Thermodynamic Studies of the Hybridization Properties of Photolesions in DNA

Yoshie Fujiwara and Shigenori Iwai*

Department of Bioorganic Chemistry, Biomolecular Engineering Research Institute, 6-2-3 Furuedai, Suita, Osaka 565, Japan

Received August 12, 1996; Revised Manuscript Received November 25, 1996[⊗]

ABSTRACT: In order to gain insight into the mechanism of mutations induced by photolesions in DNA, the thermal melting curves of oligonucleotide duplexes containing the (6-4) photoproducts of TT and TC and the Dewar isomer were measured at varying concentrations, and the thermodynamic parameters were determined. The duplexes were designed to be used as models for template-primer systems. In the case of d(CA*XY*AGCACGAC)·d(GTCGTGCT*N*), in which *XY* = T(6-4)T, T(6-4)C, or T(Dewar)T and *N* = A, G, C, or T, the $-\Delta G°$ values were the largest when *N* = G, and the difference between *N* = G and *N* = C or T was 1.1, 0.8, and 0.5 kcal/mol at 25 °C for *XY* = T(6-4)T, T(6-4)C, and T(Dewar)T, respectively. These results provide evidence of base pair formation between the 3′-pyrimidone and the opposite guanine, which was proposed previously to explain the T → C transition induced at the 3′ side of the T(6-4)T photoproduct. This interaction was weakened by isomerization to the Dewar photoproduct, which would explain the lower mutation specificity. For d(CA*XY*AGCACGAC)·d(GTCGTGCT*GN*), no obvious differences were observed regarding the base opposite the 5′-pyrimidine of the photoproducts. In contrast to the tertiary structure, determined by NMR, of a duplex containing T(6-4)T in its center [Kim, J.-K., & Choi, B.-S. (1995) *Eur. J. Biochem. 228*, 849−854], base pair formation at the 5′-pyrimidine was not detected for any photoproduct used in this study, even when the base opposite the 3′-pyrimidone of T(6-4)T was changed to adenine.

Ultraviolet (UV)[1] light causes two major types of photolesions at dipyrimidine sites in DNA, namely, cis-syn cyclobutane pyrimidine dimers and pyrimidine(6-4)pyrimidone photoproducts (Taylor, 1995). The latter is converted to its Dewar valence isomer by absorbing UV-B light with wavelengths between 290 and 320 nm (Taylor & Cohrs, 1987). The tertiary structures of oligonucleotide duplexes containing a cis-syn thymine dimer have been studied by nuclear magnetic resonance (NMR) spectroscopy (Kemmink et al., 1987a,b; Taylor et al., 1990; Lee et al., 1994), and it was found that the formation of the cis-syn dimer induces only small distortions of the B-DNA structure, and that all base pairs are formed, although the hydrogen bonds of the A·T pairs at the thymine dimer site are weakened. The thermodynamic analysis also revealed that dimer formation had little influence on duplex stability (Taylor et al., 1990). In parallel with these results, mutation experiments using a single-stranded vector containing a cis-syn thymine dimer revealed that translesion synthesis was usually accurate in a *uvrA6* mutant of *Escherichia coli* (*E. coli*) under SOS conditions (Banerjee et al., 1988). In the case of the cis-syn cyclobutane dimer at the thymine-cytosine site, deamination of the cytosine base occurs, which results in a C → T transition by faithful execution of the replicative bypass (Tessman et al., 1992; Jiang & Taylor, 1993).

On the other hand, the (6-4) photoproduct and its Dewar isomer (Figure 1) are much more mutagenic. The bypass efficiencies and the mutation spectra were analyzed using vectors containing each photoproduct at a single site. In the absence of SOS induction, replication in repair-deficient *E. coli* was blocked almost entirely by either the (6-4) photoproduct or its Dewar isomer, and in SOS-induced cells, the T → C transition was observed with an extremely high frequency at the 3′-pyrimidone of the (6-4) photoproduct of thymidylyl(3′−5′)thymidine [T(6-4)T] (LeClerc et al., 1991; Smith et al., 1996), although the mutation spectra depended upon the host species (Gibbs et al., 1995; Gentil et al., 1996) or the flanking sequences (Smith et al., 1996) to some extent. In a similar experiment using a vector containing the (6-4) photoproduct of thymidylyl(3′−5′)deoxycytidine [T(6-4)C], the predominant mutation was the 3′ C → T transition, although the frequency was low (Horsfall & Lawrence,

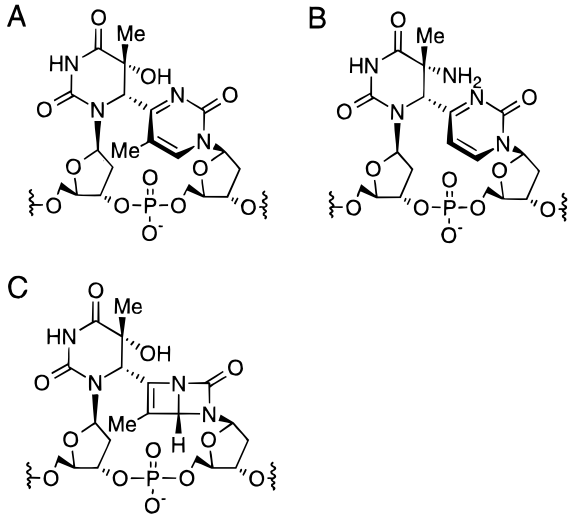

FIGURE 1: Structures of the (6-4) photoproducts of TT (A) and TC (B), and the Dewar valence isomer of A (C). The tertiary structures of these photoproducts have been reported by Rycyna and Alderfer (1985) and Taylor et al. (1988a,b).

* Author to whom correspondence should be addressed. Phone: +81-6-872-8208. Fax: +81-6-872-8219. E-mail: iwai@bioorg.beri.co.jp.

[⊗] Abstract published in *Advance ACS Abstracts,* January 15, 1997.

[1] Abbreviations: UV, ultraviolet; NMR, nuclear magnetic resonance; T(6-4)T, (6-4) photoproduct of thymidylyl(3′−5′)thymidine; T(6-4)C, (6-4) photoproduct of thymidylyl(3′−5′)deoxycytidine; T(Dewar)T, Dewar isomer of T(6-4)T; T(Dewar)C, Dewar isomer of T(6-4)C; HPLC, high-performance liquid chromatography.

S0006-2960(96)01994-0 CCC: $14.00

1994). The Dewar valence isomers of T(6-4)T and T(6-4)C [T(Dewar)T and T(Dewar)C, respectively] had lower mutation frequencies and specificities than the (6-4) photoproducts (LeClerc et al., 1991; Horsfall & Lawrence, 1994; Smith et al., 1996).

The tertiary structures of T(6-4)T (Rycyna & Alderfer, 1985; Taylor et al., 1988b), T(Dewar)T (Taylor et al., 1988a), and oligonucleotide duplexes containing these photoproducts (Kim & Choi, 1995; Kim et al., 1995; Hwang et al., 1996) have been determined using NMR data. In T(6-4)T, the 5′-pyrimidine and 3′-pyrimidone rings are perpendicular to each other, and the glycosyl bonds of the 5′-pyrimidine and the 3′-pyrimidone are in anti and high-anti conformations, respectively. The sugar conformations are also distorted from those of B-form DNA. An NMR study of a decamer duplex, d(CGCAT(6-4)TACGC)·d(GCGTAATGCG), revealed that the overall DNA helix is bent by 44°, and that hydrogen bonds are not formed between the 3′-pyrimidone and the opposite adenine base (Kim & Choi, 1995). From this NMR structure, the T → C transition at the 3′ site of T(6-4)T was attributed to the loss of the hydrogen bonding interaction at this site (Kim et al., 1995; Smith et al., 1996), whereas Lawrence and his co-workers proposed hydrogen bonds between the 3′-pyrimidone and guanine, using N3 and O2 of the pyrimidone base, to explain the 3′ T → C transition induced by the (6-4) photoproduct (LeClerc et al., 1991). Although the pyrimidone ring in the (6-4) photoproduct is flat, its counterpart in the Dewar isomer is bent at the N3−C6 bond. A model structure of a duplex containing the Dewar photoproduct was presented, which was more similar to the B-form geometry than that containing the (6-4) photoproduct (Taylor et al., 1988a), and it was suggested in an NMR study that the hydrogen bonds are lost at both sites of the Dewar photoproduct in a duplex (Hwang et al., 1996).

In spite of these structural studies, the mechanisms of mutations induced by these photoproducts have not been elucidated in detail. In this article, we describe thermodynamic analyses of base pair formation in oligonucleotide duplexes containing either the (6-4) photoproduct or its Dewar valence isomer. We undertook this study since the synthetic method using a dimer building block (Iwai et al., 1996) enabled us to prepare these oligonucleotides on the scale required for physicochemical experiments. For thermodynamic studies, the target base pairs, e.g., those containing a damaged base, are usually placed in the center of a duplex (Plum & Breslauer, 1994), but we used oligonucleotide duplexes in which the photoproducts were located at the 5′ end of the double-stranded region, as shown in Figure 2, as a model for template-primer systems. Using the thermodynamic parameters obtained in this study, mutation mechanisms are discussed.

EXPERIMENTAL PROCEDURES

Preparation of Oligonucleotides. The dodecanucleotide containing the (6-4) photoproduct of TT, d(CAT(6-4)-TAGCACGAC), was synthesized by the recently reported method using the dinucleotide building block (Iwai et al., 1996). Starting from 1.2 μmol, in total, of the 3′-terminal deoxycytidine on a polymer support, 38.5 A_{260} units of the 12-mer, with UV absorption maxima at 256 and 328 nm, was obtained after purification by high-performance liquid chromatography (HPLC). Elution profiles of the crude and purified 12-mers are shown in Figure 3A and Figure 3B, respectively. A Waters μBondasphere 5 μm C18 300 Å column (3.9 mm × 150 mm) was used with a linear gradient of acetonitrile (from 7 to 13% during 20 min) in 0.1 M triethylammonium acetate buffer (pH 7.0) at a flow rate of 1.0 mL/min. An aliquot (24.0 A_{260} units) of the T(6-4)T 12-mer was dissolved in distilled water (1.0 L), cooled in an ice bath, and irradiated for 40 min in a 1 L capacity Pyrex immersion well apparatus fitted with a 450 W high-pressure mercury lamp (Ushio UM-452). After concentration, the product without UV absorption in the long-wavelength region [the T(Dewar)T 12-mer], whose elution profile is shown in Figure 3C, was purified by HPLC under the same conditions as the T(6-4)T 12-mer. The yield was 14.9 A_{260} units. For the dodecanucleotide containing the (6-4) photoproduct of TC, d(CAT(6-4)CAGCACGAC), a 12 μM solution (75.6 mL) of the parent 12-mer, d(CATCAGCACGAC), was placed in six petri dishes, with internal diameters of 9 cm, after nitrogen purge, and was irradiated at a total 254 nm UV dose of 10 J/cm^2 in an ice bath on a Funakoshi FS-1500 UV cross-linker. After concentration, the reaction mixture was analyzed by HPLC under the same conditions as the T(6-4)T 12-mer, as shown in Figure 4A, and the product with UV absorption maxima at 256 and 314 nm [the T(6-4)C 12-mer] was isolated. The yield was 19.0 A_{260} units.

Measurement of Melting Curves. The concentrations of the oligonucleotide solutions were calculated using the equation and the coefficients described by Borer (1975). Since the molar extinction coefficients (ϵ) of the photoproducts used in this study were negligibly small at 260 nm, the sum of the ϵ values of d(CA) and d(AGCACGAC) was used for the photoproduct-containing 12-mers. Aqueous solutions (50−150 μL) containing the 12-mer (5.9 nmol) and the complementary 9- or 10-mer (5.9 nmol) were heated at 80 °C for 3−5 min and cooled gradually to room temperature. Each duplex was dissolved in a buffer (325 μL) containing 10 mM sodium phosphate, 100 mM sodium chloride, and 0.1 mM ethylenediaminetetraacetic acid (pH 7.0), at total oligonucleotide concentrations of 1.50, 2.67, 4.74, 8.43, and 15.0 μM. Melting curves (absorbance versus temperature profiles) were measured at 260 nm on a Beckman DU-600Tm spectrophotometer. The temperature was raised from 17 to 70 °C at a rate of 0.5 °C/min, and the T_m values were obtained by both the first-derivative and two-point average methods using the data processing software supplied by the manufacturer. In either method, the T_m values are hardly influenced by the rising base lines due to denaturation of the single-stranded oligonucleotides. Since reproducibility was sometimes unsatisfactory in the case of the first-derivative method probably due to the noises in the melting curves, the data sets by the two-point average method were used to determine the thermodynamic parameters.

Determination of Thermodynamic Parameters. The reciprocal of the melting temperature ($1/T_m$) was plotted against the common logarithm of the total oligonucleotide concentration [log (C_t)], as shown in Figure 5. Since the relationship between $\Delta H°$ and $\Delta S°$ is expressed with the equation, $1/T_m = (R/\Delta H°) \ln (C_t/4) + \Delta S°/\Delta H°$, in which R is the gas constant (=1.987 cal·mol^{-1}·deg^{-1}), for non-self-complementary duplexes, these values were calculated as follows: $\Delta H° = 2.303R/(\text{slope})$ and $\Delta S° = \Delta H°(y\text{-intercept}) + R \ln 4$ (Marky & Breslauer, 1987). The free energy ($\Delta G°$) at temperature T (kelvin) was calculated using the equation $\Delta G°$

Duplex TT-N	5′	C A T T A G C A C G A C	3′
	3′	N T C G T G C T G	5′
Duplex TC-N	5′	C A T C A G C A C G A C	3′
	3′	N T C G T G C T G	5′
Duplex T(6-4)T-N	5′	C A (6-4) T T A G C A C G A C	3′
	3′	N T C G T G C T G	5′
Duplex T(6-4)C-N	5′	C A (6-4) T C A G C A C G A C	3′
	3′	N T C G T G C T G	5′
Duplex T(Dewar)T-N	5′	C A (Dewar) T T A G C A C G A C	3′
	3′	N T C G T G C T G	5′
Duplex TT-AN	5′	C A T T A G C A C G A C	3′
	3′	N A T C G T G C T G	5′
Duplex TC-GN	5′	C A T C A G C A C G A C	3′
	3′	N G T C G T G C T G	5′
Duplex T(6-4)T-GN	5′	C A (6-4) T T A G C A C G A C	3′
	3′	N G T C G T G C T G	5′
Duplex T(6-4)T-AN	5′	C A (6-4) T T A G C A C G A C	3′
	3′	N A T C G T G C T G	5′
Duplex T(6-4)C-GN	5′	C A (6-4) T C A G C A C G A C	3′
	3′	N G T C G T G C T G	5′
Duplex T(Dewar)T-GN	5′	C A (Dewar) T T A G C A C G A C	3′
	3′	N G T C G T G C T G	5′

FIGURE 2: Designations and sequences of oligonucleotide duplexes used in this study. N represents A, G, C, or T.

$= \Delta H° - T\Delta S°$. The error analysis was carried out as described previously (SantaLucia et al., 1991; Persmark & Guengerich, 1994).

RESULTS

Oligonucleotides Containing a Photolesion at a Single Site. The oligonucleotide duplexes designed for the determinations of the thermodynamic parameters are shown in Figure 2, along with their designations. The photolesions were placed at the 5′ end of the double-stranded region of each duplex, to model a damaged template and its complementary strand elongated by a DNA polymerase. The photolesions analyzed in this study were T(6-4)T, T(6-4)C, and T(Dewar)T (Figure 1), and the 5′ and 3′ components of these photodimers were paired opposite each of the four normal bases in the complementary strand. Since the duplex was most stable when the 3′-pyrimidone of T(6-4)T was opposite guanine [T(6-4)T-G], as described below, this sequence was elongated for the analysis of the hybridization properties of the 5′-pyrimidine component [T(6-4)T-GN]. A set of duplexes containing adenine at this site [T(6-4)T-AN] was used for comparison.

The 12-mer containing the (6-4) photoproduct of TT, d(CAT(6-4)TAGCACGAC), was synthesized using the recently developed dinucleotide building block (Iwai et al., 1996). After deprotection, the crude sample was analyzed by reversed-phase HPLC, as shown in Figure 3A, and the main peak, with UV absorption maxima at 256 and 328 nm, was isolated (Figure 3B). Since this method afforded the oligonucleotide containing the (6-4) photoproduct on a large scale, more than half of the obtained T(6-4)T 12-mer was converted to its Dewar valence isomer, d(CAT(Dewar)-TAGCACGAC), by irradiation with Pyrex-filtered light. This conversion was completed quantitatively within 40 min, as

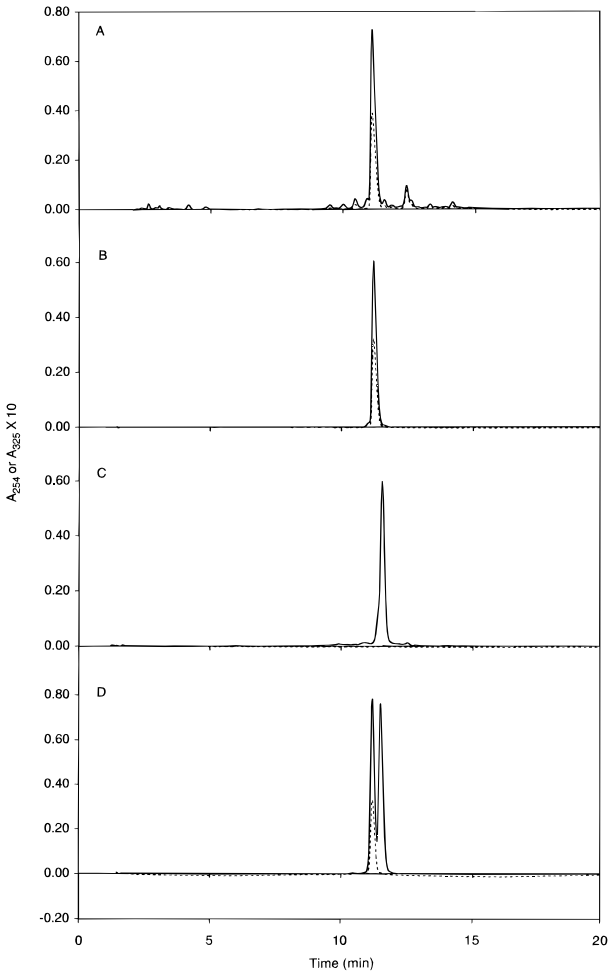

FIGURE 3: HPLC elution profiles of the crude T(6-4)T 12-mer (A), the purified T(6-4)T 12-mer (B), the crude T(Dewar)T 12-mer (C), and the purified T(Dewar)T 12-mer coinjected with the T(6-4)T 12-mer (D). The ordinates of the chromatograms monitored at 325 nm (shown by broken lines), which is specific to the (6-4) photoproduct, were magnified by a factor of 10.

shown in Figure 3C. Since small amounts of impurities were detected, the main peak, without UV absorption in the long-wavelength region, was isolated, and the separation from the (6-4) 12-mer was confirmed by coinjection onto an HPLC column, as shown in Figure 3D.

Since synthetic methods for a building block of the (6-4) photoproduct of TC have not been developed, due to the requirement of a protecting group for the amino group of cytosine, the T(6-4)C 12-mer, d(CAT(6-4)CAGCACGAC), was prepared by UV irradiation of the parent oligonucleotide, d(CATCAGCACGAC), by a procedure similar to that described by Smith and Taylor (1993). The result is shown in Figure 4A. The product with a retention time of 14.2 min had an extra UV absorption maximum at 314 nm, which is a characteristic of the (6-4) photoproduct (Franklin et al., 1982). After HPLC purification of this product, as shown in Figure 4B, a small aliquot was converted to the Dewar isomer. As the T(Dewar)C 12-mer was separated from the T(6-4)C 12-mer by our HPLC system, as shown in Figure 4C, it was confirmed that the purified T(6-4)C 12-mer (Figure 4B) was not contaminated by the Dewar isomer.

Determination of T_m Values by Measuring Melting Curves. The oligonucleotides containing the photolesions were annealed to complementary strands containing each of the four nucleosides at the 3′ end, as shown in Figure 2. Duplexes

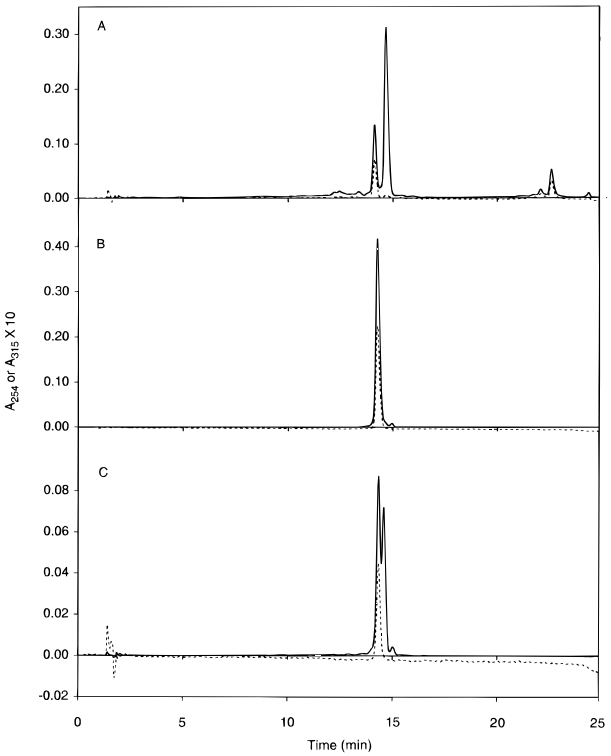

FIGURE 4: HPLC elution profiles of the UV-irradiated TC 12-mer (A), the purified T(6-4)C 12-mer (B), and the T(6-4)C 12-mer coinjected with the T(Dewar)C 12-mer (C). The chromatograms monitored at 315 nm (broken lines) were magnified by a factor of 10.

Table 1: Thermodynamic Parameters of Duplex Formation of the T(6-4)T Photoproduct

duplex	T_m (°C)[a]	$\Delta H°$ (kcal·mol^{-1})	$\Delta S°$ (cal·mol^{-1}·deg^{-1})	$\Delta G°$ (kcal·mol^{-1})[b]
TT-A	43.7	−65.3 ± 1.03	−181.0 ± 3.3	−11.28 ± 0.05
TT-G	42.2	−62.9 ± 0.31	−174.4 ± 1.0	−10.87 ± 0.01
TT-C	41.3	−51.9 ± 0.67	−140.1 ± 2.2	−10.13 ± 0.03
TT-T	41.5	−53.3 ± 0.43	−144.4 ± 1.4	−10.27 ± 0.02
T(6-4)T-A	40.9	−51.5 ± 0.50	−139.2 ± 1.6	−10.05 ± 0.02
T(6-4)T-G	41.7	−59.2 ± 0.79	−163.0 ± 2.5	−10.65 ± 0.03
T(6-4)T-C	39.1	−49.3 ± 0.54	−132.8 ± 1.8	−9.72 ± 0.02
T(6-4)T-T	38.9	−46.7 ± 0.60	−124.5 ± 1.9	−9.58 ± 0.02
TT-AA	45.8	−72.7 ± 0.45	−203.0 ± 1.4	−12.18 ± 0.03
TT-AG	43.6	−72.1 ± 0.33	−202.8 ± 1.0	−11.68 ± 0.02
TT-AC	42.4	−64.4 ± 0.31	−179.2 ± 1.0	−10.98 ± 0.01
TT-AT	43.4	−69.3 ± 0.36	−194.2 ± 1.1	−11.43 ± 0.02
T(6-4)T-GA	40.6	−57.0 ± 0.57	−156.5 ± 1.8	−10.31 ± 0.02
T(6-4)T-GG	41.9	−60.9 ± 0.32	−168.7 ± 1.0	−10.65 ± 0.01
T(6-4)T-GC	41.5	−61.8 ± 0.60	−171.7 ± 1.9	−10.64 ± 0.02
T(6-4)T-GT	41.8	−63.7 ± 0.79	−177.4 ± 2.5	−10.76 ± 0.03
T(6-4)T-AA	39.0	−54.5 ± 0.52	−150.1 ± 1.7	−9.79 ± 0.02
T(6-4)T-AG	39.3	−57.0 ± 0.30	−157.7 ± 1.0	−9.97 ± 0.01
T(6-4)T-AC	38.3	−59.2 ± 0.44	−165.3 ± 1.4	−9.94 ± 0.01
T(6-4)T-AT	39.0	−60.4 ± 0.34	−168.9 ± 1.1	−10.07 ± 0.01

[a] At the total strand concentration of 15.0 μM. [b] At 25 °C.

without any modifications of the base moieties were prepared in the same way. Thermal melting curves, monitored at 260 nm, were measured at total oligonucleotide concentrations varying between 1.50 and 15.0 μM. All curves were sigmoidal, and the T_m values were between 29 and 50 °C. The T_m values at the oligonucleotide concentration of 15.0 μM are listed in Tables 1, 2, and 3. In general, formation of the (6-4) photoproduct reduced the thermal stability of the duplexes [TT-N vs T(6-4)T-N, TC-N vs T(6-4)C-N, TT-AN vs T(6-4)T-AN, and TC-GN vs T(6-4)C-GN], but the

Table 2: Thermodynamic Parameters of Duplex Formation of the T(6-4)C Photoproduct

duplex	T_m (°C)[a]	$\Delta H°$ (kcal·mol^{-1})	$\Delta S°$ (cal·mol^{-1}·deg^{-1})	$\Delta G°$ (kcal·mol^{-1})[b]
TC-A	43.0	−55.4 ± 0.85	−150.6 ± 2.7	−10.45 ± 0.04
TC-G	50.0	−65.6 ± 0.56	−178.3 ± 1.8	−12.39 ± 0.04
TC-C	41.3	−54.9 ± 0.44	−149.7 ± 1.4	−10.24 ± 0.02
TC-T	41.4	−64.3 ± 0.69	−179.8 ± 2.2	−10.68 ± 0.03
T(6-4)C-A	39.9	−67.7 ± 0.57	−191.5 ± 1.9	−10.57 ± 0.02
T(6-4)C-G	42.3	−70.6 ± 0.42	−198.9 ± 1.4	−11.26 ± 0.02
T(6-4)C-C	38.2	−71.0 ± 0.35	−203.2 ± 1.1	−10.44 ± 0.01
T(6-4)C-T	37.1	−84.0 ± 0.44	−245.7 ± 1.4	−10.72 ± 0.01
TC-GA	51.3	−74.2 ± 0.77	−203.7 ± 2.4	−13.42 ± 0.06
TC-GG	49.9	−61.3 ± 0.43	−165.0 ± 1.4	−12.14 ± 0.03
TC-GC	49.2	−54.2 ± 0.47	−143.6 ± 1.5	−11.43 ± 0.03
TC-GT	50.1	−59.7 ± 0.56	−160.1 ± 1.8	−11.96 ± 0.04
T(6-4)C-GA	39.8	−65.3 ± 0.39	−183.6 ± 1.2	−10.51 ± 0.01
T(6-4)C-GG	41.8	−60.9 ± 0.14	−168.6 ± 0.5	−10.64 ± 0.01
T(6-4)C-GC	41.5	−64.0 ± 0.07	−178.6 ± 0.2	−10.76 ± 0.00
T(6-4)C-GT	41.6	−65.2 ± 0.32	−182.3 ± 1.0	−10.82 ± 0.01

[a] At the total strand concentration of 15.0 μM. [b] At 25 °C.

Table 3: Thermodynamic Parameters of Duplex Formation of the T(Dewar)T Photoproduct

duplex	T_m (°C)[a]	$\Delta H°$ (kcal·mol^{-1})	$\Delta S°$ (cal·mol^{-1}·deg^{-1})	$\Delta G°$ (kcal·mol^{-1})[b]
T(Dewar)T-A	40.6	−61.8 ± 0.45	−172.3 ± 1.4	−10.41 ± 0.02
T(Dewar)T-G	41.4	−63.2 ± 0.20	−176.2 ± 0.6	−10.69 ± 0.01
T(Dewar)T-C	39.5	−59.3 ± 0.14	−164.9 ± 0.5	−10.17 ± 0.01
T(Dewar)T-T	40.4	−58.7 ± 0.26	−162.3 ± 0.8	−10.27 ± 0.01
T(Dewar)T-GA	39.4	−58.6 ± 0.24	−162.7 ± 0.8	−10.08 ± 0.01
T(Dewar)T-GG	39.6	−62.9 ± 0.43	−176.1 ± 1.4	−10.35 ± 0.02
T(Dewar)T-GC	40.0	−52.5 ± 0.27	−142.8 ± 0.9	−9.90 ± 0.01
T(Dewar)T-GT	40.0	−53.8 ± 0.35	−147.0 ± 1.1	−10.00 ± 0.01

[a] At the total strand concentration of 15.0 μM. [b] At 25 °C.

duplexes containing deoxyguanosine opposite the 3′-pyrimidone of the (6-4) photoproduct [T(6-4)T-G, T(6-4)C-G, and T(6-4)T-GN] were comparatively stable. Consequently, the complementary strand containing deoxyguanosine at the 3′ end, d(GTCGTGCTG), was elongated with each of the four nucleosides, but further stabilization was not observed, regardless of the nucleoside opposite the 5′-pyrimidine component of each photoproduct [T(6-4)T-GN and T(6-4)C-GN]. In addition, no clear differences were found for the T(6-4)T-AN series. In the case of the Dewar isomer, the results were very similar to those obtained for the (6-4) photoproduct (Table 3). T(Dewar)T-G exhibited a higher T_m value than the other duplexes, and elongation of the complementary strand by one nucleotide [T(Dewar)T-GN] rather reduced the thermal stability. It should be noted that the samples were analyzed by HPLC after the measurements of the melting curves, in order to confirm that neither degradation nor photoisomerization, especially of the Dewar 12-mer, occurred during the spectrophotometric analysis.

Determination of the Thermodynamic Parameters of Duplex Formation. For a detailed investigation of the hybridization properties of DNA, a thermodynamic analysis is indispensable because the thermal stability does not necessarily correlate with the thermodynamic properties (Plum & Breslauer, 1994). Therefore, we determined the thermodynamic parameters for each duplex from the T_m data by the van't Hoff method, assuming a two-state model for duplex melting (Marky & Breslauer, 1987). This method has been used generally to study the thermodynamic stabilities of oligonucleotide duplexes containing modified bases

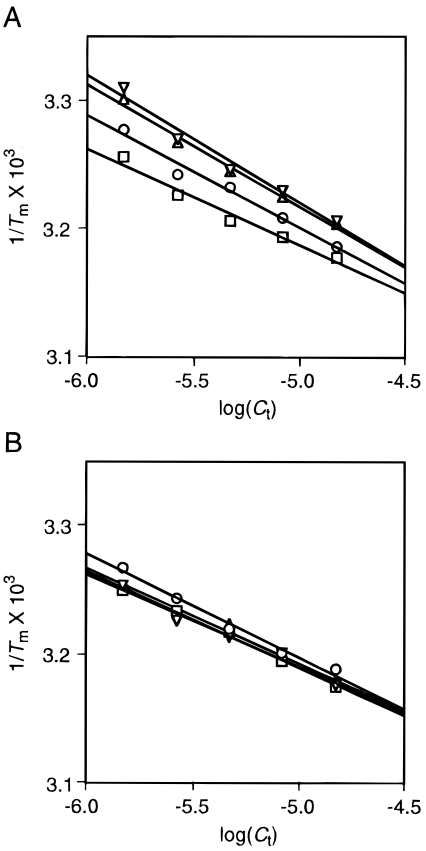

FIGURE 5: Reciprocal T_m versus log (C_t) plots for duplexes T(6-4)T-N (A: ○, N = A; □, N = G; △, N = C; ▽, N = T) and T(6-4)T-GN (B: ○, N = A; □, N = G; △, N = C; ▽, N = T).

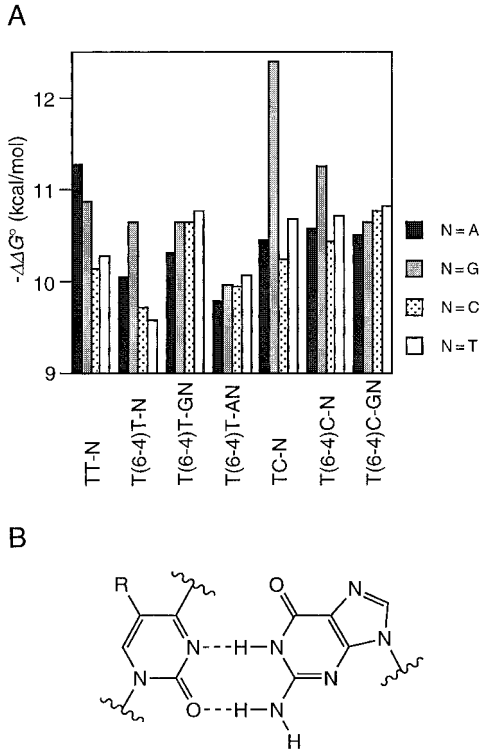

FIGURE 6: (A) Comparison of free energy increments at 25 °C. (B) Proposed hydrogen bonds between the 3′-pyrimidone of the (6-4) photoproduct and the opposite guanine. R represents CH_3 and H in T(6-4)T and T(6-4)C, respectively.

(Gaffney & Jones, 1989; Taylor et al., 1990; Plum et al., 1992; Persmark & Guengerich, 1994). Figure 5 shows the van't Hoff plots for the T(6-4)T-N and T(6-4)T-GN duplexes. The enthalpy ($\Delta H°$) and entropy ($\Delta S°$) parameters were derived from the slopes and the *y*-intercepts, respectively, of the least-squares linear regressions of these plots. The obtained thermodynamic parameters are listed in Tables 1, 2, and 3, along with the free energy at 25 °C ($\Delta G° = \Delta H° - T\Delta S°$, $T = 298.15$ K). In general, the variation in the thermodynamic stability represented by the $-\Delta G°$ values essentially paralleled that in the thermal stability (T_m). For both the (6-4) photoproducts and the Dewar isomer, the thermodynamic stability of the duplex was the largest when guanine was placed opposite the 3′-pyrimidone moiety of each photodimer [T(6-4)T-G, T(6-4)C-G, and T(Dewar)T-G], although in the case of the Dewar isomer the difference from the duplexes containing bases other than guanine at this position was smaller (0.28−0.52 kcal/mol). At the 5′-pyrimidine site, distinct stabilization by a specific base was not observed [T(6-4)T-GN, T(6-4)C-GN, and T(Dewar)T-GN], even when the nucleoside opposite the 3′-pyrimidone of T(6-4)T was changed into deoxyadenosine [T(6-4)T-AN].

DISCUSSION

Exposure of DNA to UV light yields highly mutagenic (6-4) photoproducts, as well as cyclobutane photodimers, at dipyrimidine sites. Although these photolesions are removed via the nucleotide excision repair pathway (Sibghat-Ullah et al., 1989; Wood, 1989) or repaired directly by (6-4) photolyase (Todo et al., 1993), failure of their repair induces mutations that cause cellular transformation and cell death. The major aim of this study was to find the molecular basis of such mutations. Since the synthetic method using the T(6-4)T building block (Iwai et al., 1996) provided oligonucleotides containing this photoproduct, on a scale sufficient for physicochemical experiments, we focused our study on photolesions with the TT sequence, and duplexes containing T(6-4)C were prepared for comparison. Using the oligonucleotide duplexes shown in Figure 2 as a model of replication, the possible mutations occurring at the 5′-pyrimidine and the 3′-pyrimidone of each photoproduct can be discussed separately.

Before discussion, it is necessary to examine whether the obtained thermodynamic parameters can be used for the further investigation on the hybridization properties of the photoproducts. In general, differences between the free energy changes upon dissociation ($\Delta\Delta G°$) are small when the target base pairs are placed at the end of a duplex. For example, these differences were only 0.2−0.4 kcal/mol at 37 °C for the terminal A·T pair in a 9 base pair duplex compared to mispairs, but it was reported that these differences were amplified in the complex with DNA polymerase (Petruska et al., 1988). In the present study, the $\Delta\Delta G°$ between the terminal T·A pair and the T·C or T·T mispair was about 1.0 kcal/mol with an experimental error of <0.1 kcal/mol at 25 °C. Therefore, it is concluded that this magnitude of differences in $\Delta\Delta G°$ can be used for discussion on base pair formation by the photoproducts.

For the 3′-pyrimidone side, clear results were obtained. The duplexes containing deoxyguanosine at this site [T(6-4)T-G, T(6-4)C-G, and T(Dewar)T-G] were thermodynamically more stable than the other duplexes. As shown in Figure 6A, the difference in the free energy ($\Delta\Delta G°$) between duplexes T(6-4)T-G and T(6-4)T-T was 1.07 ± 0.05 kcal/mol, which was the same, within experimental error, as that

between duplexes TT-A and TT-C (1.15 ± 0.08 kcal/mol). Incorporation of deoxyadenosine at this site [T(6-4)T-A] did not stabilize the duplex, and the $-\Delta G°$ value for this duplex was even smaller than those for the mismatched duplexes (TT-G, -C, and -T). These results strongly suggest that a stable base pair is formed between the 3′-pyrimidone of T(6-4)T and the opposite guanine base. There are two hydrogen acceptors in the pyrimidone ring, namely, N3 and the oxygen atom at C2, and the guanine base has two hydrogen donors, the imino proton at N1 and the exocyclic amino group at the C2 position. Therefore, two hydrogen bonds are proposed for this base pair, as shown in Figure 6B. This kind of base-pairing interaction was assumed previously to explain the T → C transition induced at the 3′-pyrimidone site of the T(6-4)T photoproduct (LeClerc et al., 1991). Experimental evidence for this base pair is presented in this study for the first time. A similar stabilization with guanine was observed for duplexes T(6-4)C-N and T(Dewar)T-N. Although the differences in the $\Delta G°$ values for T(6-4)C-G and the other T(6-4)C-N's were smaller (0.82 kcal/mol at most), duplex T(6-4)C-G was thermodynamically more stable than the mismatched duplexes (TC-A, -C, and -T). Since the 3′-pyrimidone of T(6-4)C differs from the counterpart of T(6-4)T only in the point that the latter has a methyl group at the C5 position, the same kind of base pairing can be proposed at this site. In comparison with the T(6-4)C-N series, duplexes T(6-4)T-N had smaller $-\Delta H°$ and $-\Delta G°$ values. This difference may be attributed to the methyl group present at the C5 position of T(6-4)T, which destabilizes the duplex due to steric hindrance, unless it is accommodated in an open space such as the major groove of DNA. In the case of T(Dewar)T-G, the $\Delta G°$ value was close to that for T(6-4)T-G, but its differences from those for the other T(Dewar)T-N's were very small (0.28−0.52 kcal/mol). Since the Dewar isomer has similar proton acceptors in its pyrimidone ring, the same types of hydrogen bonds can be expected. The thermodynamic parameters obtained in this study suggest that this base-pairing interaction is present, but it is weakened, probably because the ring bending induced unfavorable positioning of these acceptors.

In SOS-induced cells of repair-deficient *E. coli*, it has been reported that T → C transitions occur at the 3′-pyrimidone site of the T(6-4)T photoproduct (LeClerc et al., 1991; Smith et al., 1996). Although the error frequency depends upon the flanking sequences (Smith et al., 1996), the mutation specificity of 3′ T → C is extremely high (91−96%). As assumed in the previous study (LeClerc et al., 1991), this high specificity can be explained by the formation of a stable base pair between the 3′-pyrimidone moiety of T(6-4)T and the opposite guanine base shown in this study (Figure 6B). Although four components, the template strand, the extending strand, nucleoside triphosphates, and DNA polymerase, are involved in the actual replication, it is most likely, from the thermodynamic parameters obtained in the model system of this study, that deoxyguanosine is incorporated preferentially opposite the 3′-pyrimidone of T(6-4)T using the base pairing interaction. The accurate replication past the T(6-4)C photoproduct [71% in Horsfall and Lawrence (1994)] is also explained by this base pair formation. The accurate replication past T(6-4)T [11% in LeClerc et al. (1991); 27−68% in Smith et al. (1996)] and the 3′ C → T transition at T(6-4)C [28% in Horsfall and Lawrence (1994)] cannot be discussed with the thermodynamic data, because the stabilities of duplexes T(6-4)T-A and T(6-4)C-A were lower than and similar to those of the corresponding mismatch-containing duplexes, respectively. Since the bypass frequency past the (6-4) photoproduct is extremely low in the absence of SOS induction (LeClerc et al., 1991; Smith et al., 1996), the incorporation of deoxyadenosine in these cases may be attributed to the 'A-rule' (Strauss et al., 1982; Strauss, 1991). In the case of the Dewar photoproduct, both the mutation frequency and the specificity are low, but still the major mutation at the 3′-pyrimidone side is the T → C transition (LeClerc et al., 1991; Smith et al., 1996). These observations agree with our results that duplex T(Dewar)T-G was thermodynamically more stable than the other T(Dewar)T-N's, and that the difference was relatively small, as shown in Table 3. However, the low error frequency, i.e., the incorporation of deoxyadenosine at a higher rate at this site, may be the product of the A-rule.

As compared to the 3′ side, it is difficult to correlate the results obtained for the 5′-pyrimidines of the photoproducts in this study with the *in vivo* mutation spectra. Since there was no clear difference in the thermodynamic stabilities within T(6-4)T-GN, T(6-4)C-GN, or T(Dewar)T-GN, the 5′-pyrimidine bases seem to be completely noninstructional in our system. This may be a reasonable result when the 3′-pyrimidone forms a stable base pair with the opposite guanine, because the two rings composing the (6-4) photoproduct are perpendicular to each other, as shown previously (Rycyna & Alderfer, 1985; Taylor et al., 1988b). We tried to construct a tertiary structure model of duplex T(6-4)T-G with the 3′-pyrimidone·guanine pair, using the structures of canonical B-form DNA and T(6-4)T (Rycyna & Alderfer, 1985), and found that the 5′-pyrimidine ring was accommodated at the end of the major groove and covered the major groove side of the 3′-pyrimidone·guanine pair (data not shown). In an NMR solution structure of a 10-mer duplex containing the T(6-4)T photoproduct opposite the sequence of AA, the 5′-pyrimidine base retained the Watson−Crick hydrogen bonding with adenine, and the 3′-pyrimidone was distorted from the original position of thymine to maintain the perpendicularity of these two rings (Kim & Choi, 1995). Mutation mechanisms were discussed, based on this tertiary structure (Kim et al., 1995; Smith et al., 1996). Our structure containing the 3′-pyrimidone·guanine pair is obviously different from this NMR structure. Using the T(6-4)T-AN series, we examined whether the 5′-pyrimidine·adenine pair was formed when the base opposite the 3′-pyrimidone moiety was adenine, but such base pairing was not observed. Therefore, the difference between the NMR structure and ours must have arisen from the difference in the position of the photoproduct. Since the 10-mer duplex used in the NMR study contained the photoproduct in its center, the double-stranded region on the 5′ side of the photoproduct must have allowed the bending of the helix, which resulted in the base pair formation described above. Duplexes containing the photoproduct at the 5′ end of the double-stranded region, like ours, are more suitable models for the investigation of replication and mutation mechanisms.

At the 5′-pyrimidine site of the (6-4) photoproducts, accurate replication occurs predominantly (LeClerc et al., 1991; Horsfall & Lawrence, 1994; Smith et al., 1996). A possible interpretation for the difference between the observation of this accurate replication and our results showing a lack of distinct duplex stabilization by a specific nucleotide

is that the 5′-pyrimidine, which has the same hybridization properties as thymine in itself, can be utilized to incorporate deoxyadenosine in the ternary complex with DNA polymerase and dATP.

REFERENCES

Banerjee, S. K., Christensen, R. B., Lawrence, C. W., & LeClerc, J. E. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8141−8145.

Borer, P. N. (1975) in *Handbook of Biochemistry and Molecular Biology, 3rd Ed., Nucleic Acids* (Fasman, G. D., Ed.) Vol. 1, pp 589, CRC Press, Cleveland, OH.

Franklin, W. A., Lo, K. M., & Haseltine, W. A. (1982) *J. Biol. Chem. 257*, 13535−13543.

Gaffney, B. L., & Jones, R. A. (1989) *Biochemistry 28*, 5881−5889.

Gentil, A., Le Page, F., Margot, A., Lawrence, C. W., Borden, A., & Sarasin, A. (1996) *Nucleic Acids Res. 24*, 1837−1840.

Gibbs, P. E. M., Borden, A., & Lawrence, C. W. (1995) *Nucleic Acids Res. 23*, 1919−1922.

Horsfall, M. J., & Lawrence, C. W. (1994) *J. Mol. Biol. 235*, 465−471.

Hwang, G.-S., Kim, J.-K., & Choi, B.-S. (1996) *Eur. J. Biochem. 235*, 359−365.

Iwai, S., Shimizu, M., Kamiya, H., & Ohtsuka, E. (1996) *J. Am. Chem. Soc. 118*, 7642−7643.

Jiang, N., & Taylor, J.-S. (1993) *Biochemistry 32*, 472−481.

Kemmink, J., Boelens, R., Koning, T. M. G., Kaptein, R., van der Marel, G. A., & van Boom, J. H. (1987a) *Eur. J. Biochem. 162*, 37−43.

Kemmink, J., Boelens, R., Koning, T., van der Marel, G. A., van Boom, J. H., & Kaptein, R. (1987b) *Nucleic Acids Res. 15*, 4645−4653.

Kim, J.-K., & Choi, B.-S. (1995) *Eur. J. Biochem. 228*, 849−854.

Kim, J.-K., Patel, D., & Choi, B.-S. (1995) *Photochem. Photobiol. 62*, 44−50.

LeClerc, J. E., Borden, A., & Lawrence, C. W. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 9685−9689.

Lee, B. J., Sakashita, H., Ohkubo, T., Ikehara, M., Doi, T., Morikawa, K., Kyogoku, Y., Osafune, T., Iwai, S., & Ohtsuka, E. (1994) *Biochemistry 33*, 57−64.

Marky, L. A., & Breslauer, K. J. (1987) *Biopolymers 26*, 1601−1620.

Persmark, M., & Guengerich, F. P. (1994) *Biochemistry 33*, 8662−8672.

Petruska, J., Goodman, M. F., Boosalis, M. S., Sowers, L. C., Cheong, C., & Tinoco, I., Jr. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 6252−6256.

Plum, G. E., & Breslauer, K. J. (1994) *Ann. N.Y. Acad. Sci. 726*, 45−56.

Plum, G. E., Grollman, A. P., Johnson, F., & Breslauer, K. J. (1992) *Biochemistry 31*, 12096−12102.

Rycyna, R. E., & Alderfer, J. L. (1985) *Nucleic Acids Res. 13*, 5949−5963.

SantaLucia, J., Jr., Kierzek, R., & Turner, D. H. (1991) *J. Am. Chem. Soc. 113*, 4313−4322.

Sibghat-Ullah, Husain, I., Carlton, W., & Sancar, A. (1989) *Nucleic Acids Res. 17*, 4471−4484.

Smith, C. A., & Taylor, J.-S. (1993) *J. Biol. Chem. 268*, 11143−11151.

Smith, C. A., Wang, M., Jiang, N., Che, L., Zhao, X., & Taylor, J.-S. (1996) *Biochemistry 35*, 4146−4154.

Strauss, B. S. (1991) *BioEssays 13*, 79−84.

Strauss, B., Rabkin, S., Sagher, D., & Moore, P. (1982) *Biochimie 64*, 829−838.

Taylor, J.-S. (1995) *Pure Appl. Chem. 67*, 183−190.

Taylor, J.-S., & Cohrs, M. P. (1987) *J. Am. Chem. Soc. 109*, 2834−2835.

Taylor, J.-S., Garrett, D. S., & Cohrs, M. P. (1988a) *Biochemistry 27*, 7206−7215.

Taylor, J.-S., Garrett, D. S., & Wang, M. J. (1988b) *Biopolymers 27*, 1571−1593.

Taylor, J.-S., Garrett, D. S., Brockie, I. R., Svoboda, D. L., & Telser, J. (1990) *Biochemistry 29*, 8858−8866.

Tessman, I., Liu, S.-K., & Kennedy, M. A. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 1159−1163.

Todo, T., Takemori, H., Ryo, H., Ihara, M., Matsunaga, T., Nikaido, O., Sato, K., & Nomura, T. (1993) *Nature 361*, 371−374.

Wood, R. D. (1989) *Biochemistry 28*, 8287−8292.

BI9619942