Structure and function of the UVDE repair protein

Paspaleva, K.

Damage recognition by UV damage endonuclease from *Schizosaccharomyces pombe*

Chapter 4

Keti Paspaleva, Geri F. Moolenaar and Nora Goosen

Laboratory of Molecular Genetics, Leiden Institute of Chemistry, Gorlaeus Laboratories, Leiden University, Einsteinweg 55, 2300 RA Leiden, The Netherlands

SUMMARY

UV damage endonuclease (UVDE) from *Schizosaccharomyces pombe* initiates repair of UV lesions and abasic sites by nicking the DNA 5' to the damaged site. In this paper we show that in addition UVDE incises DNA containing a single-strand nick or gap, but that the enzymatic activity on these substrates as well as on abasic sites strongly depends on the presence of a neighbouring pyrimidine residue. This indicates that, although UVDE may have been derived from an ancestral AP endonuclease its major substrate is a UV lesion and not an AP site. We propose that UVDE rotates two nucleotides into a pocket of the protein in order to bring the scissile bond close to the active site and that purine bases are excluded from this pocket. We also show that in the DNA complex residue Tyr358 of UVDE penetrates the DNA helix causing unstacking of two residues opposite the lesion, thereby stabilizing the protein-DNA interaction, most likely by promoting bending of the DNA. In the absence of Tyr358 the enzyme exhibits an increased catalytic activity on UV-induced lesions, but only at a lower pH of 6.5. At physiological conditions (pH 7.5) the mutant protein completely looses its catalytic activity although it can still bind to the DNA. We propose that in addition to stabilizing the bend in the DNA the hydrophobic side chain of Tyr358 shields the active site from exposure to the solvent.

INTRODUCTION

UV damage endonuclease (UVDE) is a repair protein, which has been reported to remove a variety of structurally different DNA lesions, including UV photoproducts (CPD and (6-4)) PP, apurinic/apyrimidinic (AP) sites and nucleotide mismatches (Kanno *et al*., 1999, Avery *et al*., 1999; Kaur *et al*., 1999). The enzyme nicks the DNA 5' to the damaged nucleotide with the efficiency of the incision highly dependent on the type of lesion. Originally this nuclease was discovered in the fission yeast *Schizosaccharomyces pombe* (Bowman *et al*., 1994) and in *Neurospora crassa* (Yajima *et al*., 1995), but later a number of homologues were identified in other fungi, and several eubacterial and archaebacterial species (Takao *et al*., 1996, Earl *et al*., 2002 and Goosen and Moolenaar, 2008).

Recently the structure of UVDE from *T. thermophilus* has been solved (Paspaleva *et al*., 2007), revealing a high structural similarity with the DNA repair enzyme Endonuclease IV (Endo IV), which participates in *Escherichia coli* Base Excision Repair (BER) by nicking the DNA at the 5' side of an AP site (Hosfield *et al*., 1999). Both proteins have a TIM-barrel fold and contain three metal ions in their active sites. For Endo IV, however, these metals were shown to be Zn^{2+} ions, whereas UVDE uses Mn^{2+} ions for catalysis (Paspaleva, manuscript in preparation). Since Endo IV has been crystallised with a substrate containing an AP site, significant light was shed on the mechanism by which the enzyme specifically recognises AP sites. The DNA was seen to be bent by approximately 90°, and both the AP site and the opposing nucleotide were flipped out of the DNA duplex. The flipped-out abasic nucleotide is anchored into a pocket of the enzyme where the 5' phosphate is donated to the three Zn^{2+} ions. A normal nucleotide would sterically be excluded from this active site pocket, explaining the AP site selectivity. The opposite base is flipped away from the enzyme and completely solvent exposed. This flipped-out nucleotide is mainly stabilized by the enzyme-induced compression of the DNA backbone and not by direct interaction with the flipped-out base.

The structure of UVDE shows a deep groove that can accommodate kinked DNA similar to Endo IV. The three metal ions at the bottom of this groove are also at similar positions as in Endo IV (Paspaleva *et al*., 2007), implying that also in UVDE the damaged site must be flipped into a protein pocket in order to bring the scissile bond close to the metal ions. In case of the UVDE enzyme however, this pocket should be able to accommodate CPD's, (6-4)PP's as well as an abasic site.

In this paper we take a closer look at damage recognition by *S. pombe* UVDE by comparing the binding and incision efficiencies on different DNA substrates. Since the full-length protein is unstable (Takao *et al*., 1996) we used a 228 residue N-terminal truncation of UVDE, which has already been described to be highly active (Takao *et al*., 1996). We show that besides abasic sites, UVDE also recognises single-strand nicks and gaps, but for all three types of lesion the efficiency is highly dependent on the presence of adjacent pyrimidines. In addition by using a

fluorescent adenine analogue 2-aminopurine (2-AP) we give evidence that binding of UVDE to its DNA substrate causes significant destacking of the bases opposite the lesion.

MATERIALS AND METHODS

Proteins

The UVDE gene fragment encoding residues 229 - 599 was amplified by PCR and cloned into the *Bam*HI and *Bst*EII restriction sites of the expression vector pET16b (Novagen), generating an N-terminal fusion of the truncated gene to a 10 X His tag. The resulting plasmid (pETUVDEΔ228) was introduced into *E. coli* BL21/codon+ cells (Studier *et al*., 1990) and the Δ228-UVDE protein purified from a 2 l culture 3 h after induction by IPTG. The cells were lysed by sonication in 6 ml lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 10 mM β-mercaptoethanol, 10 % glycerol, 1 % Triton X-100) and separated into a soluble and an insoluble fraction by centrifugation at 37,000 rpm for 30 minutes. The supernatant was loaded on a HiTrap-chelating Ni column, equilibrated with 20 mM Tris pH 7.5 containing 20 mM imidazole and the protein was eluted with a gradient of 20 mM to 250 mM imidazole in the same buffer. Pooled fractions of Δ228-UVDE were loaded on a hydroxyapatite column, which was equilibrated with 10 mM KPO_4 (pH 6.5). Subsequently, the protein was eluted using a gradient from 200 mM to 400 mM KPO_4 (pH 6.5). For further purification, the UVDE fractions were applied to a P11 phosphocellulose column, equilibrated with 300 mM KPO_4 (pH 6.5) and eluted with 1 M NaCl. Finally, the UVDE containing fractions were applied to a Nap5 gel filtration column and eluted with 20 mM Tris pH 7.5, 150 mM NaCl, 10 % glycerol. The UVDE containing fractions showed more than 95 % purity.

The Y358A point mutation was constructed by PCR and verified by sequencing. The mutant UVDE protein was purified using the same purification procedure as described for the wild type enzyme and showed the same elution/purification profile.

The Endonuclease IV (Endo IV) and Endonuclease III (Endo III) enzymes were obtained commercially (New England Biolabs).

DNA substrates

The 30 bp DNA substrates used in this study are summarised in Table 1. The oligonucleotides containing CPD or (6-4)PP lesions were synthesised as described (Iwai, 2006). The 30 bp substrates containing an abasic site (via incorporation of a tetrahydrofuran-dSpacer), thymine glycol (TG) or 2-AP were obtained commercially (Eurogentec, Belgium). The DNA substrates were 5' radioactively labelled using polynucleotide kinase as described (Verhoeven *et al*., 2002). For 3' labelling the damaged top strands were annealed to a corresponding bottom strand with one additional G residue at its 5' end. Subsequently the DNA fragments were incubated with Klenow polymerase and α-^{32}P-dCTP as described (Moolenaar *et al*., 2005).

Incision assay

The labelled DNA substrates were incubated with the indicated amount of UVDE in a 20 µl reaction mix (20 mM HEPES pH 6.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM $MnCl_2$). After 15 minutes incubation at 30°C the reaction was terminated by adding 3 µl of 0.33 M EDTA, 3.3 % SDS and 2.4 µl glycogen (4 µg/µl), followed by an ethanol precipitation. For measuring the incision percentages the samples were run on a small (8 cm) 15 % acrylamide gel. For determination of the incision positions longer gels were used (20 cm) allowing separation of the different incision products.

Incision with Endo IV was performed in a 20 µl reaction mix in the presence of 50 mM Tris-HCl pH 7.9, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT) for 15 minutes at 37 °C. Incision with Endo III was performed for 15 minutes at 37°C in buffer supplied by the manufacturer.

Filter binding Assay

The filter binding assays were conducted in 20 µl samples containing 5 nM of UVDE and 4 nM of the terminally labelled DNA substrates in a reaction buffer containing 20 mM Tris pH 6.5, 100 mM NaCl. The mixes were incubated for 10 minutes at 30°C. If needed 1 mM $MnCl_2$ and 10 mM $MgCl_2$ were included in the reaction buffer. After the incubation 0.5 ml of preheated (30°C) reaction buffer was added to each sample. The mixture was then poured over a nitrocellulose filter (Millipore 0.45 µm HA) and the filter was rinsed tree times with 0.5 ml of the reaction buffer. Each sample was corrected for the amount of DNA retained on a filter in the absence of the protein. Binding is expressed as the percentage of input DNA retained on the filter by the enzymes. To determine the binding properties of UVDE under the conditions used for the 2-aminopurine measurements 4 nM of the 5' terminally labelled substrates were mixed with 0.5 µM unlabelled DNA and incubated with 2.5 µM UVDE for 10 min. at 30°C in a total volume of 30 µl.

2-AP measurements

60 µl samples containing 0.5 µM DNA and 2.5 µM UVDE were incubated in 20 mM Tris buffer (pH 6.5) for 10 min at 30°C. If needed 1 mM $MnCl_2$ and 10 mM $MgCl_2$ were included in the incubation mixture. Where indicated 300 mM acrylamide was added to the samples after incubation. The samples were transferred to a 3 mm x 3 mm quartz cuvette and placed in the fluorimeter. Fluorescence emission spectra were obtained using a PerkinElmer LS 50B fluorimeter, connected to a temperature variable water bath to maintain a temperature inside the cuvette of 30°C. The excitation wavelength was set at 310 nm, and the emission spectra were obtained by scanning from 325 to 475 nm. The excitation and emission slit widths were 5 nm and 10 nm, respectively. All spectra presented are the average of at least to experiments and were corrected for the spectra of equivalent incubation mixtures without DNA. The free 2-aminopurine signal was obtained by measuring 0.5 µM 2-aminopurine riboside-3',5'-cyclic monophosphate (BioLog) in 60 µl of 20 mM Tris buffer at 30°C.

UV survival test

The *Xba*I - *Bam*HI fragment from pETUVDEΔ228 (with or without the Y358A mutation) was inserted in pIC-19R, resulting in expression of the (mutant) UVDE under control of the Plac promoter. The plasmids were introduced in CS 5018 (Δ*uvrA,* Δ*uvrB*) (Moolenaar *et al*., 2005). Cells were grown in LB supplemented with 40 μg/ml ampicillin and 1 mM IPTG until OD_{600} reached 0.3. Subsequently 1 μl drops of a 10^{-1} dilution were spotted on an LB plate containing 40 μg/ml ampicillin and 1 mM IPTG. The drops were irradiated with the indicated dose of UV and plates were incubated overnight at 30°C.

RESULTS

Incision of an abasic site is dependent of the flanking bases

The structural similarities between UVDE and Endo IV suggest that both proteins bind the damaged DNA in a similar fashion. They both share a wide groove housing the active site

Table 1. DNA substrates used in this study.

no damage	5′CTCGTCAGCATCTTCATCATACAGTCAGTG 3′
	3′GAGCAGTCGTAGAAGTAGTATGTCAGTCAC 5′
CPD and	5′CTCGTCAGCATC**TT**CATCATACAGTCAGTG 3′
(6-4)PP	3′GAGCAGTCGTAGAAGTAGTATGTCAGTCAC 5′
CXT	5′CTCGTCAGCATC**X**TCATCATACAGTCAGTG 3′
abasic site	3′GAGCAGTCGTAGAAGTAGTATGTCAGTCAC 5′
AXT	5′CTCGTCAGCATA**X**TCATCATACAGTCAGTG 3′
abasic site	3′GAGCAGTCGTATAAGTAGTATGTCAGTCAC 5′
CXA	5′CTCGTCAGCATC**X**ACATCATACAGTCAGTG 3′
abasic site	3′GAGCAGTCGTAGATGTAGTATGTCAGTCAC 5′
AXA	5′CTCGTCAGCATA**X**ACATCATACAGTCAGTG 3′
abasic site	3′GAGCAGTCGTATATGTAGTATGTCAGTCAC 5′
C*T	5′CTCGTCAGCAT**CT**TCATCATACAGTCAGTG 3′
ss-nick	3′GAGCAGTCGTAGAAGTAGTATGTCAGTCAC 5′
C*A	5′CTCGTCAGCAT**CA**TCATCATACAGTCAGTG 3′
ss-nick	3′GAGCAGTCGTAGTAGTAGTATGTCAGTCAC 5′
A*T	5′CTCGTCAGCAT**AT**TCATCATACAGTCAGTG 3′
ss-nick	3′GAGCAGTCGTATAAGTAGTATGTCAGTCAC 5′
A*A	5′CTCGTCAGCAT**AA**TCATCATACAGTCAGTG 3′
ss-nick	3′GAGCAGTCGTATTAGTAGTATGTCAGTCAC 5′
T*T	5′CGTGTGAGGTCGTTCTGAGGTT**TT**TTTGTAATGTGCCCGTAAGTAATCCC 3′
ss-nick	3′GCACACTCCAGCAAGACTCCAAAAAAACATTACACGGGCATTCATTAGGG 5′
1 nt gap	5′AGTTCTATGCGCACCGAATTCCCACT GAACCCAAGCTTGCCGGGCCTCT 3′
	3′TCAAGATACGCGTGGCTTAAGGGTGACCTTGGGTTCGAACGGCCCGGAGA 5′
Thymine glycol	5′CTCGTCAGCATC**T**TCATCATACAGTCAGTG3′
	3′GAGCAGTCGTAGAAGTAGTATGTCAGTCAC5′

The positions of the CPD (**TT**), (6-4)PP (**TT**), AP site (**X**) and thymine glycol (**T**) are indicated in bold. The positions of the single-stranded nicks in fragments C*T, A*A and T*T are between the two bold residues.

deeply at its bottom and extensive positive charges positioned at both ends of the groove. Endo IV has been shown to flip its target, the abasic site into an extrahelical position inside a pocket of the protein. UVDE however, recognizes CPD dimers and (6-4)PPs where both thymines are covalently linked. This implies that to be able to bring the scissile phosphodiester bond close to the metals of its active site it must flip the two crosslinked nucleotides into a protein pocket. This poses the question whether upon binding of an abasic (AP) site only this AP site will be flipped into the proposed protein pocket or the AP site in consortium with its neighbouring nucleotide, similar to the crosslinked pyrimidines. If this latter would be true one might expect that the efficiency of abasic site incision might be influenced by the nature of this flanking base. To test this we used four 30 bp DNA fragments which only differ in the bases flanking the AP site on the 5'or 3'side (substrates CXT, AXT, CXA and AXA, with X representing the abasic site (see Table 1).

The highest incision was obtained with substrate CXT (Figure 1, lane 7), which has the AP site flanked by two pyrimidines (> 95 %). The incision efficiency on this AP site is similar to the incision on a CPD or (6-4)PP (lanes 1 - 6). Incision is significantly lower on an abasic site in the sequences AXT (20 %) and CXA (15 %) (lanes 8, 9) and extremely low on substrate AXA (< 5 %, lane 10) which has the lesion flanked by two purines. The AP-endonuclease IV enzyme, however, incised all four DNA constructs with equal efficiencies (not shown). Apparently, the incision activity of UVDE on an abasic site indeed strongly depends on the sequence surrounding the lesion.

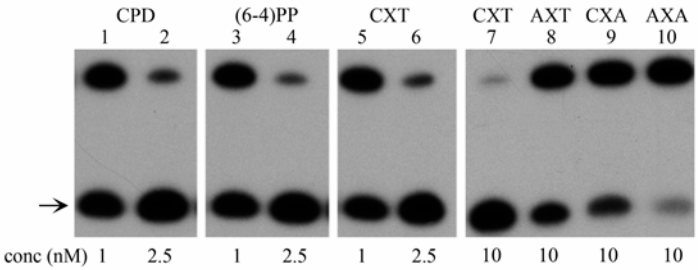

Figure 1. UVDE incision of CPD, (6-4)PP and AP sites in different sequence contexts.

The 5'-terminally labelled substrates (0.1 nM) were incubated with the indicated amount of UVDE for 15 minutes. The different substrates are shown above each lane. The arrow indicates the incision product.

We also determined the incision positions on the different substrates using constructs labelled on either the 5' (Figure 2A) or the 3' side (Figure 2B) of the damaged strand. As a control the incision product of UVDE on a CPD-containing fragment (Figure 2A, lane 2) was included in the gel, showing that on this substrate UVDE uniquely nicks the DNA immediately 5' to the CPD lesion. The minor band that migrates one position lower in the gel can be ascribed to the presence of a small amount of 29-mer in the CPD-substrate (lane 1). With substrate AXT UVDE also incises the DNA immediately 5' to the AP site (Figure 2A, lane 8), but on substrates

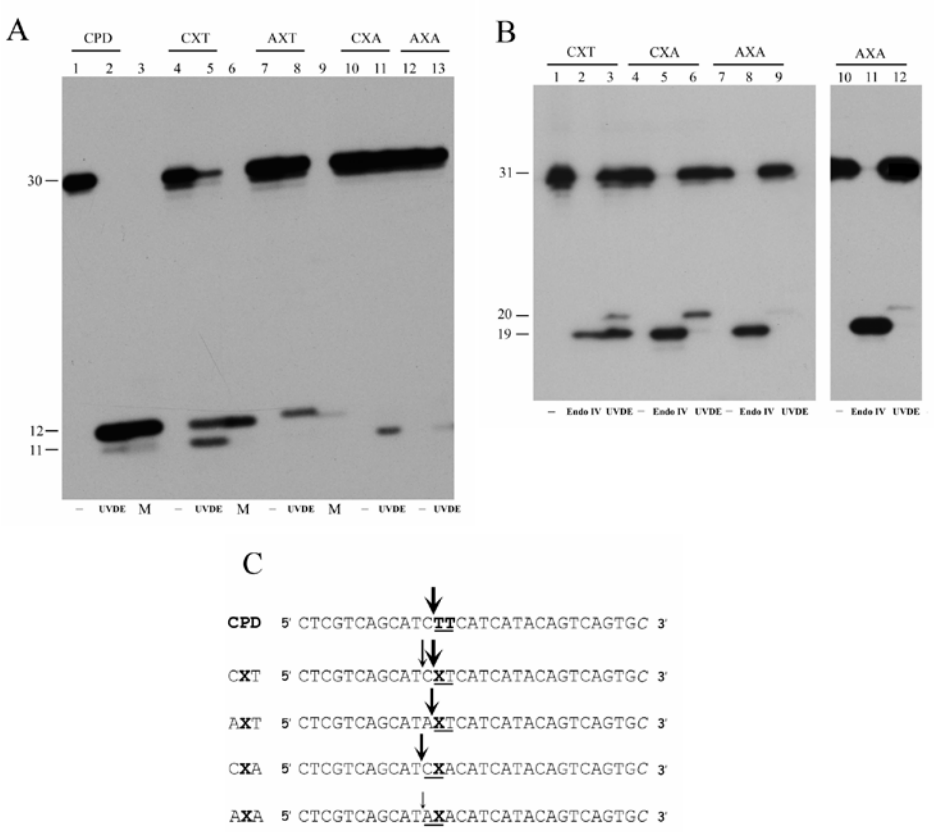

Figure 2. Determination of incision positions.

A The 5'-terminally labelled substrates (0.8 nM) were incubated without protein (lanes 1, 4, 7, 10, 12) or with 10 nM (lane 2) or 5 nM of UVDE (lanes 5, 8, 11, 13) for 15 minutes and loaded on a long acrylamide gel. The positions of the non-incised fragment (30 nt) and the incision products (11 and 12 nt) are indicated. The substrates used are indicated above the lanes. The lanes containing the 12 nt marker fragment (with the sequence corresponding to the first 12 nt of the top strand of the CPD substrate) are indicated with M.

B The 3'-terminally labelled substrates were incubated without protein (lanes 1, 4, 7, 10), with Endo IV (lanes 2, 5, 8, 11) and 2 nM (lane 3) or 5 nM UVDE (lanes 6, 9, 12) for 15 minutes. Lanes 10 - 12 represent a longer exposure of lanes 7 - 9 to visualize the incision product. The positions of the non-incised fragment (31 nt) and the incision products (19 and 20 nt) are indicated.

C Schematic representation of the incision positions. The arrows indicate the positions of the nicks made by UVDE. The sizes of the arrows correspond to the efficiencies of the direct incisions. The residues that take up the same position with respect to the nick as the CPD are underlined. Note that only the 3'-labelled fragments contain an additional C (italics) at the 3' end.

CXA and AXA the incision position is shifted one nucleotide in the 5' direction (Figure 2A, lanes 11, 13; Figure 2B, lanes 6, 12). With the CXT sequence clearly two incision positions are observed which have equal intensities with the 5'-labelled DNA (Figure 2A, lane 5). The 3' labelling, however, reveals that the major incision is at position 19, which is immediately 5' to the abasic site (Figure 2B, lane 3). There is only a minor incision on the position which is 1 nt shifted to the 5' side, indicating that the 11 nt fragment observed with the 5' labelling is mainly the result of a second incision that is made after introduction of the nick adjacent to the AP site (see also below). When we used Endo IV on the different AP site fragments only one incision product was obtained, as result of incision directly 5' to the AP site (Figure 2B, lanes 2, 5, 8). Apparently for UVDE the bases flanking the AP site not only determine the efficiency of incision but also the position of the nick.

When we align the different (major) incision positions of the AP constructs with that of the CPD substrate (Figure 2C) we can extrapolate which two residues (the AP site and a flanking nucleotide) will take up the same position as the CPD in the protein-DNA complex formed on the abasic sites. These combinations are incised with different efficiencies: XT > CX >> AX. The combination XA apparently does not lead to a productive complex, since neither in the CXA nor in the AXA fragment a significant incision is observed immediately adjacent to the AP site. Taken together our results indicate that indeed UVDE recognizes the abasic site together with a flanking nucleotide either on the 5'- or on the 3' side with a high preference for a pyrimidine as a neighbour. This strongly suggests that like for the crosslinked photoproducts also on the AP site constructs UVDE rotates two residues into a protein pocket, the abasic site together with a flanking pyrimidine. The enzyme shows a preference for having this pyrimidine on the 3' side of the abasic site since on the CXT fragment the majority of the incision is directly 5' to the AP site.

Next we tested the binding of UVDE to the four DNA fragments using a filter binding assay described in Materials and Methods. The binding was performed in the absence (preventing incision) or presence (allowing incision) of 1mM $MnCl_2$ and 10 mM $MgCl_2$ (Table 2). In both conditions the percentage of binding indeed differs for the 4 constructs, with CXT giving the highest amount of complex (comparable to the binding of a CPD lesion) and AXA the lowest. For all four substrates, however, the binding is still significantly higher than binding to undamaged DNA (Table 2). Even for the AXA substrate, which is very poorly incised the binding is still 50 % of that of the CXT fragment. Apparently the binding per se does not always lead to a productive complex. Comparing the binding data of the two incubation conditions reveal that in the presence of the metal ions DNA binding is somewhat increased for the fragments that are efficiently incised by UVDE (CPD, (6-4)PP and CXT) but remains unaltered for the substrates that are only poorly incised (CXA, AXA). This suggests that the presence of a nick stabilizes the protein-DNA complex.

Table 2. UVDE binding to the different DNA substrates.

Substrate	% binding no cofactors	% binding $Mn^{2+} + Mg^{2+}$
no damage	2 ± 1	n.d.
CPD	55 ± 3	62 ± 2
(6-4)PP	57 ± 1	66 ± 1
abasic (C**X**T)	53 ± 3	59 ± 1
abasic (A**X**T)	41 ± 2	43 ± 1
abasic (C**X**A)	38 ± 2	38 ± 1
abasic (A**X**A)	29 ± 3	27 ± 1
ss-nick (C*T)	49 ± 2	47 ± 4
ss-nick (C*A)	44 ± 2	45 ± 1
ss-nick (A*T)	32 ± 1	30 ± 1
ss-nick (A*A)	22 ± 1	23 ± 1
TG	58 ± 1	60 ± 2

X, position of AP site; *, position of nick; TG, thymine glycol
Incubation was done with 4 nM DNA and 5 nM UVDE, n.d. not determined.

UVDE incises DNA containing a nick or a gap

As shown above after nicking of the CXT substrate directly 5' to the abasic site, UVDE makes a second incision 5' to the adjacent C residue. This indicates that formation of a productive complex on the CX sequence is improved by the presence of a nick between the two residues. To test whether for this incision the AP site is still required we also determined the activity of UVDE on the same DNA fragment with a ss-nick but without the abasic site (C*T). Figure 3A (lane 2) shows that this DNA substrate is efficiently incised by UVDE (approximately 50 %). A DNA fragment with identical sequence without the nick is not incised at all (not shown). The two subsequent incisions on the CXT abasic site fragment also show that under similar incubation conditions approximately 50 % of the first incision product is additionally nicked by UVDE (note the equal intensities of the two incision products in Figure 2A, lane 5). Apparently whether there is a thymine or an abasic site on the 3' side of the nick does not significantly influence the incision. Even with a purine on the 3' side (C*A, lane 4) a similar amount of incision products is found. DNA fragments with a purine on the 5' side (A*C and A*A), however, are hardly incised by UVDE (lanes 6, 8). This means that there is a preference for a pyrimidine at the 5' side of the nick but the nature or even the presence of the base on the 3' side does not seem to be of importance. Concomitant with the incision efficiencies also the DNA binding to the C*T and C*A sequences is higher than to the nicks with a 5' flanking purine residue, but again the binding to the A*C and A*A fragments is significantly higher than to undamaged DNA (Table 2), as was observed for the abasic site fragment that was very poorly incised (AXA). UVDE incises the C*T and C*A fragments 5' of the C that is flanking the nick, but also a second incision product is observed which results from incision 1 nt further downstream (Figure 3A, lanes 2, 4). When using a 50 bp substrate containing a nick flanked by two stretches of pyrimidines (constructed for the fluorescence studies described below) even two additional incision products were obtained

(20 and 21 nt, Figure 3B, lane 1). The intensities of these incision products suggest that they result from subsequent incision events. This would imply that UVDE can also nick DNA 5' to a nucleotide that is flanked by a 1 or 2 nt gap. To test this we constructed a DNA fragment containing a 1 nt gap (Table 1). Indeed UVDE does nick this DNA resulting in two incision products of 25 and 24 nt (Figure 3B, lane 5). At a higher protein concentration the amount of the 25 nt fragment decreases whereas that of the 24 nt fragment increases showing that the 24 nt fragment results from two consecutive incision events. Both incisions are made 5' to a pyrimidine residue. A subsequent third incision is not observed, most likely because this nick would have to be made 5' to a purine residue (see sequence Figure 3B). In the T*T fragment there are three pyrimidines present explaining why on this substrate three successive incisions

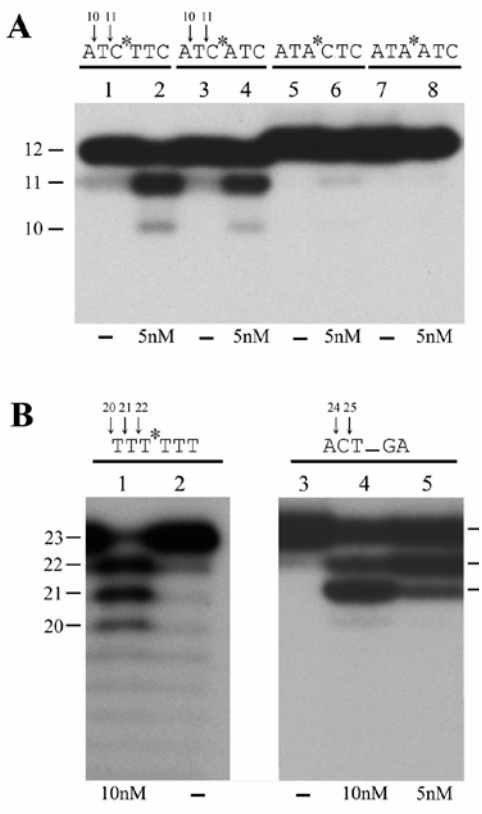

Figure 3. Activity of UVDE on a DNA fragment with a ss-nick or 1 nt gap.

A. The 5'-terminally labelled substrates (0.8 nM) were incubated with 5 nM of UVDE for 15 minutes.

B. Similar incubations containing 0.3 nM of T*T nick fragment (lanes 1, 2) or 0.8 nM of the gapped substrate (lanes 3 - 5) with the indicated amount of UVDE. The relevant sequences of the DNA substrates are shown above the lanes with the position of the nick (*) and the position of the gap (-) and the incision positions (arrows).

can be made. Taken together the results show that UVDE can also form productive complexes on a pyrimidine residue that lacks a 3' neighbour, implying that on these substrates rotation of only one nucleotide into the proposed protein pocket is sufficient. The fact that on a nicked substrate, in contrast to the abasic site constructs, the nature of the 3' flanking base is of much less importance suggests that because of the interrupted phosphodiester backbone also here only one nucleotide which is 5' of this nick will be rotated. It should be noted that although the binding of the CXT abasic fragment is similar to that of the C*T nicked DNA, the overall incision of the abasic site is still higher (compare Figure 2A, lane 5 with Figure 3A, lane 2). This implies that the catalytic reaction is more successful in complexes with two flipped bases on an intact DNA backbone than on complexes with one flipped base on a broken DNA backbone.

UVDE-mediated unstacking of nucleotides opposite the lesion

In the co-crystal structure of Endo IV with DNA not only the AP site but also the opposite base is extruded from the DNA helix (Hosfield *et al*., 1999). To test whether base flipping in the opposing strand also occurs upon binding of UVDE we made use of the fluorescent adenine analogue 2-AP that we incorporated in the different DNA substrates opposite the lesion (CPD, (6-4)PP, CXT abasic). Excitation of 2-AP with light of a wavelength of 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

Table 3. UVDE binding to 2-aminopurine (2-AP) containing DNA substrates.

Damage	2-AP position	% binding wt UVDE	% binding Y358A
No damage	1	5 ± 3	4 ± 2
No damage	2	8 ± 2	6 ± 3
CPD	no	61 ± 1	28 ± 1
CPD	1	60 ± 1	25 ± 1
CPD	2	59 ± 1	27 ± 1
(6-4)PP	no	59 ± 3	60 ± 3
(6-4)PP	1	61 ± 4	62 ± 2
(6-4)PP	2	65 ± 1	66 ± 1
abasic (C**X**T)	no	62 ± 1	16 ± 1
abasic (C**X**T)	1	61 ± 4	15 ± 2
abasic (C**X**T)	2	60 ± 1	15 ± 1
nick (T*T)	no	57 ± 1	n.d.
nick (T*T)	1	57 ± 2	n.d.
nick (T*T)	2	59 ± 1	n.d.
TG	no	60 ± 1	n.d.
TG	1	60 ± 2	n.d.
TG	2	61 ± 1	n.d.

X, position of AP site, TG, thymine glycol. Incubation was done using the same conditions as used for the fluorescence assays without metal cofactors. The positions of the 2-AP residues are as shown in Figures 4 and 5.

for base unstacking and base flipping (Allan *et al*., 1996; Christine *et al*., 2002; Holz *et al*., 1998; McCullough *et al*., 1997; Bandwar and Patel, 2001 and Malta *et al*., 2006). The incision efficiencies of the different substrates were not influenced by the presence of a 2-AP instead of a normal adenine (not shown) and also the DNA binding efficiencies are similar (Table 3).

In the absence of protein none of the DNA fragments exhibit significant fluorescence (Figure 4, A - F), showing that the different lesions do not disturb the base stacking in the opposing strand. The addition of UVDE to a non-damaged DNA fragment containing either of the 2-AP residues also did not give rise to any fluorescence signal (not shown).

Upon incubation of the (6-4)PP-containing DNA with UVDE in the absence of metal ions (preventing DNA incision) fluorescence of the 2-AP at position 1 (opposite the 5' T) as well as the 2-AP at position 2 (opposite the 3' T) increases significantly to 25 and 32 AU respectively (Figure 4, A and B). Considering that the fluorescence of the same concentration of free 2-AP is around 100 AU and that under conditions used for the fluorescence measurements about 60 % of the DNA is bound (Table 3), significant destacking of the two damage-opposing bases does occur upon UVDE binding. For the CPD lesion similar destacking of the two bases can be detected, again with the signal of the 2-AP opposite the 3' T somewhat higher than its neighbour opposite the 5' T (Figure 4, C and D). Binding of UVDE to the abasic site in the sequence CXT shows that the 2-AP residues opposite the abasic site (position 1) and the 3' flanking T (position 2) show the same fluorescence as the 2-AP's opposite the CPD or (6-4)PP lesions (Figure 4, E and F). This confirms that in this substrate UVDE is positioned on the XT sequence in a similar way as on the CPD and (6-4)PP.

We also tested fluorescence of the T*T nicked DNA with 2-AP residues opposing the thymines flanking the nick (Figure 5A, B). The T*T DNA fragment by itself (in the absence of protein) already gives rise to significant fluorescence. Apparently the presence of a nick in the A/T-rich sequence largely enhances the flexibility of this DNA. In the presence of UVDE a similar fluorescence of the 2-AP residues is observed as for the other substrates (Figure 5, A and B) showing that unstacking of the bases in the opposite strand also occurs when the 'damaged' strand is broken.

We also tested whether conformation of the DNA in the complex changes after incision. To this purpose the DNA substrates were pre-incubated with UVDE in the presence of the metal cofactors for 10 minutes to allow incision, after which the fluorescence spectra were recorded (Table 4). Incision of the different substrates under these conditions was confirmed (not shown). For none of the 2-AP residues a significant change in fluorescence could be observed (Table 4), indicating that upon incision the residues opposing the lesion remain in the same unstacked conformation.

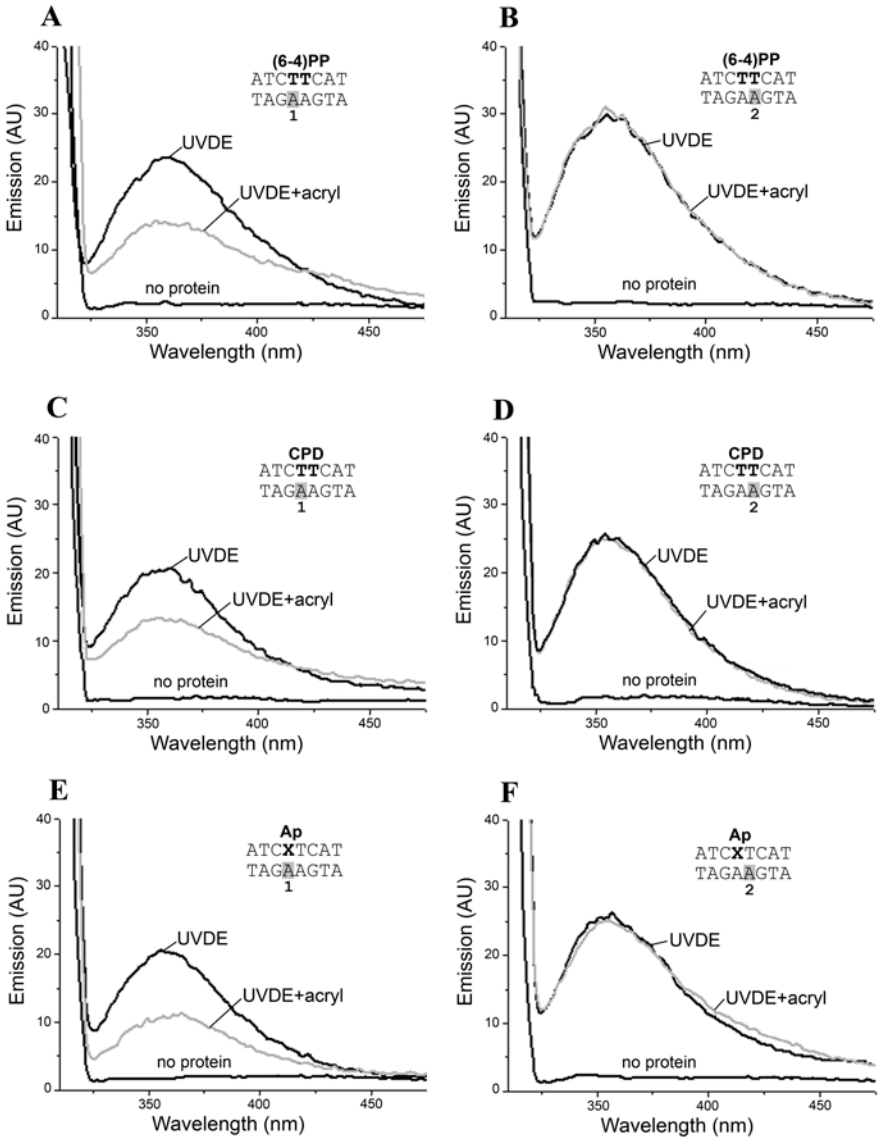

Figure 4. Fluorescence emission spectra. The DNA fragments (0.5 µM) with a (6-4)PP (A and B) a CPD (C and D) or an AP lesion (E and F) were incubated with or without 2.5 µM UVDE for 10 minutes at 30°C. After incubation the sample was transferred to a cuvette and emission spectra were recorded at 30°C (excitation at 310 nm). The relevant sequences of the different substrates are shown with the 2-AP residue at position 1 (A, C, E) or position 2 (B, D, F). The spectra of the samples without protein or after incubation with UVDE are in black as indicated. In grey are the spectra recorded in the presence of acrylamide.

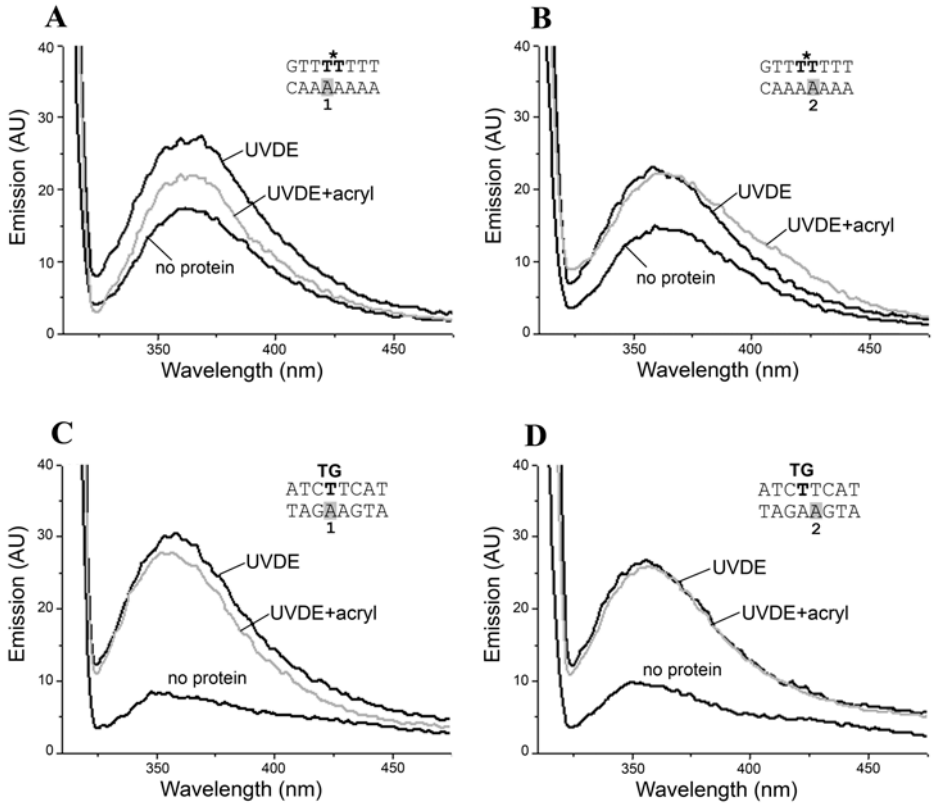

Figure 5. Fluorescence emission spectra. The DNA fragments (0.5 μM) with a ss-nick (T*T, panels A and B) or thymine glycol (TG, panels C and D) were incubated with or without 2.5 μM UVDE for 10 minutes at 30°C. After incubation the sample was transferred to a cuvette and emission spectra were recorded at 30°C (excitation at 310 nm). The relevant sequences of the different substrates are shown with the 2-AP residue at position 1 (A and C) or position 2 (B and D). The spectra of the samples without protein or after incubation with UVDE are in black as indicated. In grey are the spectra recorded in the presence of acrylamide.

Table 4. Fluorescence signals before and after incision.

Damage	2-AP position	Fluorescence no incision	Fluorescence 10 min incision
CPD	1	23 ± 1	25 ± 4
CPD	2	27 ± 2	28 ± 2
(6-4)PP	1	25 ± 2	27 ± 3
(6-4)PP	2	32 ± 1	30 ± 2

The fluorescence signal (emission at 370 nm) is shown in arbitrary units (AU).

In the Endo IV protein-DNA complex the extrahelical base opposite the AP site does not make any contacts with residues of the protein and is fully solvent exposed. To test whether UVDE extrudes the opposing bases in a similar way we measured fluorescence of the UVDE-DNA

complex in the presence of acrylamide. Acrylamide has been shown to quench the fluorescence of 2-AP but it can only do so if the 2-AP is accessible to the compound, i.e. solvent exposed (Malta *et al*., 2006, Rai *et al*., 2003). Figures 4 and 5 (grey lines) show that for all four substrates fluorescence of the 2-AP at position 2 remains unaltered upon addition of acrylamide, indicating that this residue is shielded from solution by residue(s) of the UVDE protein. The 2-AP at position 1 appears not fully protected by the protein as in all four substrates it is partly quenched by the acrylamide (Figures 4 and 5). For the T*T containing fragment the quenching of 2-AP at position 1 seems less than for the other substrates (Figure 5A), but one should bear in mind that for this particular substrate the unbound DNA that is still present in the sample also contributes to the fluorescence signal. In any case, unlike Endo IV for none of the DNA substrates the residues in the complementary strand become fully solvent exposed upon UVDE binding.

UVDE recognizes but does not efficiently incise DNA containing a thymine glycol.

It has been reported that UVDE does not incise DNA containing a thymine glycol (Kanno *et al*., 1999), but since the data of this experiment were not included in the paper, this might have been due to the presence of purine residues flanking the thymine glycol (TG) lesion. We therefore constructed a DNA fragment in which the TG is flanked by pyrimidine residues in the same sequence context as the optimal abasic site substrate CXT (Table 1). The binding of UVDE to this DNA fragment is indeed comparable to that of the CXT, CPD or (6-4)PP containing fragments (Table 2). Also under conditions of the 2-AP fluorescence experiments a similar binding is observed (Table 3) and these complexes do give rise to fluorescence of the 2-AP residues in the opposing strand (Figure 5, C and D). The addition of acrylamide again shows that these 2-AP residues do not become solvent exposed. The quenching at position 1 is somewhat less compared to the other types of lesion, indicating that the conformation of this residue might be different, shielding it more from the solution.

When we tested the incision of the TG-containing DNA by UVDE the nicking activity appeared surprisingly low. At the 'normal' concentrations (5 nM) UVDE does hardly incise the DNA and only at a 10 times higher concentration some nicking (20 %) is observed (Figure 6A, lanes 2, 3). As a control the Endo III enzyme does efficiently induce incision on the same substrate (Figure 6A, lane 1). Apparently although UVDE does bind the TG lesion and also induces destacking of the bases in the opposing strand, it cannot effectively position the scissile bond into the active site of the protein.

Residue Y358 stabilizes the extra helical bases opposite the lesion.

In the crystal structure of *T. thermophilus* UVDE an aromatic residue (Tyr105) was seen to point out from the proposed DNA binding groove into the solvent (Paspaleva *et al*., 2007). The importance of Tyr105 for the activity of the *T. thermophilus* UVDE became clear when this residue was changed into an alanine, since the incision activity of the resulting mutant was severely reduced (Paspaleva *et al*., 2007).

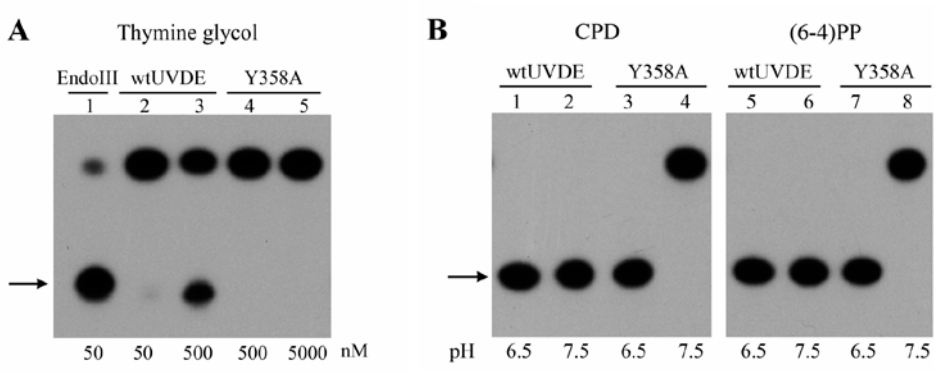

Figure 6. Incision activity on thymine glycol and effect of pH on incision.

A. The 5'-labelled 30 bp DNA fragment (0.2 nM) with a TG lesion was incubated with the indicated amount of Endo III (lane 1) and wtUVDE (lanes 2 and 3).

B. The 5'-labelled 30 bp DNA fragment (0.2 nM) with a CPD or (6-4)PP was incubated with 5 nM of UVDE at two different pH conditions as shown. The arrow indicates the position of the incision product.

To our surprise, mutating the equivalent tyrosine residue in *S. pombe* UVDE (Y358A) resulted in a protein that was severely reduced in incision of DNA containing an AP site (Figure 7A, lane 2), but the incision on the CPD or (6-4)PP was still very efficient (Figure 7A, lanes 3 - 6). Filter binding studies revealed that indeed the binding of the mutant protein to an AP site is significantly reduced (Table 3). The binding of Y358A to the (6-4)PP is similar to that of the wild type protein, but also the binding to the CPD appears reduced (Table 3). A kinetic assay with a limiting amount of protein revealed that the incisions of the CPD and (6-4)PP by the mutant protein are even much faster than for the wild type protein (Figure 7, B and C). The (6-4)PP containing DNA is more efficiently nicked by the mutant protein than the CPD, which probably reflects the difference in binding between these substrates. Apparently the absence of Tyr358 does reduce the stability of the protein-DNA complex on the CPD, but this is largely compensated by a much faster incision step. For the (6-4)PP Tyr358 is not required for stable binding and also for this DNA fragment the removal of this residue speeds up the incision reaction.

Next we tested the effect of the Y358A mutation on the 2-AP fluorescence. For this we used the DNA with the (6-4)PP lesion, since under the conditions used for the fluorescence assay the mutant protein forms a similar amount of complexes on this lesion as the wild type protein (Table 3). Position 1 no longer shows any fluorescence upon binding of the mutant protein (Figure 8A) and fluorescence at position 2 is reduced by about a factor of 3 (Figure 8B). The peak of the remaining fluorescence signal at position 2 is significantly shifted to a higher wavelength indicating that the 2-AP residue in the mutant complex is in a different environment. This was confirmed by the observation that the remaining fluorescence at position 2 can now

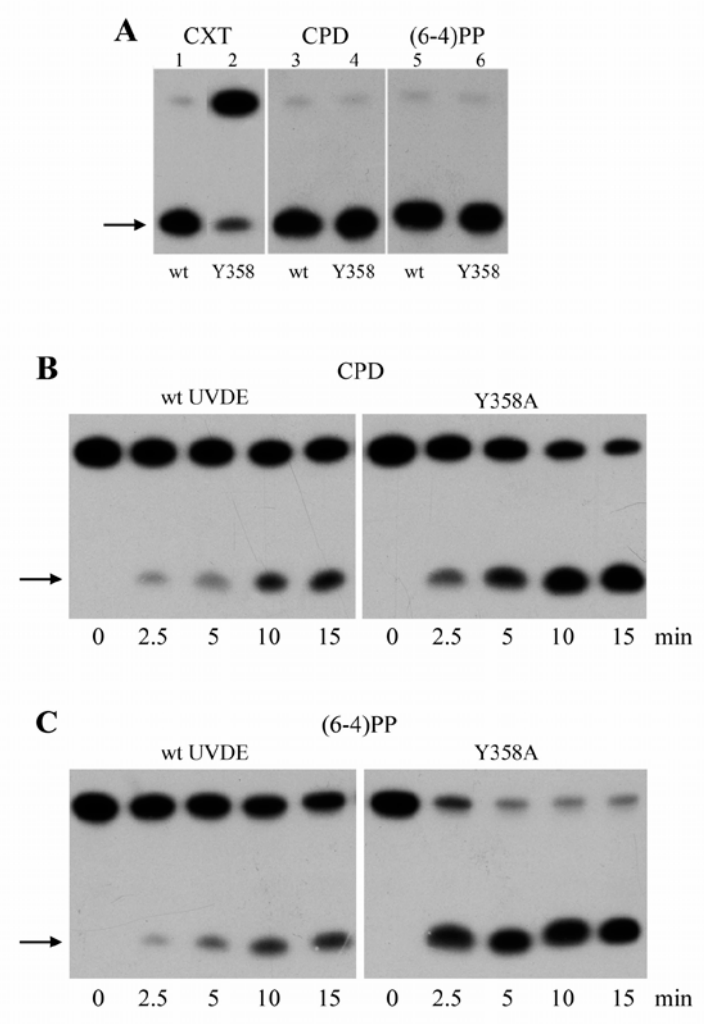

Figure 7. Incision activity of the Y358A mutant.

A. The 5'-labelled 30 bp DNA fragments (0.2 nM) with a CPD, (6-4)PP or AP site (CXT) were incubated with 5 nM UVDE or mutant Y358 for 15 minutes at 30°C (B and C). The CPD fragment (0.1 nM) and (6-4)PP fragment (0.1 nM) were incubated with 0.25 nM of (mutant) UVDE for the indicated amount of time (minutes). The incision products are indicated with an arrow.

be fully quenched by acrylamide (Figure 8B), whereas the fluorescence of the same residue in the complex formed by the wild type protein was fully protected from quenching (Figure 4B). This clearly shows that residue Tyr358 not only stabilizes the destacking of the bases in the non-damaged strand but also that in the absence of Tyr358 position 2 becomes more solvent exposed.

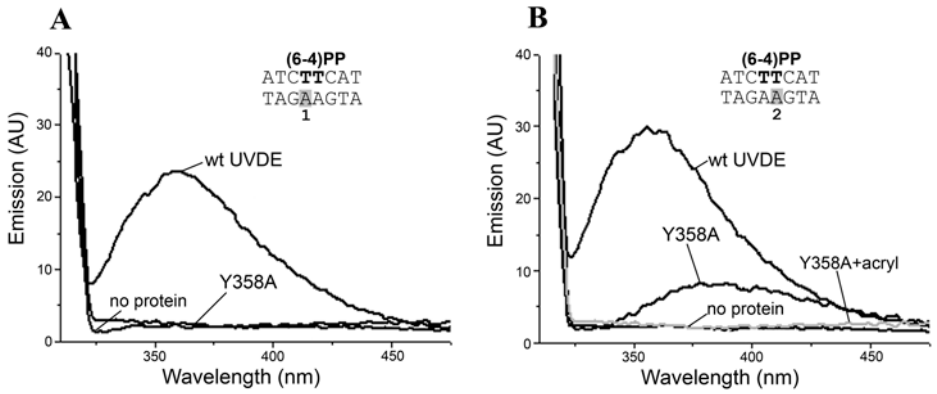

Figure 8. Fluorescence emission spectra of wt UVDE and mutant Y358A on a substrate containing a (6-4)PP.

The position of the 2-AP and the relevant sequence are shown. In panel A the 2-AP residue is incorporated at position 1, while in panel B the fluorescent base is at position 2. The spectra of the samples without protein or after incubation with wt UVDE or Y358A are in black as indicated. In grey is the spectrum recorded in the presence of Y358A and acrylamide.

Mutant Y358A does not repair UV lesions *in vivo*.

The surprising increased incision activity of the Y358A mutant on the CPD and (6-4)PP lesions prompted us to test the ability of the mutant protein to repair UV-induced damage *in vivo*. For this purpose we used an *E. coli* strain lacking the *uvrA* and *uvrB* genes, which as a consequence of the absence of nucleotide excision repair (NER) is very UV sensitive (Figure 9, row 1). Introduction of a plasmid with the wt UVDE gene under control of the Plac promoter restores UV resistance (Figure 9, rows 2 and 6). The repair of UV lesions with only the UVDE protein is almost equal to the repair in a wt *E. coli* strain by the NER proteins (compare rows 2 and 3). Apparently the UVDE not only incises the DNA adjacent to the UV-induced lesions in *E. coli*, but the bacterium can effectively process the subsequent nicks to complete repair. The presence of the NER proteins together with UVDE even further improves the UV resistance (row 4). Apparently there are lesions left by the NER system (most likely CPD's) that are repaired by UVDE. This explains why some bacterial species (like *Bacillus subtilis*), which do possess an active NER machinery have also acquired the UVDE gene (Goosen and Moolenaar, 2008). Expression of the Y358A mutant protein in a NER-deficient background, however, only gave rise to very low UV survival (Figure 9, row 7), although the wild type and mutant proteins were expressed to the same level in the cells (results not shown).

What might explain this apparent discrepancy between the *in vivo* and *in vitro* activities of the mutant protein? One explanation might be that the binding of the protein to CPD lesions, which is already reduced *in vitro* might be much further reduced *in vivo* where there is a large excess of undamaged DNA. When we tested the incision of the CPD and (6-4)PP fragments in the presence of an excess of undamaged DNA, however, the activity of the mutant protein remained

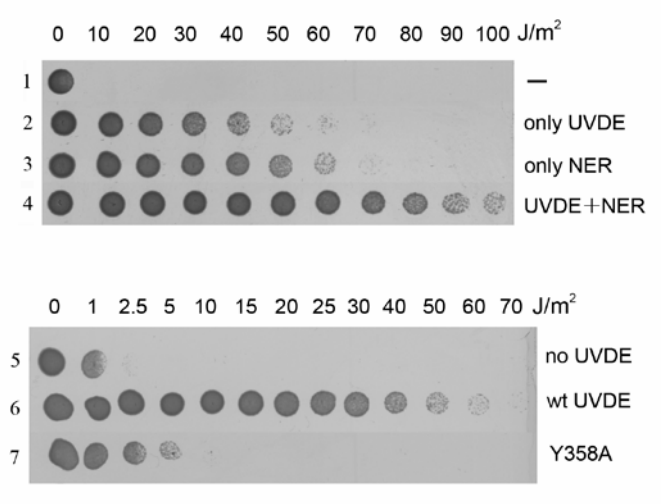

Figure 9. UV survival test.

A nucleotide excision repair deficient *E. coli* strain containing a vector plasmid without UVDE (rows 1 and 5), or plasmids expressing wild type (rows 2 and 6) or mutant Y358A proteins (row 7) were irradiated with the indicated dose of UV light. As a control also a wt *E. coli* strain (row 3) and the same strain expressing wtUVDE (row 4) were included in the test.

very high (not shown). Another difference between the *in vivo* and *in vitro* conditions is the pH. The cellular pH of *E. coli* (and *S. pombe*) is around pH 7.5 (Padan *et al*., 1981). The optimal pH for the wild type enzyme, however, is pH 6.5 (Kaur *et al*., 1999 and our lab (not shown)) and all experiments have been conducted in a buffer of this lower pH. When we tested the wild type UVDE at the more physiological pH of 7.5 the enzyme still fully incised the DNA containing a CPD or (6-4)PP at 5 nM of protein (Figure 6B). The mutant protein, however, did not show any activity at all under these conditions (Figure 6B), although the binding of the mutant protein did not alter when the pH was varied (Table 5). This means that under physiological conditions, the catalytic activity of the mutant protein is strongly reduced, explaining the impaired repair of UV damage *in vivo.* We conclude from these experiments that Tyr358 not only plays a role in stabilization of the protein-DNA complex, but it also shields the active site from the influence of a higher pH of the solvent.

DISCUSSION

One of the intriguing questions in DNA repair is how the different repair enzymes discriminate between damaged- and undamaged DNA. In some repair systems only one specific type of damage is recognized. An example of such an enzyme is uracil glycosylase where the enzyme contains a very specific uracil binding-pocket, which occludes binding of the four other bases (Parikh *et*

al., 1998 and 2000). Nucleotide excision repair (NER) is an example of a repair system with a very broad substrate specificity where multiple enzymes participate in the damage recognition process, each protein probing different characteristics of the DNA structure (Truglio *et al*., 2006, Gillet *et al*., 2006). UVDE has also shown to be more versatile in damage recognition, although in contrast to NER only one relatively small protein is responsible for both damage-specific binding and incision. In this paper we present evidence that UVDE uses different parameters to recognize a DNA damage. Initially UVDE seems to probe the DNA for its bendability. We show that not only a CPD, (6-4)PP or abasic site but also a single-strand nick or gap significantly increase the binding affinity of the protein, although some of these complexes do not result in incision. The similarity between UVDE and Endo IV predicts that in the UVDE-DNA complex the DNA will be bent by about 90°. Any alteration in the DNA that increases its flexibility is therefore expected to stabilize the protein-DNA contacts. Most likely for the same reason mismatches and small insertions can be a substrate for UVDE (Kaur *et al*., 1999, Gillet *et al*., 2006). The bendability of the DNA alone, however, is not enough. Subsequently the protein needs to present the scissile bond to the active site, which is buried inside the protein, requiring rotation of the adjacent base from the DNA helix. For the CPD and (6-4)PP this means that both covalently linked bases need to be extruded from the helix into a proposed pocket of the protein (Paspaleva *et al*., 2007). Our results with the AP site in different sequence contexts suggest that also on an AP lesion the protein will flip two residues: the abasic site together with its neighbour. Whether this will be the base at the 3' or 5' side of the AP site depends on the nature of these bases. The most preferred AP-substrate appears to be the AP site with a pyrimidine at its 3' side. The combination of an abasic site with a purine at the 3' side does not seem to be bound at all and instead on such a sequence UVDE will shift one position, now targeting the AP site together with the 5' flanking base. Apparently the protein pocket in which the two adjacent bases are proposed to flip can accommodate a normal or distorted (in the CPD and (6-4)PP) pyrimidine at the 3' side but not a purine, probably as a result of its larger size. Also for the base flanking the AP site at the 5' side a pyrimidine is preferred, although productive complexes can be formed with a 5' flanking purine as well albeit with much lower efficiency. We have presented evidence that UVDE can also form productive complexes on only one rotated pyrimidine residue if this residue is 3' of a broken backbone caused by a nick or a gap. Possibly because of the broken backbone the rotation of the two residues is no longer coupled, allowing insertion of only one pyrimidine into the protein pocket. Again here no productive complexes are formed when a purine flanks the nick in the backbone. Taken together we propose that exclusion of larger bases from the proposed protein pocket provides a second damage discrimination step.

Our results are in agreement with the reported UVDE-mediated incision of DNA containing a platinum-GG crosslink (Avery *et al*., 1999). On this DNA the incision did not occur immediately 5' to the Pt-GG adduct which can be expected since the crosslinked guanine residues would be too large to fit into the proposed protein pocket. Instead incision was made 2 nt further downstream, placing the two pyrimidine residues that flank the Pt-GG adduct into the pocket

of the protein. Surprisingly in the same paper it was reported that UVDE incises DNA with an AP site in the CXA context immediately 5' to the AP site. This is in contrast to our observation where in the same sequence context the enzyme makes the incision 5' to the adjacent C residue. An important difference between the two substrates is that in our DNA fragments the AP site consists of a tetrahydrofuran (i.e. a closed ribose ring) whereas in the substrate used by Avery *et al*. (1999) the AP site was generated by removal of a uracil with uracil DNA glycosylase, resulting in a ring-opened ribose. Possibly the open ring structure provides more conformational freedom allowing rotation of only the AP site without the 3' flanking purine. It should be noted, however, that on this CXA sequence with the ring-opened ribose incision is much less efficient (ten times lower compared to a CPD (Avery *et al*., 1999).

A thymine glycol (TG) lesion adjacent to a pyrimidine is as efficiently bound by UVDE as a CPD or (6-4)PP and the protein induces similar conformational changes in the bottom strand for all three lesions. Yet the TG-containing DNA is only poorly incised. *In silico* (Miaskiewicz *et al*., 1995) and structural studies (Kim and Choi, 1995) showed that TG is not a planar molecule and the methyl group is oriented perpendicularly with respect to the pyrimidine ring, making the TG more 'bulky' compared to a normal thymine. It is not very likely that this will completely prevent the TG from being inserted into the protein pocket, but its conformation might make it more difficult to properly position the 5' phosphodiester bond into the catalytic site.

The 2-AP fluorescence studies presented in this paper show that the two bases opposite the residues that are proposed to be flipped into the UVDE protein pocket are significantly destacked. In the crystal structure of *T. thermophilus* UVDE, Tyr358 is seen to point out of the proposed DNA binding groove and therefore it is very likely that upon binding to DNA this residue will penetrate the DNA helix. One possibility is that Tyr358 will wedge between the two residues opposite the damage thereby disrupting the base stack and stabilizing the DNA kink. As a consequence the 3' residue might be partly rotated from the helix, since it was shown to be partly accessible to the solvent. Alternatively both residues in the complementary strand might take up an extrahelical position with Tyr358 occupying the vacated space and other residues of UVDE completely shielding the 5' base. We regard this possibility as less likely, however, since it is hard to envisage how this shielding would occur, since the bases are expected to be flipped away from the protein. In either case, the situation must be different from the base-flipping in the non-damaged strand by Endo IV, since in this complex the base is fully extrahelical but it does not make any specific contacts with the enzyme.

In the absence of Tyr358 destacking of the bases in the non-damaged strand is no longer observed and as a consequence binding to the CPD lesion is reduced. On the (6-4)PP binding is not affected indicating that the contribution of Tyr358 to the stability of the UVDE-DNA complex depends on the type of damage. A (6-4)PP lesion induces a kink of 44° into the DNA (Kim and Choi, 1995) whereas CPD lesion is less kinked by about 30° (Park *et al*., 2002). Possibly if the DNA is already more kinked by the lesion itself the putative role of Tyr358 in stabilizing the kink becomes less important. The overall structure of DNA containing an AP site

does not show significant kinking (Chen *et al*., 2008), explaining that on the CXT fragment the binding of the mutant protein is even further reduced.

The incision activities of the Y358A mutant on both CPD and (6-4)PP significantly differed from the wild type protein. At pH 6.5 the mutant protein was more active than the wild type, whereas at pH 7.5 the complete opposite was found resulting in an active wild type and inactive mutant protein. Since the pH did not affect the binding of either protein this means that Tyr358 influences the catalytic step of the reaction in a pH-dependent way.

In the co-crystal structure of Endo IV with its abasic site substrate there is also a tyrosine residue (Tyr72) that inserts into the DNA helix filling the gap left by the flipped-out AP site and stacking on its 5' neighbour (Hosfield *et al*., 1999). Mutation of this residue into alanine only partially reduced the DNA binding, but caused a more dramatic decrease in catalysis. It was proposed that the hydrophobic residue shields the active site from the solvent to prevent influence on the pK_a of the catalytic Glutamate or hydroxide nucleophile (Garcin *et al*., 2008). Indeed the structure of the Y72A mutant revealed the presence of water molecules in close proximity of the scissile phosphodiester bond (Garcin *et al*., 2008). Also in UVDE Tyr358 might fulfil such a protective function. Although our fluorescence data show that this residue is more in contact with the non-damaged strand, its hydrophobic nature might similarly prevent access of the solvent to the active site. The fluorescence data show that the 5' opposing residue becomes more solvent exposed in the absence of Tyr358 and water molecules might therefore penetrate further into the active site. The pH dependence of the catalysis by the Y358A mutant, however, indicates that the solvent is not likely to influence the first step in catalysis, deprotonation of the attacking water molecule, since this would be expected to be less favourable at lower pH. Instead the mutant protein shows a higher activity at lower pH (6.5). Therefore we propose that in the mutant the solvent influences the last step of catalysis, the protonation of the departing 3'-O of the ribose. Under physiological conditions (pH 7.5) this is negatively influenced by the solvent, making the shielding effect of Tyr358 essential for the activity of the enzyme.

In conclusion we propose that although UVDE is capable of repairing different types of damage it is specialized in the removal of UV lesions (CPD and (6-4)PP) and we present the following model for its damage recognition. Initially the protein will bind to flexible sites in the DNA that allow bending of the DNA helix. Bending will be aided by insertion of the Tyr358 into the helix. Subsequently the protein will try to flip two potentially damaged bases into a pocket of the protein, which is located at the bottom of the DNA binding groove. Large bases will be excluded from this pocket, allowing entrance only of two pyrimidines or a pyrimidine together with an AP site. If insertion of these bases into the pocket is successful, the phosphodiester bond is brought in close vicinity of the metal ions in the catalytic site. The aromatic side chain of Tyr358 will shield the active site from the solvent allowing efficient catalysis at physiological pH.

REFERENCES

Allan BW and Reich NO, Targeted base stacking disruption by the *Eco*RI DNA methyltransferase, *Biochemistry* **35** (1996), pp. 14757-14762

Aller P, Rould MA, Hogg M, Wallace SS and Doublie S, A structural rationale for stalling of a replicative DNA polymerase at the most common oxidative thymine lesion, thymine glycol, *Proc. Natl. Acad. Sci. USA*. **104** (2007), pp. 814-818

Avery A, Kaur B, Taylor JS, Mello JA, Essigmann JM and Doetsch PW, Substrate specificity of ultraviolet DNA endonuclease (UVDE/Uve1p) from *Schizosaccharomyces pombe*, *Nucleic Acids Res*. **27** (1999), pp. 2256-2264

Bandwar RP and Patel SS, Peculiar 2-aminopurine fluorescence monitors the dynamics of open complex formation by bacteriophage T7 RNA polymerase, *J. Biol. Chem*. **276** (2001), pp. 14075-14082

Bowman K, Cidik K, Smith CA, Taylor JS, Doetsch PW and Freyer GA, A new ATP-independent DNA endonuclease from *Schizosaccharomyces pombe* that recognizes cyclobutane pyrimidine dimers and 6-4 photoproducts, *Nucleic Acids Res*. **22** (1994), pp. 3026-3032

Chen J, Dupradeau F-Y, Case DA, Turner CJ and Stubbe JA, DNA oligonucleotides with A, T, G or C opposite an abasic site: structure and dynamics, *Nucleic Acids Res*. **36** (2008), pp. 253-262

Christine KS, MacFarlane AW, Yang K and Stanley RJ, Cyclobutylpyrimidine dimer base flipping by DNA photolyase, *J. Biol. Chem*. **277** (2002), pp. 38339-38344

Earl AM, Rankin SK, Kim KP, Lamendola ON and Battista J, Genetic evidence that the uvsE gene product of *Deinococcus radiodurans* R1 is a UV damage endonuclease, *J. Bacteriol*. **184** (2002), pp. 1003-1009

Garcin ED, Hosfield DJ, Desai SA, Haas BJ, Björas M, Cunningham RP and Tainer JA, DNA apurinic-apyrimidinic site binding and excision by endonuclease IV, *Nature Struct. & Mol. Biol*. **15** (2008), pp. 515-522

Gillet LC and Schärer OD, Molecular mechanisms of mammalian global genome nucleotide excision repair, *Chem. Rev*. **106** (2006), pp. 253-276

Goosen N and Moolenaar GF, Repair of UV damage in bacteria, *DNA Repair* (*Amst.*) **7** (2008), pp. 353-379

Holz B, Klimasaukas S, Serva S and Weinhold E, 2-aminopurine as a fluorescent probe for DNA base flipping by methyltransferases, *Nucleic Acids Res*. **26** (1998), pp. 1076-1083

Hosfield DJ, Guan Y, Haas BJ, Cunningham RP and Tainer JA, Structure of the DNA repair enzyme Endo IV and its DNA complex: double-nucleotide flipping at abasic sites and three-metal-ion catalysis, *Cell* **98** (1999), pp. 397-408

Iwai S, Chemical synthesis of oligonucleotides containing damaged bases for biological studies, *Nucleosides Nucleotides Nucleic Acids* **25** (2006), pp. 561-582

Kanno S, Iwai S, Takao M and Yasui A, Repair of apurinic/apyrimidinic sites by UV damage endonuclease; a repair protein for UV and oxidative damage, *Nucleic Acids Res*. **27** (1999), pp. 3096-3103

Kaur B, Fraser LA, Freyer GA, Davey S and Doetsch PW, A Uve1p-mediated mismatch repair pathway in *Schizosaccharomyces pombe*, *Mol. Cell. Biol*. **19** (1999), pp. 4703-4710

Kim J-K and Choi B, The solution structure of DNA duplex-decamer containing the (6-4) photoproduct of thymidylyl(3'→5')thymidine by NMR and relaxation matrix refinement, *Eur. J. Biochem*. **228** (1995), pp. 849-854

Malta E, Moolenaar GF and Goosen N, Base flipping in nucleotide excision repair, *J. Biol. Chem*. **281** (2006), pp. 2184-2194

Miaskiewicz K, Miller J, Ornstein R and Osman R, Molecular dynamics simulations of the effects of ring-saturated thymine lesions on DNA structure, *Biopolymers* **35** (1995), pp. 113-124

McCullough AK, Dodson ML, Schärer OD and Lloyd RS, The role of base flipping in damage recognition and catalysis by T4 endonuclease V, *J. Biol. Chem.* **272** (1997), pp. 27210-27217

Moolenaar GF, Schut M and Goosen N, Binding of the UvrB dimer to non-damaged and damaged DNA: residues Y92 and Y93 influence the stability of both subunits, *DNA Repair* (*Amst.*) **4** (2005), pp. 699-713

Padan I, Zilberstein D and Schuldiner S, pH homeostasis in bacteria, *Biochim. Biophys. Acta* **650** (1981), pp. 151-166

Parikh SS, Mol CD, Slupphaug G, Bharati S, Krokan HE and Tainer JA, Base excision repair initiation revealed by crystal structures and binding kinetics of human uracil-DNA glycosylase with DNA, *EMBO J.* **17** (1998) pp. 5214-5226

Parikh SS, Putnam CD and Tainer JA, Lessons learned from structural results on uracil-DNA glycosylase, *Mutat. Res.* **460** (2000) pp. 183-199

Park H, Zhang K, Ren Y, Nadji S, Sinha N, Taylor J and Kang C, Crystal structure of a DNA decamer containing a cis-syn thymine dimer, *Proc. Natl. Acad. Sci. USA*. **99** (2002), pp. 15965-15970

Paspaleva K, Thomassen E, Pannu NS, Iwai S, Moolenaar GF, Goosen N and Abrahams JP, Crystal structure of the DNA repair enzyme UV damage endonuclease, *Structure* **15** (2007), pp. 1316-1324

Rai P, Cole TD, Thompson E, Millar DP and Linn S, Steady-state and time-resolved fluorescence studies indicate an unusual conformation of 2-aminopurine within ATAT and TATA duplex DNA sequences, *Nucleic Acids Res.* **31** (2003), pp. 2323-2332

Studier FW, Rosenberg AH, Dunn JJ and Dubendorff JW, Use of T7 RNA polymerase to direct expression of cloned genes, *Methods Enzymol.* **185** (1990), pp. 60-89

Takao M, Yonemasu R, Yamamoto K and Yasui A, Characterization of a UV endonuclease gene from the fission yeast *Schizosaccharomyces pombe* and its bacterial homolog, *Nucleic Acids Res.* **24** (1996), pp. 1267-1271

Truglio JJ, Croteau DL, Van Houten B and Kisker C, Prokaryotic nucleotide excision repair, the UvrABC system, *Chem. Rev.* **106** (2006) pp. 233-252

Verhoeven EE, van Kesteren M, Turner JJ, van der Marel GA, van Boom JH, Moolenaar GF and Goosen N, The C-terminal region of *Escherichia coli* UvrC contributes to the flexibility of the UvrABC nucleotide excision repair system, *Nucleic Acids Res.* **30** (2002), pp. 2492–2500

Yajima H, Takao M, Yasuhira S, Zhao JH, Ishii C, Inoue H and Yasui A, A eukaryotic gene encoding an endonuclease that specifically repairs DNA damaged by ultraviolet light, *EMBO J.* **14** (1995), pp. 2393-2399