^{31}P NMR study of the interactions between oligodeoxynucleotides containing (6-4) photoproduct and Fab fragments of monoclonal antibodies specific for (6-4) photoproduct

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Abstract A ^{31}P nuclear magnetic resonance (NMR) study of the interactions between oligonucleotides containing the (6-4) photoproduct and the Fab fragments of monoclonal antibodies (64M3 and 64M5) recognizing the (6-4) photoproduct is reported. The ^{31}P chemical shift data indicate that backbone conformation of (6-4) adduct is affected by the presence of flanking oligodeoxynucleotides, and (6-4) adducts with different backbone conformations are accommodated in the antigen binding sites of these antibodies. It was also revealed that epitopes for these antibodies consist of not only the (6-4) adduct but the flanking di- or tri-deoxynucleotides on both the 5′ and 3′ sides as well.

Key words: (6-4) Photoproduct; ^{31}P nuclear magnetic resonance; Antibody; Fab; DNA damage; Protein-nucleic acid interaction

1. Introduction

DNA damage, which is caused by ultraviolet light (UV), in cells leads to cell killing, mutation, and neoplastic transformation [1–3]. At adjacent pyrimidine sites, two major types of photoproducts, i.e. cyclobutane pyrimidine dimers and pyrimidine (6-4) pyrimidone photoproducts (Fig. 1), are formed. It has been reported that the (6-4) photoproduct is less tolerated in cellular genomes during replication and much more mutagenic than the cyclobutane dimer and therefore might play a major role in UV-induced DNA lesions [4,5].

Polyclonal and monoclonal antibodies specific to the cyclobutane or (6-4) photoproducts have been established to investigate the relationships between the generation of these UV-induced DNA lesions and their biological effects [6–13]. These antibodies have also been used as probes to characterize the conformations of isolated and duplex-embedded photoproducts [8,13]. In addition, it has been demonstrated that an antibody elicited against the *trans, syn* uracil cyclobutane dimer hapten catalyzes the light-dependent cleavage of the uracil dimer to normal dipyrimidine [14].

Nikaido and co-workers have established a variety of monoclonal antibodies that specifically bind to either cyclobutane or (6-4) pyrimidine dimers [9–11]. These monoclonal antibodies were elicited by immunization with UV-irradiated DNA, which is thought to contain heterogeneous antigenic determining sites, and were characterized using DNA exposed under controlled UV-irradiation conditions. These monoclonal antibodies have been widely utilized for the detection and quantitation of the DNA lesions and proved to be powerful tools for investigating cellular repair systems [15]. A detailed understanding of the mechanisms of antigen recognition of these monoclonal antibodies is required for their further application, especially as targets for antibody engineering aimed to improve their affinities and specificities and to endow them with catalytic activities for DNA repair.

In the previous papers, we have characterized a panel of anti-(6-4) photoproduct antibodies, 64M2 (IgG2a,κ), 64M3 (IgG2a,κ), and 64M5 (IgG1,κ), which were simultaneously established from the same BALB/c mouse [10]. The variable region genes from these antibodies have been cloned and sequenced [16]. It has been revealed that the sequences of the three antibodies are closely related to one another: 64M5 shares 91% and 94% identities of amino acid sequences in the variable regions with 64M2 and 64M3, respectively.

The binding affinity of 64M5 for the DNA containing (6-4) photoproduct is significantly higher than those of 64M2 and 64M3 [10]. It has been shown that the affinity of 64M5 is enhanced by the presence of oligonucleotides that flank the (6-4) adduct [17]. Similar tendencies of the affinity dependence upon the length of the DNA have been observed for other anti-DNA antibodies [18–21]. In order to gain a deeper insight into the mechanisms of molecular recognition of (6-4) photolesions by these monoclonal antibodies, it is essential to determine the binding mode of (6-4) adducts by themselves and also to clarify the contributions of the flanking oligodeoxynucleotides to their affinities based on structural aspects.

It is well known that ^{1}H-^{31}P and ^{13}C-^{31}P vicinal couplings provide us useful information on the conformation of the deoxyribose phosphate backbone of nucleotides [22–24]. However, the line-broadening of nuclear magnetic resonances (NMR), which are expected in the present anti-DNA antibodies system with molecular weight of over 50 kDa, seriously hampers the application of a strategy which heavily depends upon the ^{1}H-^{31}P and ^{13}C-^{31}P couplings.

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Abbreviations: DQF-COSY, double-quantum filtered correlated spectroscopy; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; HMBC, heteronuclear multiple-bond correlation; HSQC, heteronuclear single-quantum correlation; NOE, nuclear Overhauser effect; ROESY, rotating frame Overhauser effect spectroscopy; SPR, surface plasmon resonance; TOCSY, total correlation spectroscopy; d(T[6-4]T), pyrimidine (6-4) pyrimidone photoproduct of thymidylyl-(3′→5′)-thymidine; 2D, two-dimensional

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Here, on the basis of the chemical shift and linewidth data from ^{31}P NMR resonances, we report the epitope analysis results for 64M5 and 64M3. d(T[6-4]T) and d(GTAT[6-4]TATG) were chemically synthesized and then subjected to NMR analyses. ^{31}P resonances originating from the individual phosphate diesters of the backbone were assigned. On the basis of the perturbations of the ^{31}P chemical shift and linewidth upon binding to either the 64M5 or 64M3 Fab fragment, the epitope size and locus were determined.

2. Materials and methods

2.1. Preparation of oligonucleotides containing a (6-4) photoproduct

Oligonucleotides were synthesized using a DNA synthesizer (Applied Biosystems Model 394) and purified by HPLC. 3′-Biotinylated oligonucleotides were prepared by the same protocol on biotinylated CPG columns (Clontech, 3′Biotin-ON CPG). Oligonucleotides containing a (6-4) photoproduct were synthesized and characterized using procedures similar to those previously described [17,25,26].

2.2. Preparation of Fab fragments

The hybridoma cells were grown in NYSF 404 (Nissui) supplemented with 2% heat-inactivated FBS (JRH Biosciences) at 37°C in a humidified atmosphere of 5% CO_2/95% air. After cell growth, the supernatant was concentrated with a Millipore Minitan ultrafiltration system and then applied to an Affi-Gel protein A column (Bio-Rad). The purified 64M5 antibody was reduced by dithiothreitol and carboxymethylated, and then digested by papain as previously described [17,27]. Papain digestion of the 64M3 antibody was carried out with a modification of our previous protocol [27,28]. The purified 64M3 (4.3 mg/ml) was incubated with papain at pH 7.0, 37°C, in 75 mM sodium phosphate buffer containing 75 mM NaCl, 2 mM EDTA, and 11 μM cysteine for 8 h. The enzyme/substrate ratio (w/w) was 1:500. The reaction was terminated by the addition of 33 mM *N*-ethylmaleimide. Fab fragments were purified using a Mono Q anion exchange column (Pharmacia Biotech) as previously described [28].

2.3. Determination of binding constants of 64M3 and 64M5 for d(T[6-4]T)

For the fluorescence measurements, 64M3 and 64M5 Fab fragments were dissolved at concentrations of 1.0 μM and 30 nM, respectively, in 5 mM sodium phosphate buffer, pH 7.3, containing 200 mM NaCl and 50 μM EDTA. Emission spectra of the protein intrinsic fluorescence were recorded in the region of 310–350 nm upon excitation at 260 nm using a Shimadzu RF-5300PC spectrofluorometer. Excitation and emission band-passes were 5 and 10 nm, respectively. All measurements were carried out at 30°C. Fluorescence quenching at 334 nm upon the addition of d(T[6-4]T) was monitored and the binding constants of Fab for d(T[6-4]T) were determined from the slope of the Scatchard plot [29].

2.4. Dependency of affinity on ionic strength and chain length determined by surface plasmon resonance

Affinity of the Fab fragments for the (6-4) photoproduct-containing oligodeoxynucleotides was determined by surface plasmon resonance (SPR) measurements with a BIAcore 1000 or BIAcore 2000 instrument (Pharmacia Biotech) using the 3′-biotinylated d(AAT[6-4]TAA) and d(CAAT[6-4]TAAG) as previously described [17].

2.5. NMR measurements

For the NMR measurements, 0.2–2.3 μmol of the oligodeoxynucleotides was dissolved in 450 μl of 5 mM sodium phosphate buffer, pH 7.3, containing 200 mM NaCl, 50 μM EDTA and 3 mM NaN_3 in 90% ^1H_2O/10% ^2H_2O. ^{31}P NMR measurements were made on a Bruker AMX 400 spectrometer at 162 MHz. The probe temperature was 37°C. All spectra were recorded with the use of a WALTZ-16 composite pulse for ^1H decoupling. The free induction decay after a 40° pulse was recorded with a repetition period of 5.0 s, with 16 K data points and a spectral width of 2439 Hz. All free induction decays were multiplied by an exponential window function with a broadening factor of 1.0 Hz prior to the Fourier transformation. ^{31}P chemical shifts were given in parts per million (ppm) from the external trimethyl phosphate (0.00 ppm).

All 2D NMR spectra were recorded with spectral widths of 4800 Hz for ^1H and 1000 Hz for ^{31}P on a Bruker DRX400 spectrometer operating at ^1H frequency of 400 MHz. For the double-quantum filtered correlated spectroscopy (DQF-COSY), total correlation spectroscopy (TOCSY), and rotating frame Overhauser effect spectroscopy (ROESY) measurements, 2 K data points were used in the t_2 dimension, and 16 transients were acquired for each of the 512 t_1 points. The solvent resonance was suppressed by selective irradiation during the relaxation delay. The mixing times were set to 200 ms for TOCSY and to 800 ms for the ROESY measurements. For the ^1H-^{31}P heteronuclear single-quantum correlation (HSQC) and heteronuclear multiple-bond correlation (HMBC) measurements, 2 K data points were used in the t_2 dimension, and 16 transients were acquired for each of the 256 t_1 points. The solvent resonance was suppressed by using gradient pulses. A GARP composite pulse was used in the ^1H-^{31}P HSQC measurements for ^{31}P-decoupling. Prior to 2D Fourier transformation, the acquired data were zero-filled once along the t_1 direction and multiplied by a shifted sine square function in the t_1 and t_2 directions.

3. Results and discussion

3.1. Assignments of ^{31}P NMR resonances

Figs. 2A and 3A show the ^{31}P NMR spectra of d(T[6-4]T) and d(GTAT[6-4]TATG). The ^{31}P chemical shift of d(T[6-4]T) (−3.34 ppm) was virtually identical with that reported in the literature [30]. On the other hand, all of the ^{31}P resonances originating from d(GTAT[6-4]TATG) were shifted upfield (−4.13 to −3.65 ppm). Assignments of the ^{31}P resonances were accomplished through analyses of the ^1H-^{31}P HSQC and HMBC spectra along with DQF-COSY, TOCSY, and ROESY spectra via a sequential assignment methodology (spectra not shown). The established assignments of the ^{31}P resonances are shown in Fig. 3A, in which P_i represents the *i*th phosphorus in the phosphodiester bond between the *i*th and (*i*+1)th residues from the 5′ end.

The ^{31}P resonance originating from the (6-4) adduct (P4) overlaps those from P2 and P6 at −4.13 ppm. Thus the ^{31}P chemical shift of the (6-4) adduct in d(T[6-4]T) is significantly different from that in d(GTAT[6-4]TATG). Gorenstein and his co-workers have extensively studied the origins of variations in the ^{31}P chemical shifts of a variety of phosphorous compounds including DNA oligomers [31–35]. On the basis of the molecular orbital calculations and enormous coupling constant data, they have concluded that the variations in the ^{31}P chemical shifts of individual phosphate diesters in oligonucleotides are attributed to differences in the torsional angles (α, β, ε, and ζ) and the O–P–O bond angle in the deoxyribose phosphate backbone and that the ring current effects and other environmental effects, such as the formation of the hydrogen bond and salt bridge, upon the ^{31}P chemical

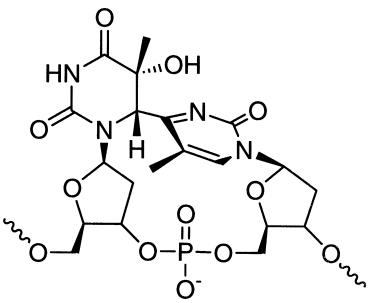

Fig. 1. Structure of d(T[6-4]T).

shifts in the nucleic acids are generally smaller than the intrinsic conformational factors.

As shown in Figs. 2A and 3A, ^{31}P resonances of the (6-4) adduct for d(T[6-4]T) and d(GTAT[6-4]TATG) are observed at different positions. Therefore, it is suggested that conformation of the deoxyribose phosphate backbone of the (6-4) adduct is affected by the flanking oligodeoxynucleotides.

Models for the structure of d(T[6-4]T) in solution have been built via a conformational search procedure using NMR-derived distance- and angle-constraints [36]. Kim and Choi have reported a solution structure of the DNA duplex-decamer, d(CGCAT[6-4]TACGC)·d(GCGTAATGCG) obtained by NOE distance and relaxation matrix refinements [37]. It was also concluded that the solution structure of the duplex-embedded (6-4) adduct exhibits a very similar overall structure compared to that of the isolated d(T[6-4]T) proposed by Taylor et al [36]. However, there exist significant differences in the torsional angles of the backbone between them. It is likely that the deoxyribose phosphate backbone of the (6-4) adduct can take different conformations in different contexts and those conformational differences might be sensitively reflected in the ^{31}P chemical shift.

3.2. Recognition of (6-4) adduct by Fab fragment

Upon the addition of either 64M5 or 64M3 Fab, the ^{31}P resonance of d(T[6-4]T) was slightly shifted downfield (Fig. 2B,C), indicating that no drastic change is induced on the backbone conformation of d(T[6-4]T) upon binding to these Fab fragments. Binding constants of the 64M3 and 64M5 Fab fragments for d(T[6-4]T) determined on the basis of the fluorescence quenching data were 1.8×10^5 M^{-1} and 5.9×10^7 M^{-1}, respectively. A significant broadness of the ^{31}P resonance was observed for the 64M3 Fab complex probably due to a faster exchange process between the free and bound forms which was a result of the lower affinity of 64M3 for d(T[6-4]T) than that of 64M5.

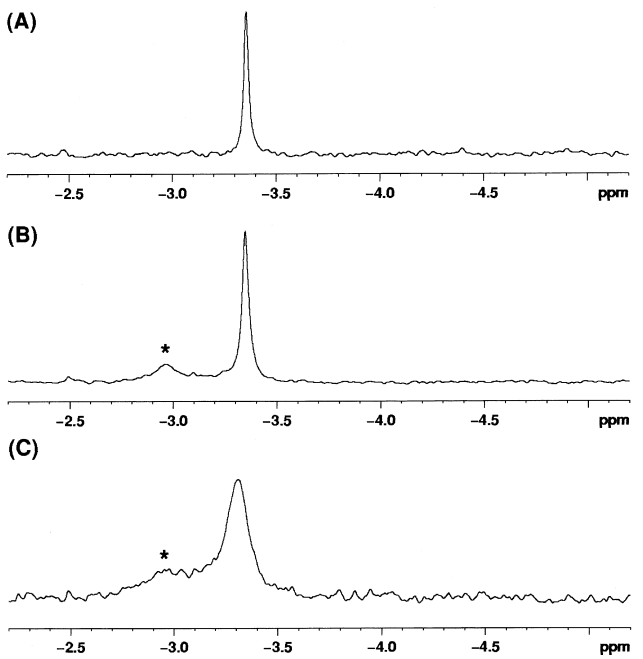

Fig. 2. ^{31}P NMR spectra of d(T[6-4]T) in the absence (A) and presence (B,C) of 0.5 molar equivalent of Fab (B: 64M5 Fab and C: 64M3 Fab). The concentrations of d(T[6-4]T) were (A) 4.5 mM and (B,C) 1.0 mM. In B and C, ^{31}P resonances originating from the Fab-bound forms of d(T[6-4]T) are indicated by asterisks.

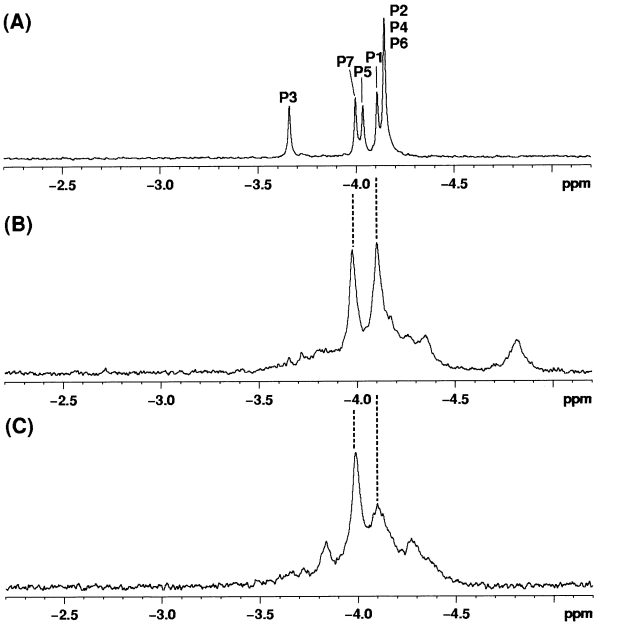

Fig. 3. ^{31}P NMR spectra of d(GTAT[6-4]TATG) in the absence (A) and presence (B,C) of 1.1 molar equivalent of Fab (B: 64M5 Fab and C: 64M3 Fab). The concentrations of d(T[6-4]T) were (A) 3.5 mM, (B,C) 0.4 mM. P_i represents the ith phosphorus in the phosphodiester bond between the ith and (i+1)th residues from the 5′ end.

On the other hand, all but P1 and P7 resonances originating from d(GTAT[6-4]TATG) were perturbed both in chemical shift and linewidth upon the addition of 64M5 Fab (Fig. 3B). This result indicates that the segment spanning P2–P6 is involved in the binding to 64M5 Fab but both of the 5′- and 3′-terminal nucleotides are not. Though the identification of the ^{31}P resonance (P4) from the (6-4) adduct of d(GTAT[6-4]TATG) in the 64M3 and 64M5 complexes is difficult due to incidental spectral overlaps and line-broadening, there is no doubt that no resonance is observed in Fig. 3B,C around the position corresponding to those of d(T[6-4]T) complexed with the Fab fragments (ca. −3 ppm). This observation indicates that the chemical shift of the (6-4) adduct in both the 64M3 and 64M5 complexes is quite different between d(T[6-4]T) and d(GTAT[6-4]TATG). On the basis of these results, it is concluded that (i) the backbone of the (6-4) adduct of d(T[6-4]T) in the complex takes a different conformation from that of the bound d(GTAT[6-4]TATG), and (ii) the (6-4) adducts with an altered backbone conformation can be accommodated in the antibody-combining sites of 64M3 and 64M5, probably due to the flexible nature of the combining sites of these antibodies.

The antibodies used in the present study recognize d(T[6-4]T) embedded in either the single- or double-stranded DNA [10,17]. Zhao and Taylor [13] have established monoclonal antibodies elicited by the isolated d(T[6-4]T) and showed that these antibodies are capable of binding to d(T[6-4]T) embedded in DNA. On the basis of these results, they have suggested that some of the conformational states of the d(T[6-4]T) in the dinucleotide and DNA are rather similar. However, the present NMR data indicate that the backbone conformation of d(T[6-4]T) can at least be altered by the presence

of the flanking sequences. We therefore point out that the conformational conservativeness of the (6-4) adduct probed by antibodies must be carefully interpreted because antibody-combining sites might adapt themselves to the (6-4) adducts exhibiting different conformations.

3.3. Contributions of the flanking oligodeoxynucleotides to antigen-antibody interactions

The results obtained in the present study showing that the regions containing P2, P3, P5, and P6 are also involved in the Fab binding are very consistent with our previous data that the dissociation rate constants (K_{diss}) of 64M5 Fab for d(T[6-4]T)-containing oligodeoxynucleotides are dependent upon the length of the sequences flanking the (6-4) adduct; longer oligodeoxynucleotides up to d6mer indicate a smaller K_{diss}, but the K_{diss} for the d8mer is virtually identical to that of the d6mer [17].

In a number of DNA-protein recognition systems, 'non-specific' electrostatic interactions significantly contribute to the binding affinities [38,39]. The contribution of electrostatic interactions has been proved or postulated for a variety of DNA-anti-DNA antibody systems [40–45]. On the basis of the results from the SPR measurement in the present study, it is concluded that the binding constant of 64M5 for the d8mer is strongly dependent upon ionic strength, i.e. 9.3×10^{9} M^{-1} and 9.2×10^{8} M^{-1} at NaCl concentrations of 10 mM and 1 M, respectively. We therefore suggest that the flanking region of the oligodeoxynucleotide containing the (6-4) adduct contributes to the binding to 64M3 and 64M5 through electrostatic interactions between the phosphate groups and basic amino acid side chains.

A significant upfield shifted resonance at −4.80 ppm is observed in the ^{31}P spectrum of d(GTAT[6-4]TATG) in the presence of 64M5 Fab (Fig. 3B), suggesting that torsional angles in the deoxyribose phosphate backbone in the flanking regions are altered upon binding to 64M5 Fab. Binding to 64M3 Fab also induced a chemical shift and linewidth changes for P2 to P6 resonances but the positions of the perturbed resonances were apparently different from those of 64M5 (Fig. 3C): the most remarkable example is the upfield peak at −4.80 ppm in the spectrum of the complex with 64M5 Fab (Fig. 2B), which is not observed for the complex with 64M3 Fab (Fig. 2C). In addition, binding to 64M3 Fab induced line-broadening of the P1 resonance, indicating that the 5′-terminal nucleotide is more restricted in motion in the complex with 64M3 than in the 64M5 complex. This indicates that (i) the backbone conformations of the flanking regions are different between the complexes with 64M3 and 64M5 Fab fragments, and (ii) the flanking region of the 5′ side is more extensively involved in 64M3 binding than in the case of 64M5 binding. The latter was supported by the data obtained from the SPR experiment showing that K_{diss} of 64M3 Fab for a d(T[6-4]T)-containing d8mer is still significantly smaller than that for the corresponding d6mer; the K_{diss} for d8mer is 6.5×10^{-4} s^{-1} and the K_{diss} for d6mer is 1.6×10^{-3} s^{-1}. On the basis of these data, we conclude that the contribution of non-specific interactions with the flanking oligodeoxynucleotides can be more significant for 64M3 Fab than for 64M5 Fab, while the affinity of 64M5 Fab for the (6-4) adduct in itself is much stronger than that of 64M3 Fab.

Although 64M5 and 64M3 share amino acid identities of 90% for the V_H and 97% for the V_L, there are three positions where substitution of a charged residue occurs: positions 99H, 50L, and 90L are occupied by Ser, Thr, and Arg in 64M5 respectively, whereas by Lys, Lys, and Gln in 64M3, respectively [16]. We suggest that differences in the charge distribution in the antibody-combining site between 64M3 and 64M5 result in a different fashion of stabilization of the flanking oligodeoxynucleotides. It is possible that the additional positive charges at 99H and/or 50L in 64M3 contribute to the interaction with the P1 phosphate group rendering the d8mer more stabilized in the complex than the d6mer. A stable-isotope-assisted NMR study has revealed that Ser-99H and Thr-50L are located in a peripheral region surrounding the (6-4) adduct-binding site of 64M5 Fab (manuscript in preparation). We propose that one possible way to achieve improvement of the affinity of 64M5 for antigenic DNA oligomers longer than d8mer is to introduce a basic amino acid residue at positions 50L and/or 99H.

The present ^{31}P NMR study has shown that epitopes for the 64M5 and 64M3 antibodies are constructed not only from the (6-4) adduct but also from the flanking di- or tri-deoxynucleotides on both the 5′ and 3′ sides. If the antigenic single-stranded DNA is bound to the antibodies in an extended form, the epitope sizes are obviously larger than that of antigen-combining sites so far reported for a variety of antigen-antibody systems [46]. In order to gain more knowledge on the binding mechanisms of these antibodies, it is necessary to obtain structural information on the antibody combining sites of these antibodies. NMR analyses using isotopically labeled Fab fragments of 64M3 and 64M5 are underway in our laboratory.

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