Nuclear Magnetic Resonance Study of the Interaction of T4 Endonuclease V with DNA

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Received September 3, 1993®

ABSTRACT: T4 endonuclease V catalyzes the DNA strand cleavage in the vicinity of a thymine dimer. In order to obtain insight into the specific recognition mechanism of this enzyme with a thymine photodimer within DNA, the conformations of five different DNA duplexes, DNA I, d(GCGGATGGCG)·d(CGCCTACCGC), DNA II, d(GCGGTTGGCG)·d(CGCCAACCGC), DNA III, d(GCGGTTGGCG)·d(CGCCAACCGC), DNA IV, d(GCGGGCGGCG)·d(CGCCCGCCGC), and DNA V, d(GCGGCCGGCG)·d(CGCCGGCCGC), with which the enzyme can interact, were studied by ^{1}H NMR. DNA I, DNA IV, and DNA V do not contain the TT sequence or a thymine dimer and hence, are expected to bind the enzyme only in a nonspecific manner. DNA II includes a single TT sequence which does not form a thymine dimer. Only DNA III is expected to bind specifically to the enzyme through a thymine photodimer. The NMR spectra of these five DNA duplexes in the absence of the enzyme clearly show that the formation of a thymine dimer within the DNA induces only a minor distortion in the structure and that the overall structure of B-type DNA is retained. The photodimer formation is found to cause a large change in chemical shifts at the GC7 base pair, which is located at the 3′-side of the thymine dimer, accompanied by the major conformational change at the thymine dimer site. The effects of T4 endonuclease V binding on these DNA duplexes were also investigated by ^{1}H NMR. The binding of this enzyme to DNA I, DNA IV, and DNA V causes no alternation in chemical shift values of the imino proton resonances, but the binding to DNA II induces a small downfield shift in the imino proton resonance of GC7. The binding of a T4 endonuclease V mutant, which lacks only the cleavage activity but retains the substrate-binding ability, to DNA III causes a strikingly large downfield shift in the imino proton resonance of GC7. Therefore, in addition to the TT moiety, this position should be either the crucial point for T4 endonuclease V recognition or the particular site of a conformational change which occurs by T4 endonuclease V binding. Photo-CIDNP experiments showed that three tyrosine residues, including tyrosine 129 located in the aromatic segment (WYKYY) near the C-terminus, lie on the molecular surface of T4 endonuclease V and that the access of the dye to these residues is completely obstructed when the enzyme binds, respectively, to the three DNA duplexes (I, II, III). These findings reveal that this aromatic segment is involved in the interaction with DNA in a TT specific or nonspecific manner. Using the wild-type and another mutant enzyme whose Arg 26 was replaced by Gln, the nonspecific binding of the enzyme with the three DNA duplexes (I–III) was investigated by observing the signal broadening of the NϵH signals of arginine side chains. The results reveal that Arg 26 is involved in nonspecific binding with the DNA duplexes presumably by electrostatic force.

The exposure of DNA to ultraviolet radiation produces cyclobutane-type pyrimidine dimers that are lethal and mutagenic for organisms. Adjacent thymine residues are known to be particularly sensitive to this photoreaction (Sancar & Sancar, 1988). T4 endonuclease V (T4 Endo V), which is encoded by the T4 *den V* gene (Yasuda & Sekiguchi, 1970), consists of 138 amino acids (M_w 16 000) and acts as a repair enzyme for the thymine photodimer lesions of double-stranded DNA. This enzyme searches the substrate site, the UV-light induced thymine dimer, by an ionic strength dependent scanning or sliding mechanism along undamaged, nontarget DNA (Dowd & Lloyd, 1989, 1990). Once the enzyme is located at the target site, it cleaves the glycosylic bond on the 5′-thymine of the dimer (glycosylase activity) and subsequently incises the phosphodiester bond between the two nucleosides of the dimer (AP-endonuclease). Recently, Hamilton et al. (1992) have reported that an enzyme functionally similar to T4 Endo V exists in *Saccharomyces cerevisiae*. This is the first report describing a T4 Endo V-like activity within a eukaryotic organism.

The three-dimensional structure of T4 Endo V was determined at 1.6-Å resolution by X-ray crystallography (Morikawa et al., 1992). Combined with the results from site-directed mutagenesis, the refined structure revealed that the catalytic center for the glycosylase comprises Glu 23 and the sur-

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® Abstract published in *Advance ACS Abstracts*, December 1, 1993.
[1] Abbreviations: T4 Endo V, T4 endonuclease V; NOE, nuclear Overhauser effect; CIDNP, chemically induced dynamic nuclear polarization; $NaDodSO_4$, sodium dodesyl sulfate; SQC, single quantum coherence; CD, circular dichroism; NMR, nuclear magnetic resonance, TT, thymine dimer.

0006-2960/94/0433-57$04.50/0

rounding basic residues (Morikawa et al., 1992; Doi et al., 1992). However, the tertiary structure of the T4 Endo V–DNA complex has not been elucidated yet. It is important to understand how T4 Endo V recognizes target sites. For the elucidation of this recognition mechanism, it is essential to know the conformational changes in the DNA molecule that are caused by photodimer formation. NMR has been effectively used to investigate the conformational change arising from the formation of a thymine dimer (Kemmink et al., 1987a, 1987b; Taylor et al., 1990). However, the details of the interactions between T4 Endo V and the substrate are not sufficiently understood at either the atomic or the molecular levels.

In order to address the detailed recognition mechanism of T4 Endo V, we focused our study on the structural alterations of the DNA duplexes that can be specifically and nonspecifically recognized by the enzyme. Furthermore, we studied the conformational changes of the DNA and the enzyme that occur during their interaction.

In the double-helical structure of DNA, imino protons are involved in the formation of Watson–Crick-type base pairs, and their signals can be observed in a NMR spectrum when their exchange rate with H_2O is sufficiently slow. Imino proton signals are good indicators of the conformational changes in DNA that occur upon protein binding because they are well isolated from the signals from proteins and their chemical shifts are sensitive to conformational changes in DNA. Therefore, we used the imino proton signals as probes for the conformational changes in the DNA that occur upon the binding of T4 Endo V.

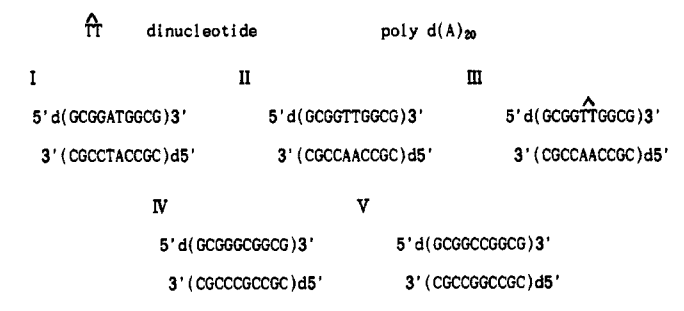
On the basis of site-directed mutagenesis experiments, the carboxyl terminal segment of T4 Endo V has been suggested to participate in the specific binding to a thymine dimer (Lloyd and Augustine, 1989; Ishida et al., 1990). The final 11 amino acid segment contains five aromatic and two basic residues and lacks acidic residues. It has been proposed that these aromatic residues may stack with bases and the basic side chains may electrostatically interact with the phosphate backbone near a thymine dimer. In this paper, we will show, on the basis of photo-CIDNP spectra, that the carboxyl-terminal segment of T4 Endo V is definitely involved in DNA binding.

T4 Endo V can scan a DNA duplex to facilitate the specific location of target sites. Scanning or sliding is electrostatic in nature and involves interactions between the acidic phosphodiester backbone of the DNA and the basic amino acid side chains of protein. Therefore, we used the signals of the arginine side chains (Arg NϵH peak) as an indicator of the DNA interaction because the NϵH group of arginine has a positive charge and its signal (at approximately 80 ppm) is well isolated from the backbone NH signals.

T4 Endo V digests DNA containing a thymine dimer in a few minutes by glycosylase and endonuclease activities. Therefore, it is difficult to directly study the structure of protein–substrate complexes of T4 Endo V–DNA (containing a thymine dimer) or T4 Endo V–DNA (with only the glycosyl bond cut). Methoxyamine completely protects the DNA (with only the glycosyl bond cut) from endonucleolytic attack at the dimer site (Liuzzi et al., 1987). Thus, we could effectively use methoxyamine in the structural study of the complex of T4 Endo V–DNA (with the glycosyl bond cut). In order to more efficiently study the structure of the complex of T4 Endo V–DNA (containing a thymine dimer), we also used a mutant of T4 Endo V (E23Q), which lacks both the glycosylase and endonuclease activities.

MATERIALS AND METHODS

Oligonucleotides were prepared by a DNA synthesizer, and those containing a thymine photodimer were synthesized by using a protected thymine dimer derivative (Taylor et al., 1987), which was prepared by a modified procedure (Murata et al., 1990). The sequences of the oligonucleotides are as follows:



Wild-type and mutant T4 Endo V proteins were purified from *E. coli* strains harboring the appropriate expression plasmids (Inaoka et al., 1989). Their purity was greater than 99%, as judged by $NaDodSO_4$–polyacrylamide gel electrophoresis. Uniform ^{15}N labeling of the protein was achieved by growing the bacteria in minimal medium, using $(^{15}NH_4)_2SO_4$ as the sole nitrogen source. $(^{15}NH_4)_2SO_4$ was obtained from Cambridge Isotope Labs.

^1H NMR spectra were recorded on Bruker AM600 and AM500 NMR spectrometers. DNA samples for ^1H NMR in D_2O were prepared by freeze–drying. Protein samples for ^1H NMR in D_2O were prepared by dialysis with D_2O. NMR measurements were mainly carried out in a neutral solution (pH 6.5) at 20 and 23 °C. Sampling buffer contained 50 mM potassium phosphate and 0.3 M KCl. 2D NOESY, DQF-COSY, and TOCSY were performed on 1.5–2.5 mM DNA solutions and 2 mM protein solutions at 23 °C. NOE mixing times were 100–200 ms. A 50-ms coherence mixing time was used in the TOCSY experiment. Spectra in H_2O were recorded by means of the 1–1 echo water suppression pulse (Sklenar et al., 1987). SQC spectra were obtained for 0.5–1.5 mM protein solution at 20 °C. Photo-CIDNP spectra were recorded on a JEOL GX 500 NMR spectrometer. For the measurement of photo-CIDNP spectra, 0.1 mM 3-*N*-(carboxymethyl)lumiflavin was added to the sample solution, and the tube was placed in a specially designed probe, into which a laser beam was introduced through a quartz rod from an NEC GLC-3300 argon ion laser. A train of several continuous saturation pulses was followed by irradiation for 0.1 s with 488-nm (1 W) prior to the detection pulse. A photo-CIDNP difference spectrum was obtained by subtracting a reference dark spectrum from a laser-irradiated spectrum. One hundred cycles were accumulated for each spectrum.

RESULTS

Assignment of the ^1H Resonances of DNAs. The sequential assignments of the ^1H resonances of the nonexchangeable protons in the 2D NMR spectra of DNA II, obtained by the NOEs between the base protons (H6 for pyrimidines, H8 for purines) and the sugar H1′ protons, are shown in Figure 1. In right-handed DNA, the distance between the base protons and the H1′ protons residing on the same residue and on the residue bound at the 5′ end is sufficiently short to give NOEs. Complete sequential assignments were made for DNA I, DNA II, and the minus strand on DNA III. The pattern of sequential

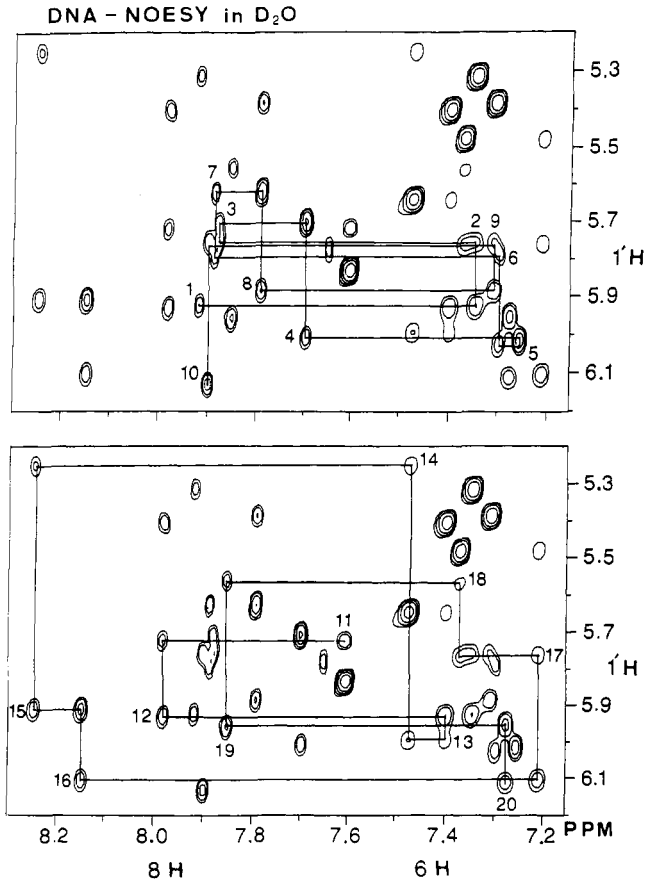


FIGURE 1: Region of 600-MHz 2D NOESY spectrum showing the cross-peak between the base H6/H8 and the sugar H1′ protons of DNA II d(GCGGTTGGCG)·d(CGCCAACCGC). Sequential assignments of the d(GCGGTTGGCG) strand (top) and d(CGCCAACCGC) strand (bottom) are indicated by lines. The spectrum was recorded at 23 °C for D_2O solution (2.5 mM DNA, 0.3 M KCl) with a mixing time of 150 ms.

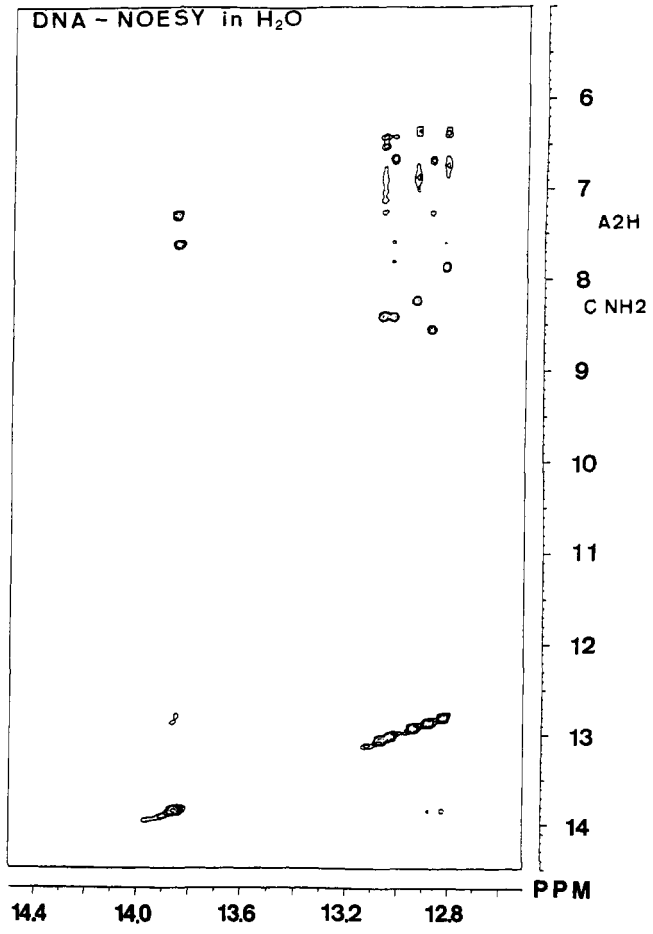


FIGURE 2: Low-field region of 600-MHz 2D NOESY spectrum of DNA II d(GCGGTTGGCG)·d(CGCCAACCGC). The spectrum was recorded at 23 °C for H_2O solution (2.5 mM DNA, 0.3 M KCl) with a mixing time of 100 ms.

NOEs of the plus strand of DNA III (–$\overset{\frown}{TT}$–) was regular from residue G1 to G4 and from G7 to G10. No NOE was observed between G4 (H1′) and T5 (H6), indicating a substantial change in the structure at this position. The assignments for the nonexchangeable protons of the three DNAs are summarized in Table I.

The 2D NMR spectrum of DNA II recorded in H_2O is shown in Figure 2. The GC1 and GC10 imino proton signals are not observed because they exchange with H_2O very fast, due to the terminal fraying effects. Complete sequential assignments can be made from CG2 to CG9. The resulting imino and amino proton assignments for the three types of DNA are summarized in Table II. DNA IV and DNA V consist of only GC pairs, and their imino proton signals overlap very severely. It was difficult to assign the signals to specific GC base pairs.

Chemical Shift Changes Induced by Thymine Dimer Formation. The root mean square differences of the chemical shifts between the proton signals of DNA II and DNA III are shown in Figure 3. The chemical shifts of both the exchangeable and nonexchangeable proton signals were used for this calculation. Although the largest change in chemical shifts is observed at the site of the thymine dimer, sizable but smaller changes occur at the sites adjacent to the dimer position. These smaller changes gradually diminish in magnitude as the distance from the site increases. The change in chemical shift is relatively large at the GC7 base pair, which is the 3′-side residue next to the dimer site. Overall, the changes in chemical shifts appear to be greater at the 3′-side of the dimer site than at the 5′-side.

Some imino proton resonances (TA5, TA6, GC7) show large upfield shifts upon thymine dimer formation (Figure 5). The major cause of these large shifts is likely to be the weakening of the hydrogen bonds in the base pairs, which is induced by the thymine dimer formation at the neighboring base pair.

Effects of T4 Endo V and Its Mutant on the Imino Proton Signals of DNA. The imino proton signals of DNA can be used as probes for conformational changes induced in the DNA structure by the binding of a protein. The effects of T4 Endo V binding on the imino proton signals were measured (Figure 4). In the case of DNA I, DNA IV, and DNA V, overall line broadening of the imino proton signals was observed upon the addition of T4 Endo V, but no change in the chemical shifts was detected. In the case of DNA II, a small downfield shift of the imino proton signal of GC7 was observed upon the addition of T4 Endo V. In the case of DNA III, which contained methoxyamine, a drastic change was observed in the spectrum upon the addition of T4 Endo V (Figure 5 (1)). This change may correspond to the conformational disruption induced within the DNA structure by the cleavage of the glycosyl bond.

In order to study the structure of the uncut DNA–T4 Endo V complex, we used a mutant of T4 Endo V (Glu 23-Gln) that lacks the glycosylase (Doi et al., 1992) and AP-endonuclease activities (Hori et al., 1992), whereas the substrate binding ability is retained. When this mutant was added to DNA III,

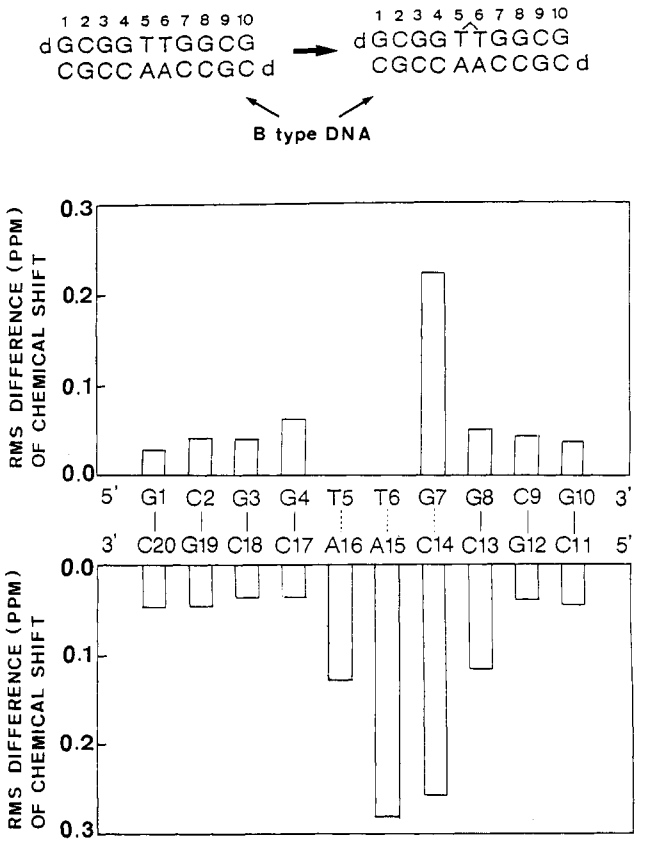


FIGURE 3: Root mean square (rms) differences of the chemical shifts between the proton signals of DNA II and DNA III (top, the plus strand, and bottom, the minus strand). The chemical shifts of the exchangeable and nonexchangeable proton signals (Table I and II) were used for the calculation of rms differences. The values for T5 and T6 are omitted because they are mainly due to changes in chemical structure, rather than conformation.

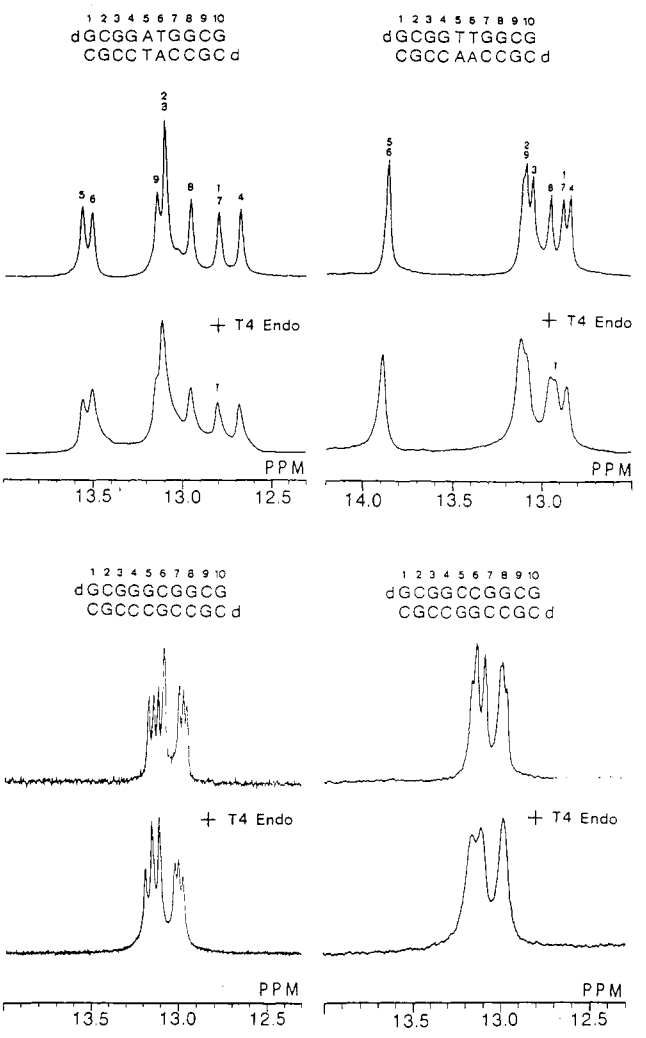


FIGURE 4: Imino proton resonances of four types of 10 mer DNAs and their complexes with T4 Endo V in H_2O. The mole ratio of T4 Endo V to DNA was 1, and the concentration of DNA was 0.2 mM. The buffer was 50 mM potassium phosphate and 0.3 M KCl (pH 6.5), and the temperature was 23 °C.

several imino proton signals showed large downfield shifts (Figure 5 (2)). One of these signals at 13.4 ppm could be assigned to GC7 by the saturation transfer experiment of the imino proton signal at 12.5 ppm (Figure 5 (3)).

Effects of the Oligonucleotide Addition on the High-Field and Low-Field Signals of T4 Endo V. In order to study the binding mode of T4 Endo V, we investigated the binding of seven different types of oligonucleotides to T4 Endo V. When DNA II was added to T4 Endo V in H_2O, new signals appeared at –0.9 and –1.25 ppm in the high-field region of the spectrum. Changes in the intensities of the signals between –0.5 and 0.5 ppm were also observed (Figure 6). In the low-field region, new signals appeared at around 9.7 ppm upon the addition of DNA II. Therefore, the exchange between the bound and free states is slower than the NMR time scale. In the case of DNA I binding, the features of the peak changes were not identical to those that occurred upon the binding of DNA II. New signals did not appear at –0.9 and –1.25 ppm, and the changes in the intensities of the signals between –0.5 and 0.5 ppm were smaller. In the case of DNA IV and DNA V binding, the features of the peak changes were similar to those that occurred upon the binding of DNA I. When DNA III and methoxyamine were added to T4 Endo V, signal changes were observed in both the high- and low-field regions. However, these changes are not similar to those observed for DNA I, DNA II, DNA IV, and DNA V binding. When a single-stranded DNA, poly(dA)$_{20}$, was added to T4 Endo V, the signal change was entirely very small. When a $\overline{TT}$ dimer (dinucleotide) was added to T4 Endo V, signal change was not observed at all in both the high- and low-field regions.

Effects of the Three Types of DNA Duplexes on the NεH Signals of the Arginine Side Chains. The SQC spectrum of ^{15}N uniformly labeled T4 Endo V is shown in Figure 7. Five well-isolated signals were observed around 80 ppm in the ^{15}N chemical shifts, which can be assigned to the NεH signals of the arginine side chains. These signals can be used as the probes for the local interaction with DNA on the protein surface, as these signals do not generally overlap with the backbone amide signals of the protein.

When either DNA I or DNA II was added to T4 Endo V, some signals at approximately 80 ppm disappeared. The missing signals corresponding to the binding are different between DNA I and DNA II. In the case of DNA I binding, the missing signal could be assigned to the NεH signal of Arg 26, since this signal is not observed in the spectrum of the mutant T4 Endo V (R26Q) (data not shown). When DNA III was added to T4 Endo V together with methoxyamine, the five signals did not disappear at all.

Photo-CIDNP Spectra of T4 Endo V. The photo-CIDNP difference spectrum of T4 Endo V exhibits three strong negative peaks, which are derived from the 3, 5 protons of three tyrosine residues (Figure 8). On the basis of NOE data, one of these peaks, at 6.2 ppm, could be assigned to the 3, 5

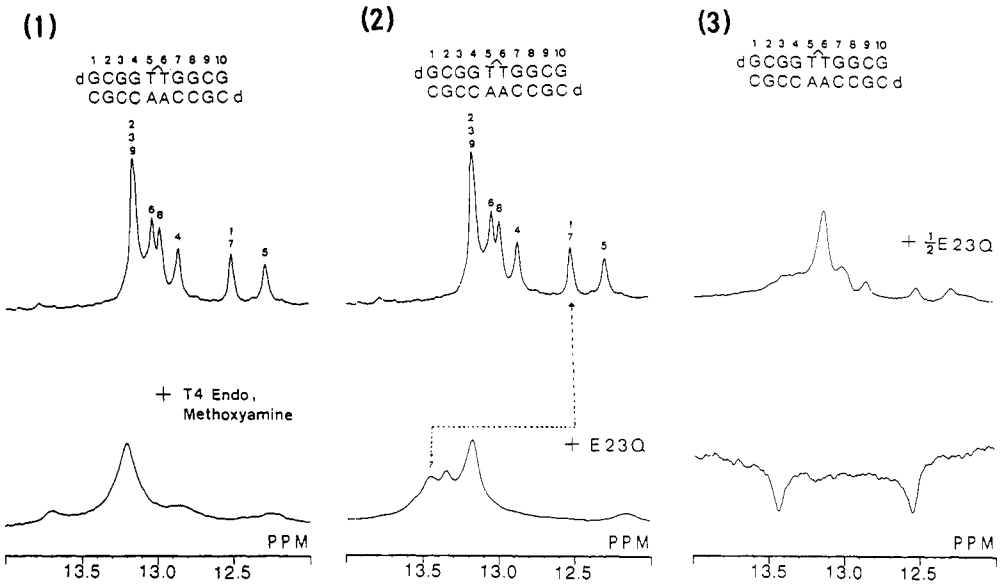


FIGURE 5: (1) Imino proton resonances of DNA III and their complexes with T4 endonuclease V in the presence of methoxyamine. The mole ratio of T4 Endo V to DNA was 1, and the concentration of DNA was 0.2 mM. The concentration of methoxyamine was 1 mM. (2) Effects on the imino proton resonances of DNA III (containing a pyrimidine dimer) by the addition of a mutant T4 Endo V, E23Q. (3) NOE difference spectrum with a mole ratio of protein to DNA of 0.5.

Table 1: Chemical Shifts of the Assigned Nonexchangeable Protons of Three Types of DNA, Measured at pH 6.5

		5′-G1	C2	G3	G4	T(A)5	T6	G7	G8	C9	G10-3′
H6/H8	TT	7.93	7.34	7.87	7.78	7.25	7.29	7.88	7.79	7.31	7.91
	⊓ TT	7.96	7.37	7.91	7.77	4.50	4.11	7.92	7.68	7.34	7.94
	AT	7.94	7.34	7.85	7.78	8.19	7.04	7.82	7.77	7.31	7.91
1′	TT	5.94	5.75	5.71	6.01	6.04	5.80	5.62	5.88	5.76	6.14
	⊓ TT	5.98	5.78	5.71	6.14	5.65	5.43	5.91	5.88	5.81	6.19
	AT	5.93	5.72	5.56	5.71	6.22	5.72	5.62	5.91	5.77	6.15
H2/H5/CH3	TT		5.33			1.34	1.65			5.39	
	⊓ TT		5.37			0.64	1.50			5.36	
	AT		5.34			7.75	1.34			5.39	
H2′	TT	2.60	1.98	2.73	2.56	2.07	2.02	2.69	2.56	1.90	2.39
	⊓ TT	2.63	2.01	2.74	2.51	2.03	2.04	2.55	2.53	1.93	2.41
	AT	2.61	1.97	2.68	2.60	2.62	1.89	2.66	2.57	1.91	2.40
H2″	TT	2.76	2.42	2.78	2.81	2.55	2.40	2.76	2.70	2.33	2.61
	⊓ TT	2.77	2.43	2.79	2.80	2.65	2.37	2.77	2.69	2.35	2.65
	AT	2.77	2.37	2.78	2.81	2.94	2.32	2.74	2.69	2.34	2.64
		3′-C20	G19	C18	C17	A(T)16	A15	C14	C13	G12	C11-5′
H6/H8	TT	7.32	7.85	7.37	7.21	8.14	8.24	7.47	7.40	7.98	7.61
	⊓ TT	7.37	7.91	7.41	7.24	8.05	8.24	7.51	7.45	8.00	7.64
	AT	7.29	7.87	7.43	7.53	7.20	8.37	7.54	7.42	7.99	7.62
1′	TT	6.14	5.96	5.57	5.77	6.10	5.91	5.25	5.99	5.93	5.73
	⊓ TT	6.17	6.00	5.60	5.77	6.17	5.97	5.77	5.98	5.97	5.78
	AT	6.13	5.97	5.56	5.98	5.92	6.33	5.43	6.04	5.93	5.75
H2/H5/CH3	TT	5.21		5.49	5.17	7.67	7.32	5.64	5.41		5.85
	⊓ TT	5.30		5.54	5.20	7.70	6.74	5.62	5.46		5.90
	AT	5.17		5.62	5.60	1.45	7.67	5.67	5.41		5.85
H2′	TT	2.22	2.58	1.97	1.95	2.61	2.79	2.00	2.06	2.69	1.95
	⊓ TT	2.23	2.61	1.99	2.02	2.56	2.59	2.23	2.25	2.73	1.99
	AT	2.23	2.61	2.03	2.15	2.08	2.74	2.17	2.11	2.70	1.95
H2″	TT	2.22	2.71	2.34	2.39	2.83	2.91	2.28	2.44	2.74	2.42
	⊓ TT	2.23	2.76	2.37	2.37	2.82	2.79	2.57	2.53	2.78	2.44
	AT	2.23	2.73	2.36	2.47	2.48	3.00	2.43	2.50	2.76	2.42

protons of Tyr 129, as this residue is very close to Trp 128 and His 16 in the crystal structure (data not shown). This CIDNP result implies that three of the seven tyrosine residues are located on the molecular surface of T4 Endo V. Furthermore,

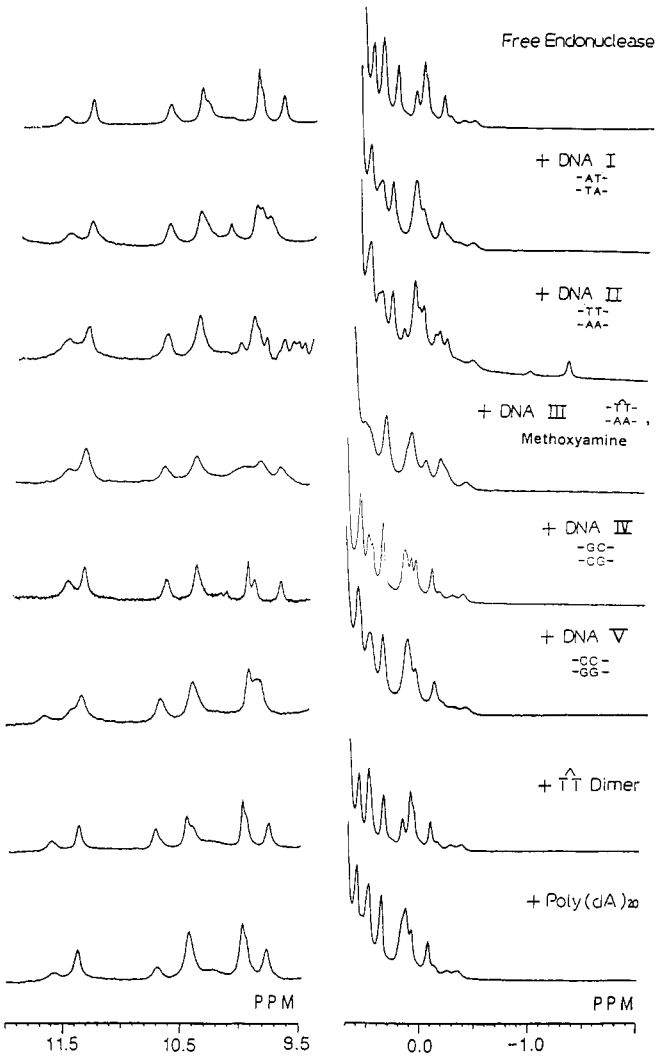


FIGURE 6: High-field and low-field resonances of T4 Endo V and its complexes with DNAs in H_2O. The mole ratio of T4 Endo V to DNA was 1, and the concentration of T4 Endo V was 0.2 mM. The concentration of methoxyamine was 1 mM. The buffer was 50 mM potassium phosphate and 0.3 M KCl (pH 6.5), and the temperature was 23 °C.

Table 2: Chemical Shifts of the Assigned Exchangeable Protons of Three Types of DNA, Measured at pH 6.5

	imino(guanine/thymine)			amino(cytosine)		
base pair	TT	TT	AT	TT	TT	AT
GC1						
CG2	13.10	13.15	13.10	6.54/8.48	6.62/8.52	6.55/8.41
GC3	13.06	13.14	13.10	6.71/8.48	6.75/8.52	6.82/8.52
GC4	12.85	12.85	12.67	6.39/7.93	6.43/7.90	6.47/8.30
TA(AT5)	13.87	12.28	13.56			
TA6	13.87	13.02	13.50			
GC7	12.89	12.51	12.80	6.71/8.59	6.93/8.61	6.78/8.50
GC8	12.96	12.97	12.95	6.39/8.29	6.54/8.44	6.36/8.21
GC9	13.10	13.15	13.14	6.54/8.48	6.62/8.52	6.51/8.45
GC10						

the photo-CIDNP signals of the three tyrosine residues disappeared upon the addition of any of the three DNA duplexes (I–III). This finding suggests that the three tyrosine residues may be covered by the DNA duplexes, regardless of specific or nonspecific binding.

DISCUSSION

There is considerable evidence that the presence of cyclobutane-type pyrimidine dimers produces some distortion

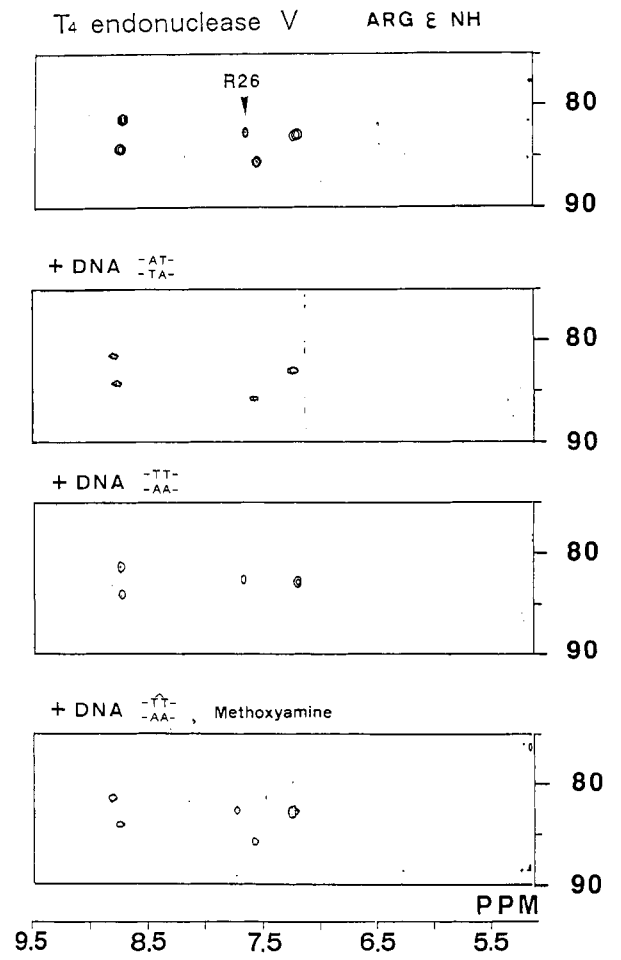


FIGURE 7: Arginine NεH signals in the SQC spectra of ^{15}N-labeled T4 Endo V and its complexes with DNAs. The concentration of ^{15}N-labeled T4 Endo V was 0.3 mM, and the concentration of methoxyamine was 1.5 mM. The buffer was 50 mM potassium phosphate and 0.3 M KCl (pH 6.5), and the temperature was 20 °C.

in a DNA structure. The introduction of dimers lowers the melting temperature (Hayes et al., 1971), increases the reactivity to form aldehydes (Shafranovskaya et al., 1973), and increases the sensitivity to a single strand-specific endonuclease (Legerski et al., 1977; Heflich et al., 1977). The data presented here reveal that, although the local environment around the thymine dimer is altered, the gross structural changes are relatively small. The DNA duplex containing a thymine dimer seems to exhibit a small distortion of the B-form. A previous NMR study of a DNA octamer, which contained a thymine dimer, showed that the structural change occurred only at the site of the cyclobutane-type thymine dimer (Kemmink et al., 1987). Our present NMR data show that the change is transmitted from the dimer site to neighboring, and the effect is greater at the 3′-side of the dimer site than at the 5′-side. Taylor and colleagues also investigated the conformational change induced by the formation of a thymine dimer (Taylor et al., 1990). They calculated the RMSD of chemical shifts using only those of nonexchangeable protons and obtained results similar to ours. However, our data should be more accurate than previous ones, since we used both exchangeable and nonexchangeable proton chemical shifts.

From the chemical shifts of the imino proton signals of the DNA, we knew that the hydrogen bonds of three base pairs (TA5, TA6, and GC7) in the DNA III containing a thymine dimer were weakened but not broken. The GC7 imino proton signal is largely downfield shifted with the addition of the

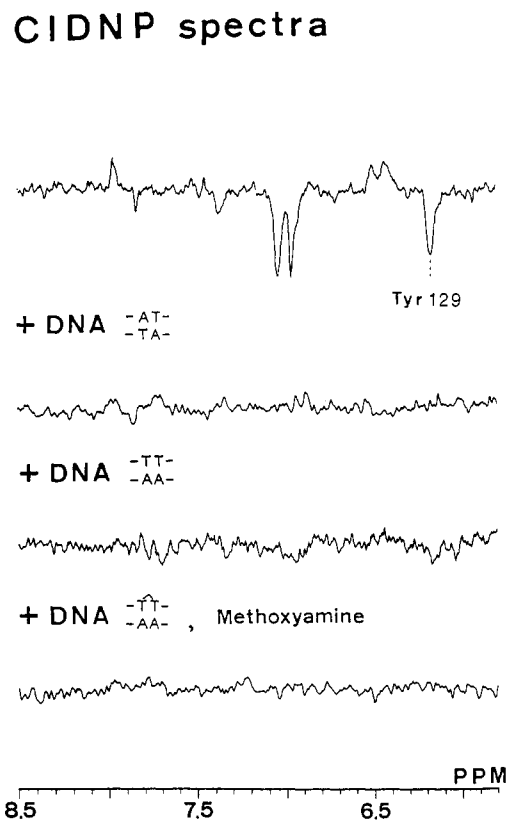


FIGURE 8: Photo-CIDNP spectra of T4 Endo V and T4 Endo V–DNA complexes. The concentration of protein was 0.4 mM, and the mole ratio of DNA to protein was 1. The buffer was 50 mM potassium phosphate and 0.3 M KCl, and the temperature was 23 °C.

E23Q mutant of T4 Endo V. This position (GC7) should be either the crucial point for T4 Endo V recognition or the particular site of a conformational change which occurs by T4 Endo V binding.

Although both DNA I and DNA II were nonspecific DNAs, NMR measurements show that T4 Endo V discriminates the difference between DNA I and DNA II. Therefore, thymine–thymine (–TT–) arrangement also appears to be important for the binding with T4 Endo V. The affinity of the enzyme for the TT duplex without a photodimer (DNA II) was much smaller than that for the $\overset{\sqcap}{TT}$ duplex (DNA III) (Inaoka et al., 1989). The differences in NMR signal changes between DNA I and DNA II may be explained by somewhat distinct binding schemes of the two complexes. In this connection, it should be noted that NMR measurements require a much higher concentration of proteins and DNA duplexes than that for the filter-binding assay (Inaoka et al., 1989). This particular solution condition for NMR experiments may lead to the detection of slight variation of nonspecific binding.

In order to study the structure of the protein–DNA complex, it is desirable to employ the wild-type enzyme. However, such an experiment is impossible because T4 Endo V digests DNA containing a thymine dimer in only a few minutes. The substitution of Gln for Glu 23 causes the complete loss of both catalytic activities (glycosylase and AP-endonuclease) (Doi et al., 1992; Hori et al., 1992). The NMR and CD spectra of the mutant and wild-type proteins showed features very similar to each other (data not shown). The E23Q mutant was found to retain specific DNA-binding ability (Doi et al., 1992). This mutant also discriminates the difference between DNA I and DNA II because this mutant binding causes chemical shift changes in the imino proton of GC7 in DNA II but not DNA I (data not shown). Therefore, the DNA-binding mode of this mutant appears to be almost the same as that of the wild type.

The glycosylase activity of T4 Endo V is greater on double-stranded DNA than on single-stranded DNA (Gordon et al., 1980). The affinity of T4 Endo V for a $\overset{\sqcap}{TT}$ duplex is known to be much higher than that for single-stranded $\overset{\sqcap}{TT}$ oligonucleotides or duplexes without $\overset{\sqcap}{TT}$ (Inaoka et al., 1989). The spectral change of T4 Endo V caused by single-stranded DNA binding (Poly $(dA)_{20}$) is smaller than that caused by the double-stranded DNA binding (Figure 6). Furthermore, the spectral change arising from the binding of DNA II, which contains the TT sequence, is larger than that occurring upon the binding of DNA I, DNA IV, and DNA V, which does not contain the –TT– sequence. This result is consistent with the fact that T4 Endo V binding causes chemical shift changes in the imino proton of GC7 in DNA II, but not in DNA I, DNA IV, and DNA V.

When T4 Endo V binds to the DNA with a cleaved glycosyl bond (DNA III containing methoxyamine), the signal changes of this protein are different from those induced by DNA I, IV, V, or II binding. When a $\overset{\sqcap}{TT}$ dimer (dinucleotide) was mixed with T4 Endo V, no changes were observed for both the signals of T4 Endo V (Figure 6) and the $\overset{\sqcap}{TT}$ dimer (data not shown). This finding demonstrates that the $\overset{\sqcap}{TT}$ dimer itself does not bind to T4 Endo V and that a sufficient length of the double stranded DNA is necessary for the induction of an observable structural change of T4 Endo V by which the enzyme can recognize the $\overset{\sqcap}{TT}$ sites.

As usually expected for a DNA-binding protein, T4 Endo V contains a lot of arginine and lysine residues. The graphic display of the electrostatic potential on the protein accessible surface clearly demonstrates the biased distribution of positive charges contributed by arginine and lysine (Morikawa et al., 1992). This feature of the enzyme is in agreement with the proposal that the enzyme scans the DNA duplex prior to recognition of a $\overset{\sqcap}{TT}$ dimer (Dowd and Lloyd, 1989), since its binding to nontarget DNA occurs through electrostatic interactions, Arg 26 is known to be responsible for the ability of the enzyme to scan nontarget DNA in search of a thymine dimer (Dowd et al., 1990). The recent study, using site-directed mutagenesis, has also revealed that the replacement of Arg 26 by Gln greatly reduces the glycosylase activity (Doi et al., 1992). This result is consistent with the present NMR data, as the NϵH Arg 26 is affected by the binding of DNA I. Arg 26 is located on the presumed recognition helix (H1) in the crystal structure of T4 Endo V (Morikawa et al., 1992).

The photo-CIDNP results suggest that the aromatic segment WYKYY of T4 Endo V, which locates at the C-terminal and involves Tyr 129, constitutes the binding site rather than the active site of the enzyme. This enzymatic reaction appears to require the precise positioning of functional groups in the active site of the protein with respect to the substrate, i.e. the modified chemical groups (or bonds) of nucleic acid base. Site-directed mutagenetic and biochemical data support the hypothesis that the C-terminal portion of T4 Endo V is involved in thymine dimer specific binding (Stump et al., 1988) and that Glu 23 is associated with active site (Doi et al., 1992). This aromatic segment near the C-terminus may play a role in the correct positioning of the protein on its target.

ACKNOWLEDGMENT

We thank Dr. K. Katayanagi for his help in obtaining the molecular graphics.

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