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Strand breakage of a (6–4) photoproduct-containing DNA at neutral pH and its repair by the ERCC1–XPF protein complex†

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The (6–4) photoproduct is one of the major UV-induced lesions in DNA. We previously showed that hydrolytic ring opening of the 5′ base and subsequent hydrolysis of the glycosidic bond of the 3′ component occurred when this photoproduct was treated with aqueous NaOH. In this study, we found that another product was obtained when the (6–4) photoproduct was heated at 90 °C for 6 h, in a 0.1 M solution of *N*,*N*′-dimethyl-1,2-ethanediamine adjusted to pH 7.4 with acetic acid. An analysis of the chemical structure of this product revealed that the 5′ base was intact, whereas the glycosidic bond at the 3′ component was hydrolyzed in the same manner. The strand break was detected for a 30-mer oligonucleotide containing the (6–4) photoproduct upon treatment with the above solution or other pH 7.4 solutions containing biogenic amines, such as spermidine and spermine. In the case of spermidine, the rate constant was calculated to be 1.4×10^{-8} s^{-1} at 37 °C. The strand break occurred even when the oligonucleotide was heated at 90 °C in 0.1 M sodium phosphate (pH 7.0), although this treatment produced several types of 5′ fragments. The Dewar valence isomer was inert to this reaction. The product obtained from the (6–4) photoproduct-containing 30-mer was used to investigate the enzymatic processing of the 3′ end bearing the damaged base and a phosphate. The ERCC1–XPF complex removed several nucleotides containing the damaged base, in the presence of replication protein A.

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Introduction

DNA undergoes chemical reactions with endogenous and exogenous factors. Ultraviolet (UV) light produces cross-links between two adjacent base moieties, and the major products are referred to as a cyclobutane pyrimidine dimer (CPD) and a pyrimidine(6–4)pyrimidone photoproduct ((6–4) photoproduct).[1] The CPD is produced by the [2 + 2] cycloaddition between the two C5–C6 double bonds. In the case of the (6–4) photoproduct, the four-membered ring containing an oxygen or nitrogen atom, formed by a similar reaction, is so unstable that it opens to produce the C6–C4-linked compound (**1**) shown in Fig. 1. Although the (6–4) photoproduct is repaired more efficiently by the nucleotide excision repair (NER) pathway,[2] it is more mutagenic than the CPD.[3–5] DNA polymerase η that can incorporate two adenines opposite the CPD is not able to bypass the (6–4) photoproduct,[6] whereas DNA polymerase ζ reportedly replicates DNA containing this photoproduct in an error-free manner.[7]

An important chemical property of the (6–4) photoproduct is its alkali lability. Since a strand break occurs at the (6–4) photoproduct site upon hot piperidine treatment, this property was used to detect the formation of this type of lesion in DNA.[8–12] We previously elucidated the mechanism of this alkali degradation. The first reaction is the hydrolytic ring opening of the 5′ base,[13] as shown in Fig. 1. This intermediate (**2**) was isolated, and the biochemical properties of DNA containing this hydrolyzed photoproduct were analyzed.[13] The second reaction is the hydrolysis of the glycosidic bond at the 3′ component. The product without the sugar moiety (**3**) was obtained even when the parent compound lacked the 3′-flanking nucleotide as a leaving group.[14] When the (6–4) photoproduct resides in DNA, the abasic site produced by the second reaction leads to strand breakage by β,δ-elimination under alkaline conditions.

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†Electronic supplementary information (ESI) available: UV absorption spectra of peaks i, ii, and iii, NMR spectra of compound **4**, and a MALDI-TOF mass spectrum of peak v. See DOI: 10.1039/c3ob00012e

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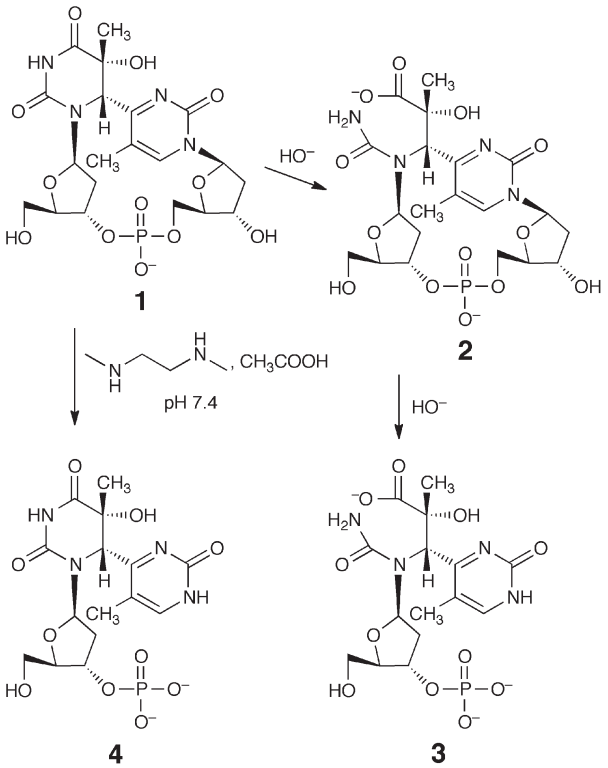

Fig. 1 Degradation of the (6–4) photoproduct under alkaline and neutral conditions.

The (6–4) photoproduct is converted to its Dewar valence isomer by absorbing UV-A/B light.[15–17] This isomer contains a Dewar pyrimidinone as the 3′ base, which does not have the 326 nm absorption maximum typical of the aromatic pyrimidinone structure, and is reportedly more labile than the (6–4) photoproduct under alkaline conditions.[18] In our previous study,[14] the hydrolytic ring opening of the 5′ base of the (6–4) photoproduct occurred quickly even at a low NaOH concentration. However, the strand break caused by the glycosidic bond cleavage at the 3′ component of the (6–4) photoproduct was much slower than that observed for the Dewar isomer when oligonucleotides containing these photoproducts were treated with NaOH. In other words, the 5′ base of the (6–4) photoproduct and the 3′ glycosidic bond of the Dewar isomer are very alkali-labile.

A previous report showed that *N*,*N*′-dimethyl-1,2-ethanediamine (DMED) cleaved an abasic site-containing or UV-irradiated DNA at pH 7.4.[19] This pH value is important because it is a physiological pH. Although the products obtained by this treatment and those generated with hot piperidine were compared by gel electrophoresis, neither the lesion type nor the reaction mechanism was elucidated in the case of the UV-irradiated DNA. In this study, we determined the chemical structure of the product and the rate constant of this reaction, analyzed the reactions in several types of solutions, and showed the removal of the 3′ blocking end by repair proteins, using synthetic oligonucleotides containing the UV-induced lesions. Our results demonstrated that the 3′-side glycosidic bond of the (6–4) photoproduct was thermally more labile than those of the normal bases under neutral conditions, while that of the Dewar valence isomer was relatively stable.

Results

DMED treatment of a (6–4) photoproduct-containing tetramer

The tetramer d(AT(6–4)TG), which was used in our previous study,[14] was dissolved in a 0.1 M solution of DMED with the pH value adjusted to 7.4 with acetic acid (AcOH). After the mixture was heated at 90 °C for various periods of time, the products were analyzed by reversed-phase HPLC. The reaction rate was unexpectedly slow, but as shown in Fig. 2, two peaks (ii and iii) were detected in addition to the starting material (peak i) after 6 h. From the UV absorption spectrum and the co-injection experiment, the product that yielded peak ii was identified as 2′-deoxyguanosine 5′-phosphate, which was released from the 3′ end of the tetramer. The other product (peak iii) generated a UV absorption spectrum with maxima at 260 and 317 nm, while those of the parent tetramer were 256 and 327 nm, as shown in Fig. S1.† This spectrum was similar to that observed for the product obtained by the degradation of d(AT(6–4)TG) with NaOH, in which 2′-deoxyadenylic acid is attached to the 5′-OH of **3**,[14] but the retention times of these two compounds were different.

Structure of the product

To determine the chemical structure of this product, compound **1**, which was prepared by deprotection of an intermediate in the synthesis of the oligonucleotide building block of the (6–4) photoproduct,[20] was treated with the same solution on a large scale, and NMR spectra of the product were measured. The ^{1}H and ^{31}P NMR spectra revealed that one of the sugar moieties was lost, although the phosphate remained. The NOESY spectra indicated that the missing sugar belonged to the 3′ component, in the same manner as the reaction with NaOH.[14] This is supported by the blue-shift of the UV absorption maximum (from 327 nm to 317 nm) in the

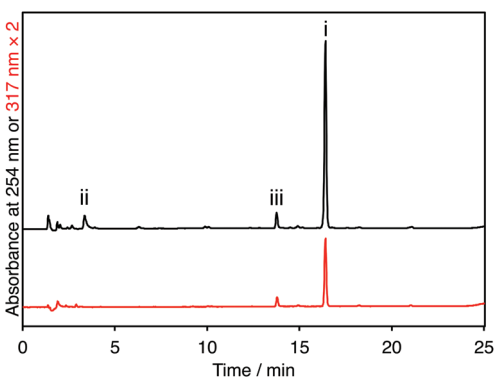

Fig. 2 HPLC analysis of the solution containing d(AT(6–4)TG) in 0.1 M DMED–AcOH (pH 7.4), after heating at 90 °C for 6 h. The chromatogram monitored at 317 nm, shown in red, is magnified by a factor of 2.

Table 1 ^{13}C chemical shifts

	5′ Base					3′ Base					5′ Sugar				
Comp. (ref.)	C2	C4	C5	C6	CH_3	C2	C4	C5	C6	CH_3	C1′	C2′	C3′	C4′	C5′
1 (21)	156.4	176.7	74.9	60.4	27.7	159.9	176.9	119.0	146.8	16.2	84.9	37.5	72.3	84.8	61.4
2 (13)	160.7	179.3	86.3	61.7	21.4	159.7	175.2	117.5	148.1	15.7	84.4	36.9	72.7	84.8	62.3
3 (14)	161.4	179.8	87.1	61.8	21.0	161.6	174.8	117.3	152.5	15.9	86.8	39.4	77.0	87.9	64.8
4	156.9	177.1	74.9	60.6	28.0	161.0	178.2	118.6	149.1	16.3	87.2	38.2	77.1	87.7	64.6

abovementioned experiment, which also demonstrated that the 3′ pyrimidone ring was intact. The structure of the 5′ base was determined by ^{13}C NMR spectroscopy. The ^{13}C signals were assigned by using the HSQC and HMBC spectra, and a difference in the chemical shifts of the 5′ base carbons was detected between the products obtained by the reactions with DMED–AcOH (**4**) and with NaOH (**3**), as shown in Table 1. The values obtained for **4** in the present study were very close to those reported for the parent compound (**1**).[21] This observation and the mass spectrometry result indicated that the hydrolytic ring opening of the 5′ base, which was found in the alkali degradation of the (6–4) photoproduct,[13] did not occur under the conditions used in this study.

Treatment of a 30-mer with amine solutions

To investigate whether the reaction shown in Fig. 3 occurs in longer oligonucleotides, a chemically-synthesized 30-mer containing the (6–4) photoproduct, d(CTCGTCAGCATCT(6–4)TCATCATACAGTCAGTG),[20] was treated with 0.1 M DMED–AcOH (pH 7.4), in the same manner as the tetramer, and the result is shown in Fig. 4A. From the long-wavelength UV absorption, peak v was identified with the 5′-side fragment containing the (6–4) photoproduct at the 3′ end. This product, d(CTCGTCAGCATCX) in which X was expected to have the structure of **4**, was purified by HPLC. Its structure including the intact 5′ base was confirmed by MALDI-TOF mass spectrometry, as shown in Fig. S9.† Peak vi corresponded to the 3′-side fragment, namely, 5′-phosphorylated d(CATCATACAGTCAGTG). The structure of the break site is shown in Fig. 3. Although the conditions were the same, the strand break apparently occurred more efficiently with the 30-mer than with the tetramer.

McHugh and Knowland[19] used DMED, because the pK_a value of one of the protonated amino groups of this compound (7.43) is lower than that of the other one (10.23). At pH 7.4, about half of the molecules are singly-protonated. We expected that the same reaction might occur when biogenic amines, such as spermidine and spermine (Fig. 3), are used instead of DMED. Although the lowest pK_a was not close to 7.4 (for example, that of spermine is reportedly 7.95[22]), the solutions of spermidine and spermine were adjusted to pH 7.4 with AcOH, and the 30-mer was heated in each solution at 90 °C for 6 h. The results of the HPLC analyses of the reaction mixtures are shown in Fig. 4B and 4C. Although the product yields were slightly lower, the elution profiles were similar to that obtained for the reaction using DMED, and the identification was performed by co-injection experiments. To assess the biological relevance of the results, the rate constants of the strand break in 0.1 M spermidine–AcOH (pH 7.4) were measured at temperatures between 70 and 90 °C, and the value at 37 °C was estimated to be 1.4×10^{-8} s^{-1} from the Arrhenius plot (Fig. 5).

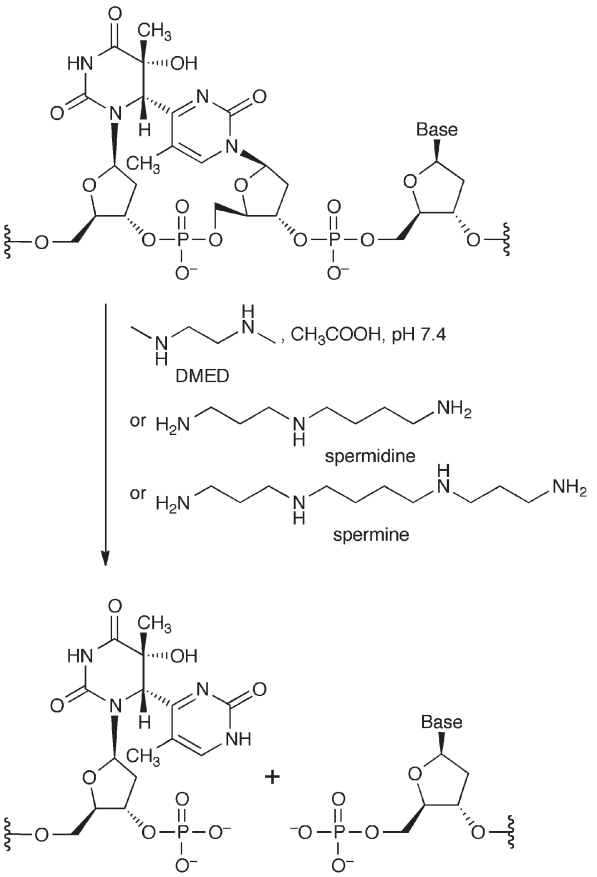

Fig. 3 Strand breakage at the (6–4) photoproduct at pH 7.4.

Strand breaks in other solutions

Next, we tested the reaction using ethylamine, which is a simple primary amine. A solution of 0.1 M ethylammonium acetate (pH 7.4) was prepared, and the 30-mer containing the (6–4) photoproduct was treated with this solution at 90 °C for 6 h. As shown in Fig. 6A, two peaks were detected at the retention time of the 5′ fragment, while the peak of the 3′ fragment looked the same as that shown in Fig. 4A. A co-injection

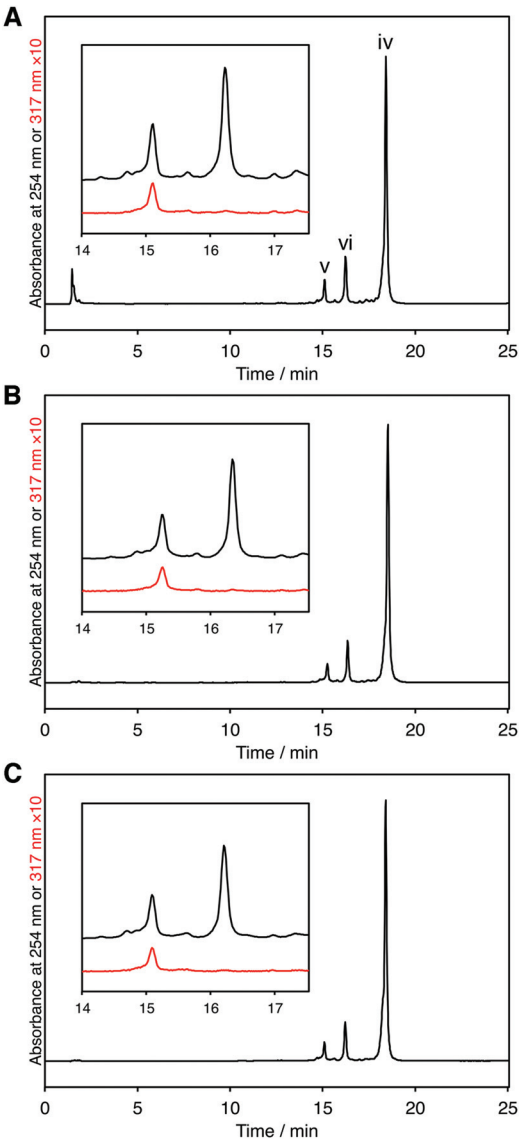

Fig. 4 HPLC analysis of the solutions containing the (6–4) 30-mer in 0.1 M DMED–AcOH (pH 7.4) (A), in 0.1 M spermidine–AcOH (pH 7.4) (B), and in 0.1 M spermine–AcOH (pH 7.4) (C), after heating at 90 °C for 6 h. The insets are magnifications of the product peaks. The vertical scales of the chromatograms monitored at 317 nm, shown in red, are magnified by a factor of 10.

experiment revealed that one of the two smaller peaks and the larger product peak coincided with the 5′ and 3′ fragments obtained in the experiment using DMED, respectively. Finally, we tried to confirm whether amines were required for the strand break at the (6–4) photoproduct. The same oligonucleotide was treated with 0.1 M ammonium acetate (pH 7.4) and 0.1 M sodium phosphate (pH 7.0) in an identical manner. Contrary to our expectations, the same 3′ fragment produced by the strand break at the (6–4) photoproduct was detected even in the sample heated in the phosphate buffer, although these treatments yielded further additional peaks assigned to the 5′ fragment from the UV absorption spectra (Fig. 6B and 6C). The results indicated that the strand break at the

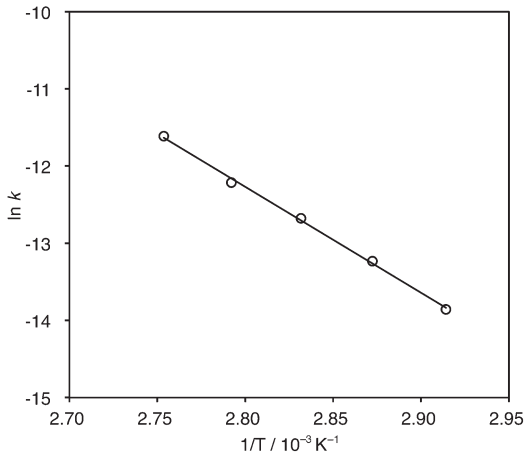

Fig. 5 Arrhenius plot for the strand-break reaction of the (6–4) 30-mer in 0.1 M spermidine–AcOH (pH 7.4).

(6–4) photoproduct was caused by the thermal instability of the glycosidic bond of the 3′ component of this lesion, but not by the reaction with the amines.

Dewar valence isomer

A chemically-synthesized 30-mer, containing the Dewar valence isomer of the (6–4) photoproduct in the same sequence as above,[23] was treated with 0.1 M DMED–AcOH (pH 7.4) and 0.1 M sodium phosphate (pH 7.0) at 90 °C for 6 h. As shown in Fig. 7, only very small peaks of the 3′ fragment were detected, and the 5′ fragment was hardly identifiable. These results showed that the glycosidic bond of the Dewar isomer is thermally stable, although it is more alkali-labile than that of the (6–4) photoproduct.[14,18]

Since this finding is important, we further investigated whether the same holds true for the photoproducts formed at the thymine–cytosine sequence, which is the major (6–4) photoproduct site.[10] A tetramer, d(ATCG), was irradiated with 254 nm light to form the (6–4) photoproduct, and after HPLC purification, the (6–4) photoproduct was converted to its Dewar valence isomer by irradiation with Pyrex-filtered light from a high-pressure mercury lamp. The glycosidic bond of the Dewar isomer was obviously more labile in alkaline solutions than that of the (6–4) photoproduct (Fig. 8A), in the same manner as the photoproducts formed at TT shown in our previous study.[14] However, the stability against heat degradation at pH 7.0 was the opposite, as shown in Fig. 8B and 8C, and the results obtained for the 30-mers containing the TT photoproducts (Fig. 6C and 7B) were reproduced.

Enzymatic removal of the 3′ blocking end

When this type of strand break occurs in cells, the 3′ end bearing the cross-linked base and the phosphate is produced at the gap, and this nonconventional structure blocks the chain elongation by DNA polymerase. We investigated whether this 3′-blocking residue could be removed enzymatically. The excision repair cross-complementation group 1 (ERCC1)–

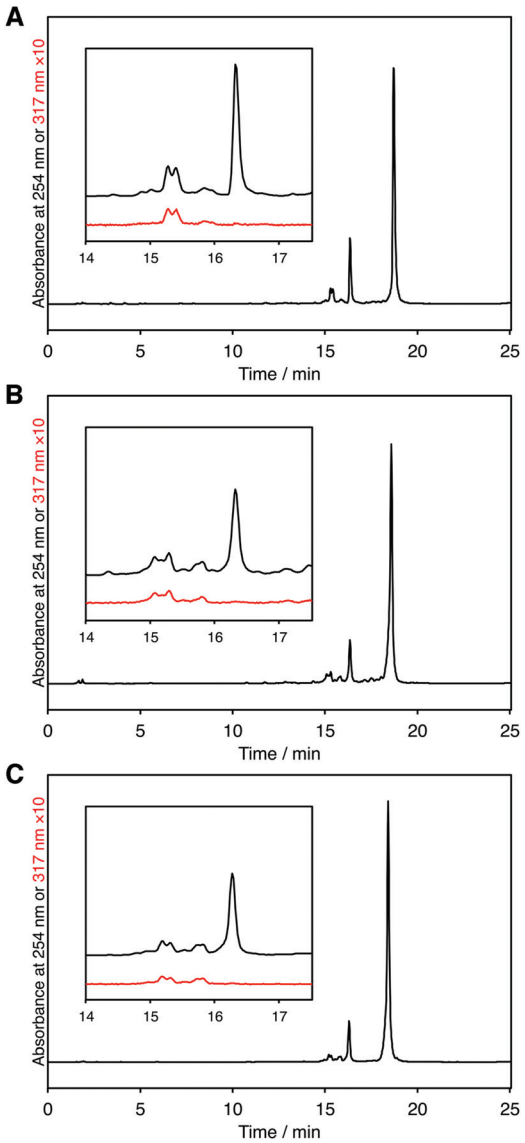

Fig. 6 HPLC analysis of the solutions containing the (6–4) 30-mer in 0.1 M ethylammonium acetate (pH 7.4) (A), in 0.1 M ammonium acetate (pH 7.4) (B), and in 0.1 M sodium phosphate (pH 7.0) (C), after heating at 90 °C for 6 h. The insets are magnifications of the product peaks. The vertical scales of the chromatograms monitored at 317 nm, shown in red, are magnified by a factor of 10.

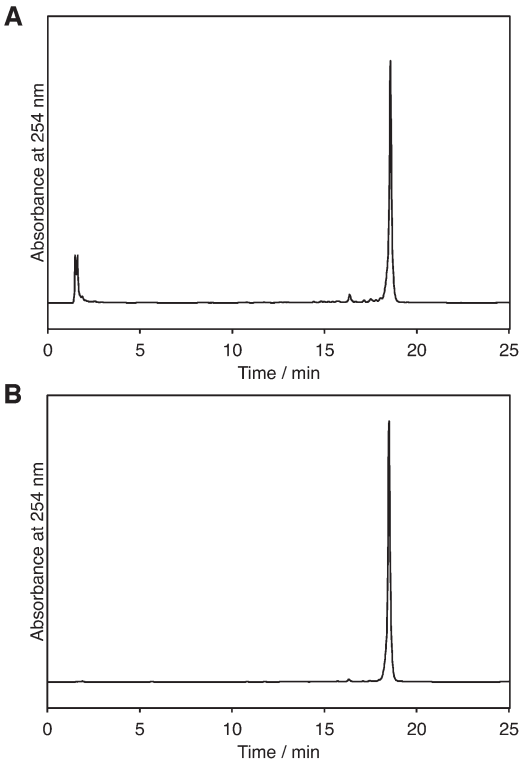

Fig. 7 HPLC analysis of the solutions containing the Dewar 30-mer in 0.1 M DMED–AcOH (pH 7.4) (A) and in 0.1 M sodium phosphate (pH 7.0) (B), after heating at 90 °C for 6 h.

xeroderma pigmentosum complementation group F (XPF) complex, which is an essential endonuclease for NER of UV-damaged DNA, was chosen because it reportedly removes 3′-phosphoglycolate,[24,25] another type of 3′-blocking end. After ^{32}P-labeling at the 5′ end of the 5′ fragment obtained in the experiment shown in Fig. 4A, a duplex with a gap shown in Fig. 9A was prepared as the substrate. This duplex was incubated with the recombinant ERCC1–XPF complex,[26] and the product was analyzed by polyacrylamide gel electrophoresis (PAGE). Since replication protein A (RPA) reportedly stimulates the endonuclease activity of the ERCC1–XPF complex,[27] its effect was tested, and the D731A XPF mutant, which lacks the nuclease activity (this mutant was referred to as D720A in the original report),[28] was also used. As shown in Fig. 9B, several nucleotides at the 3′ end of the ^{32}P-labeled fragment were removed in the presence of RPA, in a similar manner to the 3′-phosphoglycolate substrate.[24,25] The major products were d(CTCGTCAG) and d(CTCGTCA), and the product bands were not detected when the mutant XPF protein was used. The dependence of the endonuclease activity on the RPA concentration was confirmed, as shown in Fig. 9C.

Discussion

The (6–4) photoproduct formed in DNA by UV irradiation is known to be alkali-labile. Strand breaks occur when UV-irradiated DNA is treated with hot alkali, and we previously elucidated the degradation mechanisms.[13,14] The first reaction is the hydrolytic ring opening of the 5′ base, and subsequently, the glycosidic bond at the 3′ component is cleaved to yield an abasic site, which leads to the strand breakage by β,δ-elimination. These two reactions occur independently, but the stabilities of the bonds are different. Since extremely alkaline conditions, such as 1 M piperidine or 0.1 M KOH, and a very high temperature, *e.g.* 90 °C for a 30 min treatment, are required for the strand break,[9,29,30] these reactions are unlikely to occur in the cells of living organisms. However, this method is often utilized for experiments to determine the photoproduct sites in UV-irradiated DNA.[8–12] On the other hand,

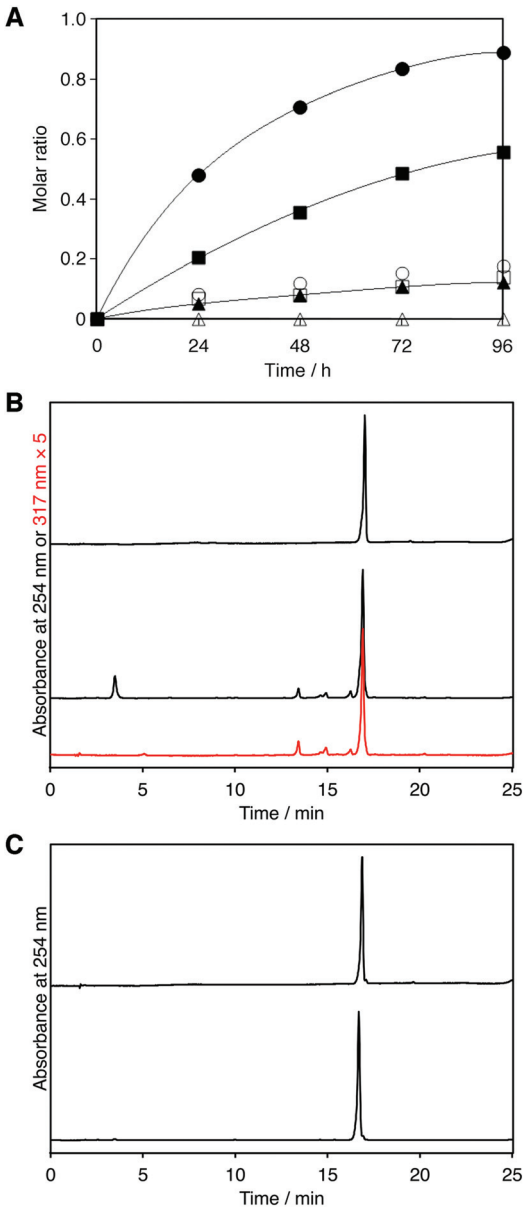

Fig. 8 Analysis of the strand breaks at the (6–4) photoproduct and its Dewar valence isomer formed between thymine and cytosine. (A) Strand breaks in alkaline solutions. Tetramers containing the (6–4) photoproduct (open symbols) and the Dewar isomer (filled symbols) were incubated at 37 °C at pH 10 (triangles), 11 (squares), and 12 (circles), and the released 2′-deoxyguanosine 5′-phosphate was quantified. (B and C) HPLC analysis of the solutions of the tetramers containing the (6–4) photoproduct (B) and the Dewar isomer (C) in 0.1 M sodium phosphate (pH 7.0), before and after heating at 90 °C for 6 h. The peak detected at 3.5 min in B is 2′-deoxyguanosine 5′-phosphate.

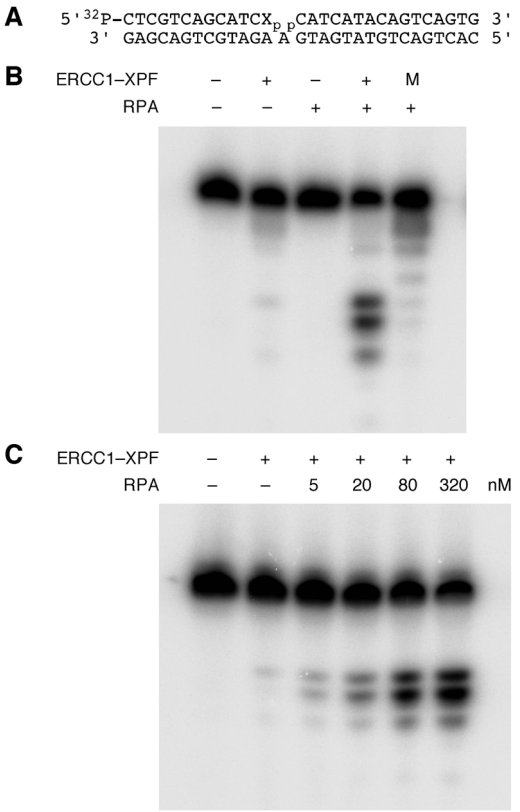

Fig. 9 Analysis of the ERCC1–XPF reaction. (A) The structure of the substrate. X represents the (6–4) photoproduct without the 3′ sugar moiety. (B) PAGE analysis of the products. M represents the D731A mutant of the XPF protein. (C) Dependence of the nuclease activity of the ERCC1–XPF complex on the RPA concentration.

McHugh and Knowland[19] reported that strand breaks were detected when UV-irradiated DNA was treated with DMED–AcOH at pH 7.4. Although the reaction temperature was 90 °C, we were interested in this report because of the physiological pH value.

The first finding in this study was that a strand break, similar to that caused by the hot alkali treatment, occurred when a tetramer containing the (6–4) photoproduct was treated with 0.1 M DMED–AcOH (pH 7.4) at 90 °C for 6 h (Fig. 2). However, the 5′ fragment was not identical to that produced by the alkali treatment in our previous study.[14] Analyses of this product by NMR spectroscopy and mass spectrometry revealed that the structure of the (6–4) photoproduct was intact after the strand break, while the 5′ base underwent hydrolytic ring opening by hot alkali.[13,14] The chemical structures of these products are shown in Fig. 1. A similar strand break occurred in a 30-mer oligonucleotide, apparently with higher efficiency (Fig. 4A), and the same products were detected when biogenic amines, *i.e.* spermidine and spermine, were used instead of DMED (Fig. 4B and 4C). The rate constant of this strand breakage in 0.1 M spermidine–AcOH (pH 7.4) at 37 °C was determined to be 1.4×10^{-8} s^{-1}. This value is larger than the rate constants of cytosine deamination at 37 °C (1×10^{-10} and 7×10^{-13} s^{-1} in single- and double-stranded DNA, respectively)[31] and depurination at 37 °C and pH 7.4 (3×10^{-11} s^{-1}),[32] which are both important types of spontaneous DNA damage. We used a single-stranded oligonucleotide to determine the rate constant, while DNA is usually in the double-stranded form. However, the (6–4) photoproduct reportedly destabilizes the duplex to a great extent,[33] and the hydrogen bonding is lost at the 3′ component of this photoproduct.[34] Therefore, it

is assumed that the strand break at the (6–4) photoproduct in double-stranded DNA occurs at a similar rate.

When the same oligonucleotide was treated with 0.1 M ethylammonium acetate (pH 7.4), the 5′ fragment was split into two peaks with the same UV absorption spectrum (Fig. 6A). We assumed that a DNA fragment containing an α,β-unsaturated aldehyde at the 3′ end, which was formed by β-elimination, was obtained, in addition to the β,δ-elimination product that was exclusively produced by the DMED treatment. The protonated form of DMED has pK_a values of 10.23 and 7.43. The value of one of the protonated amino groups of this compound is lower, due to the protonation of the other one. Therefore, about half of the molecules were singly-protonated at pH 7.4, and the free amine in this species was expected to abstract a proton in the elimination reaction.[19] Since the pK_a value of the conjugate acid of ethylamine is 10.63,[35] this amine is almost completely protonated at pH 7.4, and thus the elimination reaction must proceed less efficiently. It was also proposed that the positive ammonium group in the singly-protonated DMED might interact with the phosphate group in DNA.[19] However, based on our results, this interaction is not required, at least for the strand break.

To our surprise, the strand break was detected when the 30-mer oligonucleotide was treated with ammonium acetate (pH 7.4) or sodium phosphate (pH 7.0), although the 5′ fragment became a mixture of several species (Fig. 6B and 6C). In all of the reactions, the 3′ product was the same 5′-phosphorylated 16-mer oligonucleotide. These results suggested that the strand break was caused by thermal cleavage of the glycosidic bond at the 3′ component of the (6–4) photoproduct. The amines that contained a low-pK_a amino group, such as DMED, spermidine and spermine, probably facilitated the δ-elimination reaction, which resulted in the formation of a single-type 5′ fragment containing the structure shown in Fig. 3.

The glycosidic bond of the Dewar valence isomer is more alkali-labile than that of the parent (6–4) photoproduct,[14,18] but it was quite stable under the conditions used in this study (Fig. 7 and 8). These two types of photoproducts cannot be distinguished by the hot piperidine treatment, because the DNA strand is cleaved at both sites. However, only the (6–4) photoproduct, but not the Dewar isomer, is expected to yield fragment bands in a PAGE analysis after 5′-^{32}P-labeled UV-irradiated DNA is heated at 90 °C in 0.1 M sodium phosphate at pH 7.0.

Although the reaction rate is slow at 37 °C, the 3′ end of the 5′ fragment bearing the (6–4) photoproduct and a phosphate, shown in Fig. 3, blocks replication when the strand break found in this study occurs in cells. We demonstrated its removal by the ERCC1–XPF complex (Fig. 9), and RPA had a stimulatory effect on the processing of this novel blocking end. Since the (6–4) photoproduct is efficiently recognized by the NER proteins, it is repaired by the NER pathway in normal cells. However, this end processing may function in cells defective in the NER activities.

Experimental

Materials

Compound **1** was prepared as described previously,[36] using the intermediate in the synthesis of the oligonucleotide building block of the (6–4) photoproduct (compound **2** in ref. 20). A tetramer and a 30-mer containing the (6–4) photoproduct, d(AT(6–4)TG) and d(CTCGTCAGCATCT(6–4)TCATCATACAGTCAGTG), were synthesized using the dinucleotide-type building block, as described previously.[20] A 30-mer containing the Dewar valence isomer of the (6–4) photoproduct, in the same sequence as above, was synthesized in a similar manner.[23] The ERCC1–XPF complex and RPA were prepared according to the methods described previously.[26,37]

Amine treatment

The tetramer containing the (6–4) photoproduct (3.5 nmol) was dissolved in a 0.1 M solution (50 μL) of DMED–AcOH (pH 7.4), and this mixture was heated at 90 °C for 6 h. The HPLC analysis was performed on a Gilson gradient-type analytical system equipped with a Waters 2996 photodiode array detector, using a Waters μBondasphere C18 5 μm 300 Å column (3.9 × 150 mm) with a linear gradient of acetonitrile (from 0 to 10% for 20 min) in 0.1 M triethylammonium acetate (TEAA, pH 7.0), at a flow rate of 1.0 mL min^{-1}.

For the NMR study, **1** (0.1 mmol) was dissolved in the above solution (10 mL), and the mixture was heated at 90 °C under a reflux condenser. The reaction was monitored by reversed-phase HPLC, using a GL Science Inertsil ODS-3 5 μm column (4.6 × 250 mm) with a linear gradient of 0–7.5% acetonitrile in 0.1 M TEAA (pH 7.0). After 8 hours, the mixture was concentrated on a rotary evaporator equipped with a vacuum pump, and the residue was dissolved in water (1 mL). The product (**4**) was purified by HPLC, and the cation was exchanged to Na^+ using a Bio-Rad AG 50W-X2 resin. The structure of **4** was determined by NMR spectroscopy and mass spectrometry. The NMR measurement revealed that the sample was contaminated with sodium acetate, which yielded a signal with a chemical shift of 1.86 ppm in the ^{1}H NMR spectrum and signals with shifts of 184.2 and 26.1 ppm in the ^{13}C NMR spectrum. In the high-resolution mass analysis, the *m*/*z* value obtained for **4** was 449.1074 ($[M + H]^+$; calculated for $C_{15}H_{22}N_4O_{10}P$: 449.1074).

The 30-mer containing the (6–4) photoproduct or its Dewar valence isomer (1 nmol) was dissolved in 50 μL of each solution described in the figure legends, and the mixtures were heated at 90 °C for 6 h. For the HPLC analysis, a Waters μBondasphere C18 5 μm 300 Å column (3.9 × 150 mm) was used at 60 °C, with a 2.5 to 12.5% acetonitrile gradient over 20 min. The product (peak v in Fig. 4A) was purified under the same conditions, and was characterized by MALDI-TOF mass spectrometry ($[M - H]^-$ *m*/*z* 4091.42; calculated for $C_{130}H_{167}N_{45}O_{83}P_{13}$: 4088.68). To determine the reaction rates, the 30-mer was treated with 0.1 M spermidine–AcOH (pH 7.4) at 90, 85, 80, 75, and 70 °C, and the product yields were obtained from the peak areas, using the molecular extinction

coefficients calculated by the reported method.[38] The rate constant at 37 °C was estimated from the Arrhenius plot (Fig. 5).

NMR measurement of 4

^{1}H, ^{13}C, and ^{31}P NMR spectra were measured at 30 °C on a Varian Unity-INOVA 500 spectrometer. The ^{1}H chemical shift was calibrated with internal HDO (4.70 ppm) in D_2O. In the ^{13}C measurement, the ^{13}C reference frequency was obtained by calculation from the ^{1}H reference frequency, as reported previously.[14] The ^{31}P chemical shift was calibrated with external trimethyl phosphate. Two-dimensional NMR spectra were recorded on a 5 mm pulse field gradient probe for indirect detection. For the NOESY measurement, the mixing time was set to 700 ms.

^{1}H NMR (500 MHz) δ (ppm): 7.79 (s, 1H, pT-H6), 6.25 (dd, J(H,H) = 5.2, 9.7 Hz, 1H, H1′), 5.06 (s, 1H, Tp-H6), 4.52 (m, 1H, H3′), 4.05 (m, 1H, H4′), 3.75 (m, 2H, H5′), 2.18 (s, 3H, pT-CH_3), 1.92 (dd, J(H,H) = 5.2, 13.7 Hz, 1H, H2′), 1.68 (s, 3H, Tp-CH_3), 1.55 (m, 1H, H2″). ^{13}C NMR (125 MHz) δ (ppm): 178.2 (pT-C4), 177.1 (Tp-C4), 161.0 (pT-C2), 156.9 (Tp-C2), 149.1 (pT-C6), 118.6 (pT-C5), 87.7 (d, J(C,P) = 4.6 Hz, C4′), 87.2 (C1′), 77.1 (d, J(C,P) = 4.8 Hz, C3′), 74.9 (Tp-C5), 64.6 (C5′), 60.6 (Tp-C6), 38.2 (C2′), 28.0 (Tp-CH_3), 16.3 (pT-CH_3). ^{31}P NMR (202 MHz) δ: −0.468 ppm.

Photoproducts formed at the TC sequence

A 40 μM aqueous solution (12.7 mL) of a tetramer, d(ATCG), was placed in a Petri dish with an internal diameter of 9 cm, and was irradiated at a total 254 nm UV dose of 15 J cm^{-2} in an ice bath on a SpectroLinker XL-1500 UV crosslinker (Spectronics Corporation). The (6–4) photoproduct-containing tetramer with absorption maxima at 256 and 317 nm was purified by HPLC, using a Waters μBondasphere C18 5 μm 300 Å column (3.9 × 150 mm) with a linear gradient of acetonitrile (from 0 to 10% for 20 min) in 0.1 M TEAA (pH 7.0). TEAA was removed by evaporation and repeated coevaporation with water. Aliquots of the (6–4) photoproduct-containing tetramer (11.6 nmol) were dissolved in water (8.9 mL) and irradiated in the same dish on an ice bath with a 450 W high-pressure mercury lamp (Ushio UM-452) through a Pyrex jacket for 5 h. A small amount of the starting material remaining after the irradiation was removed by HPLC purification.

Alkali degradation of the tetramers containing the (6–4) photoproduct and its Dewar valence isomer was analyzed by incubating the oligonucleotides (3 nmol) in 50 mM sodium phosphate (pH 10, 11, and 12; 100 μL) at 37 °C, followed by HPLC analysis at intervals of 24 h. Thymine was added as an internal standard for the quantification of 2′-deoxyguanosine 5′-phosphate produced by the strand break, and a GL Science Inertsil ODS-3 column (4.6 × 250 mm) was used in this case. Heat degradation of the tetramers at pH 7.0 was analyzed in the same manner as the DMED treatment of d(AT(6–4)TG).

Enzymatic reactions

The 5′ fragment produced from the (6–4) photoproduct-containing 30-mer (20 pmol) was incubated with T4 polynucleotide kinase (20 units) and [γ-^{32}P]ATP (0.93 MBq), in a buffer (10 μL) containing 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, and 5 mM dithiothreitol, at 37 °C for 10 min. After heating at 95 °C for 3 min, the ATP was removed by using a GE Healthcare MicroSpin G-25 column. The substrate shown in Fig. 9A was prepared by mixing the three strands (each 20 pmol) in water (33 μL), heating the solution at 85 °C for 2 min, and cooling it to 20 °C. This substrate (0.4 pmol) was incubated with ERCC1–XPF (0.2 pmol) and RPA (1 pmol in Fig. 9B, and 0.05–3.2 pmol in Fig. 9C), in a buffer (10 μL) containing 50 mM Tris-HCl (pH 8.0), 0.5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 0.1 mg mL^{-1} bovine serum albumin, at 30 °C for 90 min. A 96% formamide solution, containing 9.6 mM ethylenediaminetetraacetic acid (EDTA), 48 μg mL^{-1} bromophenol blue, and 48 μg mL^{-1} xylene cyanol FF (10 μL), was added to the mixture. After heating the solution at 95 °C for 5 min, the products were separated by electrophoresis on a 12.5% denaturing polyacrylamide gel, in a buffer containing 89 mM Tris base, 89 mM boric acid, and 2 mM EDTA, at 35 W for 75 min. The bands were detected with a GE Healthcare Typhoon FLA 7000 image analyzer.

Conclusions

The results of this study indicated that the glycosidic bond of the (6–4) photoproduct was heat-labile at neutral pH, and the reaction rate of the strand breakage was higher than those observed for cytosine deamination and depurination. Analyses of the chemical structure of the products revealed that the hydrolytic ring opening of the 5′ base, which was found for the alkali degradation, did not occur at pH 7.4. Several types of amines were effective in yielding the single product, probably by facilitating δ-elimination at the abasic site. On the other hand, the glycosidic bond of the Dewar valence isomer of this photoproduct was stable under the same conditions. The 3′-blocking end formed by the strand break could be removed by the ERCC1–XPF complex in the presence of RPA. These findings will extend our understanding of the properties of UV-induced DNA damage.

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