX. For all UvrB mutants emission at 370 nm was measured after 10 min of incubation in the presence of CK and 20 mM CP (Table 1). First we used a mutant that was defective in its ATPase activity, UvrB(K45A). This mutant has an alanine substitution in the Walker A motif of the ATPase site, resulting in a protein still capable of interacting with UvrA but unable to hydrolyze ATP (12). UvrB(K45A) does not form UvrB-DNA complexes (Fig. 7*A*, *lanes 6* and *13*) and there is no detectable incision by UvrC or Cho (Fig. 7, *B* and *C*, *lanes 5* and *11*). On substrate IX, fluorescence was comparable with the amount found for UvrA alone (Table 1). On substrate II, the signal in the presence of UvrA and UvrB(K45A) is somewhat higher than the signal of UvrA alone, but still much lower than with UvrA and wtUvrB. This confirms that base flipping of both residues can only be observed after UvrB has been loaded onto the damaged site.

UvrB can bind to the DNA damage as a dimer, with one subunit stably associated with the damaged site and the second more loosely associated (5, 23). Mutant UvrB630 lacks the C-terminal domain involved in UvrB dimerization and as a consequence the second UvrB subunit readily dissociates from the UvrB-DNA complex (5, 23). The amount of UvrB preincision complexes formed by UvrB630 was comparable with wild type (Fig. 7*A*, *lanes 7* and *14*) and they were efficiently incised by Cho (Fig. 7, *B* and *C*, *lane 12*). Incision by UvrC, however, does not occur (Fig. 7, *B* and *C*, *lane 6*) because the CTD also constitutes an important UvrC-binding domain (37). The fluorescence in the presence of UvrA + UvrB630 appears similar to that of the wild type proteins for both DNA substrates (Table 1). For substrate II, the signal even seems somewhat higher. This means that the second UvrB subunit is not required for stabilization of the extrahelical bases.

The β-hairpin of UvrB plays an important role in damage-specific binding by UvrB (21, 22, 38). It can therefore be expected that residues in this hairpin structure are involved in the base flipping that we observe here. We have analyzed three different mutants with substitutions in the highly conserved tyrosine residues of the β-hairpin. The double mutant Y92A/Y93A has been described before (22, 23). Removal of the two aromatic residues results in a protein that can form stable complexes with undamaged DNA. On damaged DNA, however, the double mutant is still capable of finding the damage.

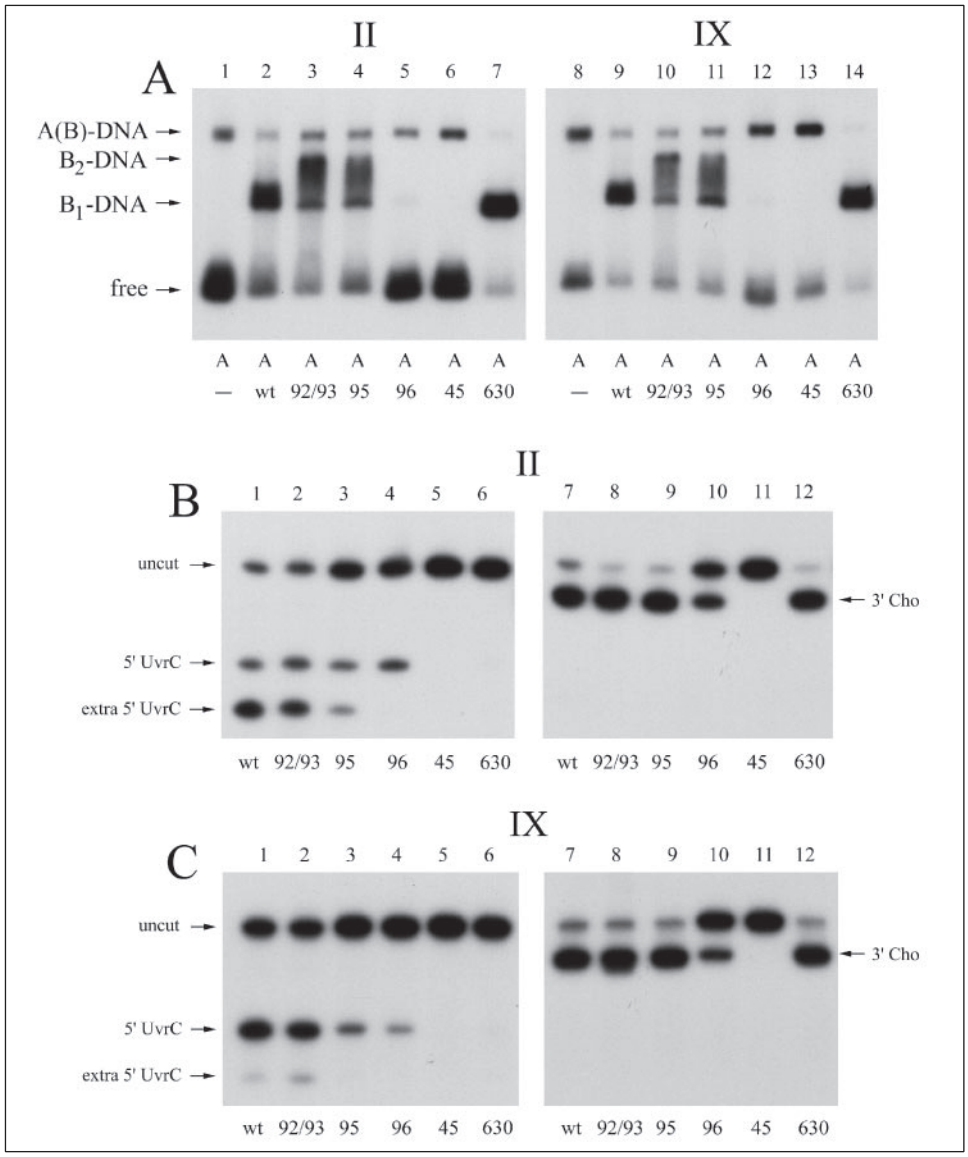

FIGURE 7. **Complex formation and incisions in the presence of the mutant UvrB proteins.** *A*, substrates II and IX were incubated with 1.25 nM UvrA and 100 nM (mutant) UvrB for 10 min at 37 °C. The mixtures were analyzed on 3.5% native polyacrylamide gels containing 1 mM ATP. *Lanes 1* and *8*, no UvrB; *lanes 2* and *9*, wtUvrB; *lanes 3* and *10*, UvrB(Y92A/Y93A); *lanes 4* and *11*, UvrB(Y95A); *lanes 5* and *12*, UvrB(Y96A); *lanes 6* and *13*, UvrB(K45A); *lanes 7* and *14*, UvrB630. The positions of the different protein-DNA complexes are indicated with *arrows*. $UvrB_1$-DNA has a UvrB monomer bound to the DNA and $UvrB_2$-DNA contains the UvrB dimer. *B* and *C*, substrates II and IX were incubated with 2.5 nM UvrA, 100 nM (mutant)UvrB, and 25 nM UvrC (*lanes 1–6*) or Cho (*lanes 7–12*) at 37 °C for 30 min. The incision products were analyzed on a 15% denaturing polyacrylamide gel. *Lanes 1* and *7*, wtUvrB; *lanes 2* and *8*, UvrB(Y92A/Y93A); *lanes 3* and *9*, UvrB(Y95A); *lanes 4* and *10*, UvrB(Y96A); *lanes 5* and *11*, UvrB(K45A); *lanes 6* and *12*, UvrB630. The incision products are indicated with *arrows*.

The incisions of substrates II and IX by UvrC or Cho in the presence of the double mutant are comparable with those in the presence of wild type UvrB (Fig. 7, *B* and *C*, *lanes 2* and *8*). As shown before (22) the second UvrB subunit of the Y92A/Y93A mutant is more firmly associated with the complex through interaction with the DNA flanking the damage and therefore the bandshift assay predominantly shows the dimeric $UvrB_2$-DNA complex (Fig. 7*A*, *lanes 3* and *10*). Overall complex formation of the double mutant is similar to that of wtUvrB. The fluorescence levels of mutant Y92A/Y93A (with a stable second UvrB subunit) are comparable with those of UvrB630 (with a very loose second subunit) on both substrates (Table 1). This confirms that the second UvrB does not influence base flipping. Moreover, the high fluorescence levels of the Y92A/Y93A protein on the two substrates show that residues Tyr-92 and Tyr-93 are not directly involved in pushing either of the bases in an extrahelical position.

We previously reported that the double mutant Y95A/Y96A was no longer capable of damage-specific binding (22). To test whether one of these β-hairpin residues is involved in base flipping we have isolated the single mutants Y95A and Y96A. Mutant Y96A appears very disturbed in UvrB-DNA complex formation (Fig. 7*A*, *lanes 5* and *12*). In accordance, incision by UvrC (Fig. 7, *B* and *C*, *lane 4*) or Cho (Fig. 7, *B*, and *C*, *lane 10*) is also much reduced. Fluorescence levels of both substrates II and IX do not exceed those obtained by the UvrB(K45A) mutant (Table 1), indicating that solution complexes formed by Y96A are too unstable to be able to detect any change in fluorescence signal. These results are in good agreement with the data obtained by Skorvaga *et al.* (16) who showed that also the Y96A mutant of *B. caldotenax* UvrB is not capable of forming a stable UvrB-DNA complex.

Protein Y95A very efficiently forms UvrB-DNA complexes (Fig. 7*A*, *lanes 4* and *11*). Like for the Y92A/Y93A double mutant these complexes are predominantly in the $UvrB_2$-DNA form, indicating that the Y95A mutation also stabilizes the second B-subunit. Cho incision of the Y95A complexes is very efficient, like for the wild type complexes (Fig. 7, *B* and *C*, *lane 9*). In contrast UvrC incision is significantly reduced (Fig. 7,*B* and *C*, *lane 3*). It is in particular the 3′ incision that was reduced, because no uncoupled 3′ incision was observed. Moreover on a substrate pre-nicked at the 3′ site, the 5′ incision with Y95A is like wild type (not shown). The reduced 3′ incision cannot be ascribed to the more stable UvrB dimer, because the Y92A/Y93A double mutant in which the second UvrB is also stabilized gives normal incision by UvrC. It is more likely that the Y95A protein fails to induce a specific conformation in the

DNA that is needed for UvrC incision. What this conformation might be becomes clear from the fluorescence data (Table 1). On substrate II, the level of fluorescence is comparable with that induced by wtUvrB, indicating that base flipping adjacent to the damage still occurs when residue Tyr-95 is missing. On substrate IX, however, fluorescence with Y95A does not exceed the level of Y96A or K45A. This strongly suggests that Tyr-95 is directly involved in "pushing" the base in the non-damaged strand. In its turn the extrusion of this base might be important for targeting UvrC, explaining the reduced UvrC incision with mutant Y95A.

DISCUSSION

Our working hypothesis, based upon previous studies with UvrB (22, 23), is that the UvrB protein probes the DNA for the presence of damage by trying to flip potentially damaged bases out of the helix. When base stacking is reduced as a consequence of the presence of damage, flipping will be facilitated, leading to formation of a stable UvrB-DNA complex. The studies presented here do not allow direct detection of the conformation of the damage itself upon UvrB binding, but we do show that the base adjacent to the damage at the 3′ side is flipped into an extrahelical conformation by UvrB.

What could be the reason for flipping of this non-damaged base? One possible explanation might be that the nature of the lesion used in these studies determines that the adjacent base is pushed out of the helix. The cholesterol damage used is directly coupled to the DNA backbone via a carbon-linker (9), and consequently there is no ribose or base present at this position. By lack of a nucleotide, UvrB might "take" the next available base that is not properly stacked, which is the base adjacent to the damage. On the other hand, the fluorescence data in the presence of acrylamide strongly suggest that the extrahelical base adjacent to the damage inserts into a pocket of the UvrB protein that is too small to allow access of the acrylamide. It is not very likely that such a tight pocket would permit the entrance of a base that may carry a large variety of bulky adducts. Therefore flipping of the adjacent base might be a general mechanism for damage recognition, also on other types of lesion. For a lesion-flanking base it can also be expected that the stacking interactions are reduced, thereby facilitating the flipping process and contributing to damage recognition.

That indeed in the DNA substrates used in this study the conformation of the cholesterol lesion might not be altered upon DNA binding is supported by the fluorescence data of the 2-AP residues directly 5′ to the lesion (substrate IV) and in the non-damaged strand opposite the lesion (substrate VIII) and at position +1 (substrate VII). In all three cases the lesion itself causes destacking of these 2-AP residues in the unbound DNA. Upon UvrB binding, the fluorescence signals of these residues do not change, which could mean that the cholesterol moiety is still in the same position, *i.e.* not flipped in an extrahelical position

The opposing base in the non-damaged strand (−1) is also significantly destacked upon UvrB binding. The lower signal indicates that for this residue base flipping is less pronounced, suggesting that stacking with its neighbors is only partly removed. It has been shown that a 2-AP residue opposite an abasic site is significantly less stacked than in a corresponding normal duplex (39). The destacking of the base in the non-damaged strand that we observe here could therefore be the direct result of flipping the opposing base adjacent to the damage, because the result of this flipping mimics an abasic site. With the UvrB(Y95A) mutant, however, we could show that destacking of the two opposing bases are two independent events. UvrB(Y95A) shows base flipping of the residue adjacent to the damage like wild type, whereas destacking of the base in the non-damaged strand no longer occurs. Residue Tyr-95 therefore seems to be directly involved in destacking of this base in the non-damaged strand, either by pushing it or by preventing it to "fall back" to its original position. Ma and Zou (40) have shown using acrylamide-mediated quenching of the introduced tryptophan in a Y95W mutant that upon binding of UvrB to DNA residue Tyr-95 becomes significantly less exposed. These results also point to direct involvement of this residue 95 in DNA interaction. It can be expected that a tryptophan residue can stabilize an extruded base in a similar way as a tyrosine residue.

The preincision complexes formed by mutant Y95A can subsequently be incised by Cho with the same efficiency as the wild type complexes. The 3′ incision by UvrC, however, is strongly reduced. It is not likely that residue Tyr-95 is directly involved in binding to UvrC because changing this residue into tryptophan did not affect the UvrC-mediated incision (38). Possibly it is the extruded base in the non-damaged strand itself, or other conformational changes in the UvrB-DNA complex induced by the extrusion that form an important recognition target for the UvrC protein. The cofactor studies have shown that the base in the non-damaged strand is more exposed when UvrB is bound to ATP or ATPγS than when it is in the ADP-bound form. In accordance with this we have shown in a previous study that the 3′ incision by UvrC is much higher when the UvrB preincision complex contains ATP or ATPγS than with ADP (17).

The fluorescence results with mutant Y92A/Y93A show that these two residues are not required for flipping of the base adjacent to the damage. Another candidate for being involved in stabilizing the flipped configuration of this damage flanking base is Tyr-96. Indeed no significant increase in fluorescence was measured with this mutant, but this was related to the inability to form a stable preincision complex. One should bear in mind, however, that it is very likely that a mutation that prevents base flipping also prevents stable UvrB binding. Therefore Tyr-96 still remains a good candidate for stabilizing the flipped base.

Monitoring fluorescence in time showed that within 5 min of mixing the substrate with the proteins a maximal level of UvrB binding was obtained. After about 30 min (depending on the capacity of the ATP-regenerating system) most of the ATP in the system appears to be consumed. It is not clear from our experiments whether this is because of the damage-bound UvrB that continues hydrolyzing ATP or to the remaining free proteins that keep searching for damage. Eventually all the damage-bound UvrB ends up in the ADP-bound form, either by hydrolyzing the ATP itself or by exchanging it for excess ADP in solution. The level of fluorescence of the two opposing residues varies with ADP or ATP. Fluorescence of the base adjacent to the damage becomes even higher when UvrB is in the ADP-bound form. Possibly this ADP-induced conformation reflects the extent of the base flipping that occurs during loading of UvrB. When UvrB detects damage, it hydrolyzes its ATP (17). After this hydrolysis UvrB is in the ADP-bound form, in which possibly the base is extrahelical but not yet fully inserted into the protein pocket. In the ADP-bound conformation the complex is not yet stable and depending on the sequence context this can lead to dissociation of the protein from the DNA. In a normal situation, however, the ADP is quickly exchanged with ATP that might cause complete insertion of the base into the protein pocket, resulting in a more stable UvrB-DNA complex

It has been proposed by Theis *et al.* (18) that UvrB binds to the DNA as a "padlock," clamping one of the DNA strands between the β-hairpin and domain 1b of the protein. If we view our results in light of this padlock model it would mean that initially upon UvrB binding to a damaged site, when its ATP has been hydrolyzed and UvrB is in the ADP-bound form, this padlock is still open, allowing the DNA to slip

from behind the β-hairpin, and dissociation of the complex. The subsequent exchange of ADP with ATP might close the padlock resulting in a very stable complex, in which the base in the non-damaged strand is now sufficiently extruded to allow incision by UvrC.

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