Investigation of the Cyclobutane Pyrimidine Dimer (CPD) Photolyase DNA Recognition Mechanism by NMR Analyses*

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The cyclobutane pyrimidine dimer (CPD) is one of the major forms of DNA damage caused by irradiation with ultraviolet (UV) light. CPD photolyases recognize and repair UV-damaged DNA. The DNA recognition mechanism of the CPD photolyase has remained obscure because of a lack of structural information about DNA-CPD photolyase complexes. In order to elucidate the CPD photolyase DNA binding mode, we performed NMR analyses of the DNA-CPD photolyase complex. Based upon results from 31P NMR measurements, in combination with site-directed mutagenesis, we have demonstrated the orientation of the bound DNA relative to the active site. The FAD is excited by light, and then transfers one electron to the CPD bases (6–8). After the electron transfer, the cyclobutane ring splits and then one electron is transferred back to the FAD (9, 10). In order to understand the mechanism based on structural analyses, the crystal structures of the CPD photolyases from Escherichia coli, Anacystis nidulans, and Thermus thermophilus have been solved without the substrates, i.e. CPD-containing DNAs (11–15). The x-ray studies revealed that the enzyme substrate share a similar global fold, which consists of an a/b domain and a helical domain. The helical domain is composed of clusters I and II, and a cavity is formed between the clusters, where the FAD is deeply buried. It has been suggested that the cavity is used for the CPD binding, because the asymmetric polarity of the cavity fits well with that of the CPD (11–15). Based upon the crystal structures without the substrates, two research groups have proposed computer models of the DNA-CPD photolyase complex, in which the relative orientations of the DNA chain are different from each other (11–15). However, no crystallographic or NMR structure information on the complexes is presently available.

Here, we report NMR analyses of the DNA recognition mechanism by T. thermophilus CPD photolyase with a molecular weight of 48 kDa. Initially, we analyzed DNA complexed with the CPD photolyase and its mutants by 31P NMR. These analyses revealed the orientation of the bound DNA relative to the enzyme. Next, more detailed analyses of the CPD lesion, utilizing stable isotope-labeled DNA, revealed that the CPD base is buried in the cavity in the DNA-CPD photolyase complex, and that the CPD is flipped out of the DNA helix in the complex. We discuss the correlation between the structural information and activity on the basis of the present NMR data.

**EXPERIMENTAL PROCEDURES**

Preparation of Oligonucleotides and CPD Photolyase from T. thermophilus—Oligonucleotides labeled with 13C and 15N were prepared according to a published method (16). The other oligonucleotides without the CPD lesion were purchased from ESPEC Oligo Service Corp. d(CGCAAT[CPD]TAAGCCG) was chemically synthesized as previously reported (17). The other oligonucleotides containing CPD lesions were prepared by UV irradiation (0.9 J/cm²) with a FUNA-UV-LINKER FS-800 (Funakoshi) and were purified in the same way as previously reported (17). The CPD photolyases from T. thermophilus HB87 and its mutants were prepared, according to an established procedure (18). The enzymes labeled with 3H were also obtained in the same way, except that the E. coli strains expressing them were cultured for 24 h in M9 medium containing d-glucose-3H₂ and 13C, 15N instead of d-glucose and H₂O, respectively.

Surface Plasmon Resonance (SPR) Measurements—The binding constants of the CPD photolyase and its mutants to d(GTAT[CPD]TATG),...
contains a biotin at the 5’-end, were determined using a BIAcore 1000 instrument (BIAcore AB) in the same way as reported previously (17), except that the enzyme concentration range was 10–100 nm.

**NMR Measurements**—Four different buffers were used for the NMR measurements. Buffer I: 5 mM sodium phosphate buffer, pH 7.3, containing 200 mM NaCl, and 5 mM NaN$_3$ in $^2$H$_2$O. Buffer II: Buffer I plus 10 mM dithiothreitol, 1 mM EDTA, and 5% ethylene glycol, with the $^2$H$_2$O reduced to 10%. Buffer III: Buffer I plus 10 mM dithiothreitol-$^2$H$_2$O and 5% ethylene glycol-$^2$H$_2$O with a 100 mM concentration of NaCl. Buffer IV: the pH of Buffer III was changed to 6.5, and the 2H$_2$O was reduced to 10%.

The experiments to assign the $^{31}$P resonances of the oligodeoxynucleotides in the absence of the enzymes were carried out at a concentration of 0.5–2 mM in 420 μl of Buffer I, in the same way as reported previously (19). Buffer II was used for one-dimensional $^{31}$P NMR measurements in the absence and presence of the enzymes and for $^{31}$P-$^{31}$P exchange spectroscopy (EXSY) experiments. The experiments described above were performed on a Bruker DRX400 spectrometer, and those described below were monitored on a Bruker DRX600 spectrometer. One-dimensional $^1$H, $^1$H-$^1$C heteronuclear single quantum coherence (HSQC) (20), HCCH type- (21), HCN (22), HCP (23), and single-stranded DNA (ssDNA) was not detected under our experimental conditions. The binding constants of the imino resonances of dsDNA and UV/visible absorption spectra, respectively (data not shown). The binding constants of the mutants are summarized in Table II. The binding constants of the other mutants were almost identical to that of the wild type. The binding constants of the CPD photolyase from *T. thermophilus* for ssDNA containing CPD were determined by SPR measurements with 10–100 mM protein concentrations and running buffer (10 mM HEPES, pH 7.3, 3.5 mM EDTA, 0.005% Tween-20, 10 mM 2-mercaptoethanol, and NaCl).

**Table I**

| NaCl (mM) | $K_a$ (nM) |
|----------|------------|
| 10$^{-6}$ | 9.4 $\times$ 10$^6$ |
| 50       | 2.8 $\times$ 10$^6$ |
| 100      | 6.2 $\times$ 10$^7$ |
| 150      | 2.6 $\times$ 10$^7$ |
| 200      | 1.4 $\times$ 10$^7$ |
| 300      | 4.8 $\times$ 10$^6$ |
| 500      | < 10$^6$  |
| 1,000    | < 10$^6$  |

$^a$ Binding between the CPD photolyase and non-damaged DNA was also observed under these conditions.

**Table II**

| K$_a$ (nM) | $^{31}$P NMR$^c$ |
|------------|-----------------|
| Wild type  | 2.6 $\times$ 10$^7$ |
| R134A      | 4.0 $\times$ 10$^6$ |
| R141A      | 1.1 $\times$ 10$^7$ |
| R142A      | 2.1 $\times$ 10$^7$ |
| R198A      | 4.8 $\times$ 10$^6$ |
| R201A      | 9.4 $\times$ 10$^6$ |
| R203A      | 3.3 $\times$ 10$^7$ |
| R232A      | 2.6 $\times$ 10$^7$ |
| R233A      | 2.1 $\times$ 10$^7$ |
| R239A      | 2.2 $\times$ 10$^7$ |
| K240A      | 7.4 $\times$ 10$^6$ |
| E244A      | 2.3 $\times$ 10$^7$ |
| W247A      | 8.0 $\times$ 10$^6$ |
| R311A      | < 10$^6$ |
| W353A      | < 10$^6$ |
| R366A      | < 10$^6$ |
| L372A      | 4.1 $\times$ 10$^7$ |
| R376A      | 9.8 $\times$ 10$^6$ |
| R382A      | 5.2 $\times$ 10$^7$ |
| R397A      | 3.9 $\times$ 10$^7$ |
| R407A      | 2.1 $\times$ 10$^7$ |
| R419A      | 2.6 $\times$ 10$^7$ |

$^a$ The ss$^7$mer phosphate groups that had different chemical shifts between the complex of the mutants and the wild type are shown. The numbering of the phosphate groups was shown in Fig. 2.

$^b$ Responses were not observed for these mutants.

$^c$ Bound resonances were not obtained because of low affinities.

$^{31}$P NMR analyses of ssDNA-CPD Photolyase Complexes—In order to determine the binding site of the CPD photolyase on ssDNA, we measured the $^{31}$P NMR spectra of d(TAT(CPD)TATG) (ss$^7$mer) in the absence and presence of the CPD photolyase (Fig. 2, a–c). Assignments of $^{31}$P resonances derived from the ss$^7$mer in the free form were accomplished through the analysis of $^3$H-$^{31}$P HSQC spectra, along with total correlation spectroscopy (TOCSY) and rotational Overhauser effect spectroscopy (ROESY) spectra, via a sequential assignment methodology (spectra not shown) (19). The established assign-
Fig. 2. $^{31}$P NMR analysis of the CPD photolyase-ssDNA complex. $^{31}$P NMR spectra of d(TAT[CPD]TATG) (ss7mer) in the absence (a) and presence (c) of 1.5 molar equivalents of the wild-type CPD photolyase. b, $^{31}$P-$^{31}$P EXSY spectrum of the ss7mer in the presence of a 0.67 molar equivalent of the wild type. The mixing time was set to 50 ms. d, $^{31}$P NMR spectra of the ss7mer in the presence of 1.5 molar equivalents of the R201A CPD photolyase.

Table II.

To examine the effect of the aromatic groups in the CPD binding site, the $^{1}$H-$^{13}$C HSQC spectra of the ss5mer in the presence of 1.5 molar equivalents of the wild type. The mixing time was set to 50 ms. d, $^{31}$P NMR spectra of the ss7mer in the presence of 1.5 molar equivalents of the R201A CPD photolyase.

To examine the effect of the aromatic groups in the CPD binding site, the $^{1}$H-$^{13}$C HSQC spectra of the ss5mer in the presence of 1.5 molar equivalents of the wild type. The mixing time was set to 50 ms. d, $^{31}$P NMR spectra of the ss7mer in the presence of 1.5 molar equivalents of the R201A CPD photolyase.
FIG. 3. NMR analysis of $^{13}$C-labeled DNA in complex with the CPD photolyase. $^1$H-$^{13}$C HSQC spectra of dAT(CPD)/TAC containing $^{13}$C-labeled CPD (ss5mer) after the addition of about 1 molar equivalent of the wild type (a, b, and c) and W247A (d). a, deoxyribose region; b, 6-position region; c and d, methyl regions.

FIG. 4. $^1$H NMR spectra of ssDNA in complex with the deuterated CPD photolyase. a, d(AT(CPD)/TAC) (ss5mer) in complex with the wild-type CPD photolyase; b, pT(CPD)/Tp in complex with the wild type; and c, ss5mer in complex with W247A.

DISCUSSION

The CPD Photolyase Binding Site on DNA and the DNA Binding Site on CPD Photolyase—The dependence of the DNA binding affinity of the CPD photolyase upon the ionic strength of the solution (Table I) suggested that electrostatic interactions, between phosphate groups in DNA and basic residues in CPD photolyase, significantly contribute to the DNA binding of the enzyme. The $^{31}$P NMR analysis showed that $P_{-1}$, $P_0$, $P_1$, and $P_2$ were perturbed, in terms of both the chemical shift and the line broadening, upon the addition of d(TAT(CPD)/TATG) (Fig. 2, a and b). These results indicate that a phosphate group of the CPD, and one phosphate group flanking at the 5'-side and two flanking at the 3'-side of CPD, are responsible for binding to CPD photolyase.

In order to investigate the basic residues that are involved in the electrostatic interaction, we determined the affinities of the mutants with substitutions of the basic residues, especially the Arg residues, to Ala (Table II). Fig. 7 shows the mapping of the affected residues identified in the mutant experiments on the crystal structure of the CPD photolyase from *T. thermophilus* without the substrates (13). The mapping indicates that the residues that participate in the binding are located in the region surrounding the cavity formed by clusters I and II, which could be a possible binding pocket for the CPD (13, 15).

Orientation of DNA Relative to the DNA Binding Site—The $^{31}$P NMR spectra of the CPD-containing ssDNA complexed with a variety of the mutants were measured. Some of the resonances from the ssDNA-mutant complexes have different chemical shifts from those from the ssDNA-wild type complex, indicating that the phosphate groups of the ssDNA with different chemical shifts are spatially close to the mutation position. Fig. 2d is a representative spectrum, in which the chemical shifts of $P_{-1}$ and $P_0$ of the ssDNA-R201A complex were different from those of the ssDNA-wild type complex, suggesting that the $P_{-1}$ and $P_0$ sites are located in the vicinity of Arg201. In a similar way, we obtained the spatial relationship between the residues of the photolyase and the phosphate groups of the ssDNA complexed with the deuterated CPD.
DNA (Table II). The results obtained from the present experiments are also summarized in Fig. 7. As shown in Fig. 7, the mapping reveals that the 5'-side of the DNA lies on cluster I, the 3'-side is on cluster II, and the CPD resides at the cavity formed by both of the clusters.

On the basis of molecular dynamics simulations, the binding modes of the complex have been proposed (11–15). In the model proposed by Sanders and Wiest, the orientation of the DNA is opposite to that in our results. On the other hand, the model by Vande Berg and Sancar has the same DNA direction of DNA as in our model.

The CPD Bases Are Buried in the Cavity on the DNA Binding Surface—To investigate the environment of the CPD bound to the enzyme, we measured the resonances derived from a CPD lesion labeled with 13C and 15N in a single-stranded pentamer (ss5mer), in both the free and bound forms. Interestingly, the resonances from the 6-position and the methyl groups of the base showed remarkable chemical shift changes along the 1H dimension, upon complexation with the CPD photolyase (Fig. 3, b and c).

In the spectrum of the pT[CPD]Tp-CPD photolyase complex (Fig. 4b), two methyl resonances were observed at the same positions as those from the ss5mer-CPD photolyase complex (Fig. 4a). This result indicates that the drastic chemical shift changes are induced, not by the flanking nucleotides, but by the CPD photolyase.

Considering the facts that CPD exists in the cavity in the bound form, as described in the previous section, and that the cavity is composed of some aromatic groups, such as Trp247, Trp353, and FAD (Fig. 7), it is most likely that the drastic chemical shift change is caused by the ring current shift of the aromatic groups. In order to define the position of the CPD relative to the cavity, we performed NMR analyses, using W247A.

As a result, the chemical shifts of the CPD methyl resonances were significantly different between the wild-type complex (Fig. 3c) and the W247A complex (Fig. 3d). Among them, the resonance from the 3'-methyl group of CPD showed a remarkable chemical shift difference between the wild-type complex and the W247A complex. These results suggest that
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Trp<sup>247</sup> mainly and partially causes the chemical shift change of the methyl resonances of the 5'- and 3'-sides, respectively. Therefore, we concluded that the bases of CPD are buried in the cavity and the side chain of Trp<sup>247</sup> is in the closest vicinity of the 5'-side methyl group of the bases in the ss5mer-CPD photolyase complex. This is consistent with the conclusion from the <sup>31</sup>P NMR analyses described above: the 5'-side of DNA lies on cluster I, where Trp<sup>247</sup> exists.

### Flipping of CPD from the DNA Duplex in the Complex

The CPD photolyase is reportedly able to recognize and repair a CPD lesion embedded in dsDNA as well as that in ssDNA (24–30), although McAttee et al. (30) reported that a CPD in dsDNA exists within the DNA helix and forms Watson-Crick type hydrogen-bonds with the complementary strand. One possible recognition mode for the buried CPD in dsDNA by the CPD photolyase is that the enzyme unwinds the dsDNA and then recognizes the CPD in dsDNA.

On the other hand, it has been established that one of the important recognition modes for a damaged base is that repair enzymes recognize the flipped out damaged base. Several groups of crystallographers have revealed the structures of DNA protein complexes with the base flipping (31–38). However, in the case of the CPD photolyase, no structural evidence of CPD-flipping has been shown.

In order to investigate the mode of either CPD-flipping or DNA-unwinding, we performed a structural analysis of the DNA protein complexes with the base flipping (31–38). However, in the case of the CPD photolyase, no structural evidence of CPD-flipping has been shown.

In the previous section, we showed that the stable duplex exists even in the vicinity of the CPD in the dsDNA-CPD photolyase complex. This base-flipping mechanism is also required for minimizing the structural changes in the dsDNA and avoiding perturbations of the transcriptional regulation. Base-flipping may be the most efficient and universal repair system by the CPD photolyases.

### Process Leading to CPD-flipping

In the previous section, we concluded that the CPD is flipped out in the bound form. However, as described above, the bases of the CPD exist inside the CPD-containing dsDNA in the free form (30). The difference between the DNA structures in the free and bound forms raises the question of how the CPD photolyase recognizes the CPD in dsDNA.

In addition to the chemical structure of the CPD base, the B<sub>11</sub> conformation of the backbone flanking the CPD on the 3'-side is the most pronounced characteristic of the CPD-containing dsDNA structure (30). Thus, the CPD photolyase may recognize the B<sub>11</sub> conformation of the backbone in the first step of DNA recognition. Vande Berg and Sancar demonstrated that Arg<sup>452</sup> in the CPD photolyase from yeast, which corresponds to Arg<sup>311</sup> from *T. thermophilus*, is essential for the enzyme to distinguish CPD from other normal bases (15). The present study revealed that Arg<sup>311</sup> is located on the 3'-side of CPD in the complex (Fig. 7). Therefore, we suggest that the region containing Arg<sup>311</sup> is necessary for the CPD photolyase to search for the distortion of the backbone, and as described above, to form an ion pair with the 3'-phosphate group adjacent to the CPD upon binding.

### Biological Significance of CPD-flipping

The CPD photolyases repair CPD lesions by an electron transfer between FAD and CPD. The efficiency of the electron transfer depends on the distance between the FAD and the CPD. If the CPD bases are inside the DNA helix in the complex, then the CPD should be more than 20 Å away from the FAD, which would make it difficult to transfer the electron from FAD to CPD with high efficiency. Therefore, CPD-flipping is required for the efficient CPD repair reaction by the enzyme.

The CPD photolyases repair both active and inactive genes (39). Therefore, the enzyme should search for and repair CPD lesions without interfering with the transcriptional regulation. In the present study, we found that the stable duplex exists even in the vicinity of the CPD in the dsDNA-CPD photolyase complex. This base-flipping mechanism is also required for minimizing the structural changes in the dsDNA and avoiding perturbations of the transcriptional regulation. Base-flipping may be the most efficient and universal repair system by the CPD photolyases.

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