Cyclobutane pyrimidine dimer (CPD) photolyses, which contain FAD as a cofactor, use light to repair CPDs. We performed structural analyses of the catalytic site of the *Thermus thermophilus* CPD photolyase-DNA complex, using FAD-induced paramagnetic relaxation enhancement (PRE). The distances between the tryptophan residues and the FAD calculated from the PRE agree well with those observed in the x-ray structure (with an error of <3 Å). Subsequently, a single-stranded DNA containing 13C-labeled CPD was prepared, and the FAD-induced PRE of the NMR resonances from the CPD lesion in complex with the CPD photolyase was investigated. The distance between the FAD and the CPD calculated from the PRE is 16 ± 3 Å. The FAD-induced PRE was also observed in the CPD photolyase-double-stranded DNA complex. Based on these results, a model of the CPD photolyase-DNA complex was constructed, and the roles of the CPD photolyase-double-stranded DNA complex. The PRE would be useful for determining the distance between donors and acceptors determining the efficiency of electron transfer reactions. Therefore, a structural analysis of the catalytic site of the enzyme is important for understanding the mechanism of the high efficiency of the reaction.

The crystal structures of the CPD photolyses from *Escherichia coli*, *Anacystis nidulans*, and *Thermus thermophilus* HB8 were recently solved (8–10). These structures show a similar global fold, and they have an FAD-containing cavity in the C-terminal helical domain. The crystal structure of the *T. thermophilus* CPD photolyase-thymine complex has also been solved (10). In this structure, the thymine molecule resided in the cavity, suggesting that this cavity forms the CPD-binding site. The structure of a CPD photolyase in complex with CPD-containing DNA has not yet been determined.

Unpaired electrons induce paramagnetic relaxation enhancement (PRE) in NMR resonances from neighboring atoms in a distance-dependent manner, and the relaxation data are calculated with the Solomon-Bloembergen equation (11). PRE has been used for analyses of denatured proteins (12–14), protein-ligand complexes (15–18), and large proteins (19), by introducing an unpaired electron into the proteins or the ligands through site-directed spin labeling or metal-chelating tags. PRE would be useful for determining the distance between FAD and CPD in the CPD photolyase-DNA complex, because the FAD in CPD photolyase can assume the radical semiquinone form (4).

We have been using NMR to study the DNA recognition and repair mechanism of CPD photolyase from *T. thermophilus* HB27 with a molecular mass of 48 kDa. Our previous NMR analysis revealed the orientation of a CPD-containing ssDNA on CPD photolyase (20). We also proposed that the CPD lesion is flipped out of the dsDNA in the CPD photolyase-DNA complex. In the present study, we have determined the distance between FAD and CPD in the CPD photolyase-DNA complex, using FAD-induced PRE.

**MATERIALS AND METHODS**

**Sample Preparation**—The *E. coli* strains expressing CPD photolyase from *T. thermophilus* HB27 were cultured as described previously (21). Mutagenesis of the enzyme was performed, using the QuickChange™ site-directed mutagenesis kit (Stratagene). Selective 15N labeling of the tryptophan residues was performed as described previously (22). The enzyme was purified by polyethyleneimine, P11 chromatography, DNA-cellulose chromatography, and DEAE-Sephadex chromatography. Details of the purification procedure will be published elsewhere. DNA oligomers containing CPD were prepared as described previously (20).}

**Photoreactivation Activity**—Three nanomoles of the enzyme and 0.75 nmol of the CPD-containing ssDNA (dTAT/CPD/TATG) were added to 1.5 ml of 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 mM 2-mercaptoethanol, 100 mM NaCl, and 10% ethylene glycol, in the dark, and the reaction mixture was irradiated with monochromatic light by a fluoresc
ence spectrometer (Shimadzu RF 5300PC) equipped with a UV-350 filter (Shimadzu) under aerobic conditions at 25 °C. The bandwidth and irradiation time were set to 1.5 nm and 20 min, respectively. The light intensity was measured with an optical power meter (Newport C-5840). The enzyme was removed from the reaction mixture by phenol extraction. The reaction mixture was lyophilized and fractionated by Pegasil ODS 7µ (8.0 × 300 mm, Senshu Scientific) high-performance liquid chromatograph with a 40-min 8.0–14.0% gradient of CH3CN in 0.1 M triethylamine acetate, pH 7.0, at a flow rate of 1.5 ml/min. Photolytic cross-section values (eb) were calculated as described previously (23, 24).

**NMR Measurements**—Three kinds of buffers were used for the NMR measurements; buffer I, 50 mM NaF, pH 6.5, 100 mM NaCl, 3 mM NaN3, 1 mM EDTA, 10 mM dithiothreitol, 5% ethylene glycol, 10% D2O, was used for 1H/15N heteronuclear single quantum coherence (HSQC) experiments; buffer II, in which the pH of the buffer I was changed to 7.3, was used for 1H one-dimensional experiments; buffer III, 50 mM NaF, pH 7.3, 100 mM NaCl, 3 mM NaN3, 10 mM dithiothreitol-D2O, 5% ethylene glycol-D2O, and 100% D2O was used for 1H-13C HSQC experiments.

For measuring the radical semiquinone form spectra, sodium dithionite was added to the purified enzyme to a final concentration of 20 mM, and the buffer was exchanged to the NMR buffers. The solution was concentrated to 0.1–0.2 mM using a Centriprep YM-30 (Millipore) filter, and lyophilized DNA was added to the enzyme solution. The mixture was transferred to an NMR tube in a nitrogen atmosphere, in the dark. Sodium dithionite was added to the NMR samples to a final concentration of 20 mM, to keep the enzyme in the reduced form. All NMR experiments were performed at 37 °C on a Bruker DRX 400 or Avance 500 spectrometer.

**Distance Determination Using PRE**—NMR spectra were processed and analyzed with the program XWIN-NMR version 3.1 (Bruker). The distances between the observed tryptophan atoms and FAD were measured from the crystal structure of T. thermophilus CPD photolyase in the substrate-free form (10). The unpaired electron in FAD is distributed from the crystal structure of T. thermophilus CPD photolyase (25), and the effective magnetization in HSQC measurements (19), with the program Kaleidograph (Synergy Software).

### RESULTS

**FAD-induced PRE of NMR Resonances from CPD Photolyase**—Prior to the determination of the intermolecular FAD-CPD distance by FAD-induced PRE, we investigated the FAD-induced intramolecular PRE to evaluate the distance dependence of the PRE. We used the enzyme with tryptophan selective labeling for the following reasons: (i) tryptophan residues are widely distributed throughout the three-dimensional structure of the enzyme, and the distance between the trypto-phan NH groups and the FAD ranges from 4.3 to 32.1 Å; (ii) the enzyme has 15 tryptophan residues, and the number of resonances is adequate for observing them without signal overlapping; and (iii) no metabolic migration occurs in the amino acid (tryptophan)-selective labeling.

We measured the [H,15N] HSQC spectra of the CPD photolyase labeled with [α,ε-15N]tryptophan (Fig. 1A, inset), in complexes with CPD-containing ssDNA, in the reduced and radical semiquinone forms, respectively (Fig. 1). In the spectrum of the enzyme in the reduced form (Fig. 1A), 30 resonances derived from 15 tryptophan residues were separately observed. Assignments of the cross-peaks were established by site-directed mutagenesis. In the spectrum of the mutant W351F (Fig. 1B), two resonances were not observed, in comparison with the wild type spectra, and thus the resonances were assigned as Trp-351.

The resonances from Nα and Nε were distinguished by measuring the spectra of the enzyme labeled with [α-15N]- and [ε-15N]tryptophans, respectively (data not shown). In a similar way, all of the resonances in Fig. 1A were assigned.

In the spectrum of the enzyme in the radical semiquinone form, the main-chain HN resonances of Trp-247, Trp-351, and Trp-353 and the side-chain HN resonances of Trp-241, Trp-247, Trp-351, and Trp-353 had disappeared, and those from HNα of Trp-49, Trp-228, Trp-241, Trp-260, Trp-285, Trp-383, and Trp-387 and HNε of Trp-260, Trp-275, Trp-328, and Trp-383 were significantly reduced in intensity, as compared with the spectrum of the enzyme in the reduced form (Fig. 1A and C, and Table I). Based upon the spectra, the intensity reduction ratios of each residue were calculated. Fig. 2 shows the correlation between the intensity reduction ratio and the distance between the observed atoms and FAD in the crystal structure of the enzyme. All of the atoms with resonances absent from the radical form spectrum were within 13 Å of FAD, and all of the atoms with resonances significantly reduced in intensity in the radical form spectrum were 13–21 Å away from FAD.

The radical-induced intensity reduction of the resonances in the HSQC spectra can be converted into the distance between the observed hydrogen atoms and an unpaired electron by the equations proposed by Battiste et al. (19). For the conversion, the τc and RC values should be determined. However, the direct determination of accurate τc and RC values is difficult in large proteins. In the present study, these values were evaluated by fitting the experimental values of the intensity reduction ratios and the distances in the three-dimensional structure to the equations. The evaluated τc and RC values were 13 ns and 1.5 × 104 s⁻¹, respectively. The RC value was reasonable for the molecular mass of the enzyme (47.8 kDa), whereas the RC value was much larger than what we expected. The extraordinarily large RC value indicates that RC(RC + R2p) can be approximated to 1, and the intensity reduction is mainly derived from relaxation during the insensitive nuclei enhanced by polarization transfer. Therefore, the equation was simplified and the intensity reduction ratio was correlated with the distance between the observed atoms and FAD by Equation 3.
from the FAD-induced PRE in the CPD resonances using Equation 3. We prepared a single-stranded DNA (ssDNA) containing 13C-labeled CPD, d[AT(CPD)TAC], and measured the 1H-13C HSQC spectra in complexes with deuterated CPD photolyase for the reduced and radical semiquinone forms, respectively (Fig. 3). Comparing the two spectra, the intensities of the resonances of the base (methyl and 6H) and the 1H11032 positions in CPD were significantly decreased in the spectrum of the radical form complex, whereas those of the other deoxyribose resonances were decreased by 10% (Table II). The intensity reduction ratio indicates that the FAD-CPD distance in the CPD complex, [W49N,H9251, W285N,H9280]-15N tryptophan, complexed with a CPD-containing ssDNA, (TAT[CPD]TATG). Assignments based on site-directed mutagenesis are shown in the spectra.

**Fig. 2.** Plots of the FAD-induced intensity reduction ratio against the distance from FAD in the crystal structure. A fitting curve to the Solomon-Bloembergen equation is shown in the plot. The resonances from W49N, W285N, and W387N were not included, because of the overlap. The resonance from W328N was not included because the resonance was so weak that we could not measure its intensity precisely. The error bars represent the root sum square of the reciprocal of the signal-to-noise ratio of the resonances in reduced and radical semiquinone forms.

from the FAD-induced PRE in the CPD resonances using Equation 3. We prepared a single-stranded DNA (ssDNA) containing 13C-labeled CPD, d[AT(CPD)TAC], and measured the 1H13C HSQC spectra in complexes with deuterated CPD photolyase for the reduced and radical semiquinone forms, respectively (Fig. 3). Comparing the two spectra, the intensities of the resonances of the base (methyl and 6H) and the 1H-position in CPD were significantly decreased in the spectrum of the radical form complex, whereas those of the other deoxyribose resonances were decreased by <10% (Table II). The intensity reduction ratio indicates that the FAD-CPD distance in the CPD complex, [W49N,H9251, W285N,H9280]-15N tryptophan, complexed with a CPD-containing ssDNA, (TAT[CPD]TATG). Assignments based on site-directed mutagenesis are shown in the spectra. A, spectrum of the wild type complex in the reduced form. Upper inset shows the chemical structure of tryptophan. Lower inset shows one-dimensional slices of representative resonances in the spectrum. B, spectrum of the W351F complex in the reduced form. C, spectrum of the wild type complex in the radical semiquinone form. Inset shows one-dimensional slices of representative resonances in the spectrum. In B and C, the resonances that disappeared in comparison with A are enclosed in squares.

**Table I**

| Residue | r_{xray}^{a} | Δr_{int}^{b} | r_{calc}^{c} | |r_{xray}^{a} - r_{calc}^{c}| |
|---------|-------------|-------------|-------------|-----------------|
| W49N_a  | 14.5        | 0.30        | 15.9        | 1.4             |
| W228N_a | 18.6        | 0.15        | 18.2        | 0.4             |
| W241N_a | 14.9        | 0.54        | 13.9        | 1.0             |
| W260N_a | 18.0        | 0.18        | 17.5        | 0.4             |
| W260N_b | 20.5        | 0.16        | 18.0        | 2.5             |
| W275N_a | 16.3        | 0.18        | 17.5        | 1.2             |
| W285N_a | 16.3        | 0.18        | 17.5        | 1.2             |
| W328N_a | 13.8        | 0.52        | 14.1        | 0.3             |
| W383N_a | 19.6        | 0.23        | 16.7        | 2.9             |
| W387N_a | 14.6        | 0.62        | 13.4        | 1.2             |
| W387N_b | 19.7        | 0.22        | 16.8        | 2.9             |

*a* The distances between the observed atoms and the closest heavy atoms of the isoalloxazine ring of FAD were measured in the crystal structure of *T. thermophilus* CPD photolyase in the substrate free form (10).

*b* Intensity reduction ratio (1 - I_{rad}/I_{red}).

*c* The distances were calculated from the intensity reduction ratio, using Equation 3 in the text.
shown in the spectra. Resonance assignments performed in the previous NMR study (20) are CPD photolyase in the reduced and radical forms, respectively. Resonances with chemical shifts similar to those of the methyl groups in DNA lesion in photolyase-ssDNA complex are enclosed in squares. The intensity reduction ratios of the resonances were 0.5.

**FIG. 3.** FAD-induced PRE in the NMR resonances from the CPD lesion in the CPD photolyase-ssDNA complex. A and B show 1H-13C HSQC spectra of a ssDNA containing 13C-labeled CPD, d(AT[CPD]TAC), in complexes with excess amounts of the deuterated form complex. The distances between FAD and CPD in the CPD photolyase-DNA complex is 16 ± 3 Å (Table II).

**TABLE II**

The distances between the CPD and FAD in the CPD photolyase-DNA complex calculated from FAD-induced PRE

Residues with resonances reduced in intensity by more than 10% are shown.

| Residue | Int | \( r_{\text{calc}} \) |
|---------|-----|-------------------|
| CH₃ (5' side) | 0.27 | 16.2 |
| CH₃ (3' side) | 0.25 | 16.4 |
| 6H | 0.26, 0.31 | 16.3, 15.7 |
| 1'c | 0.10, 0.24 | 17.8, 16.5 |

\( a \) Intensity reduction ratio \((= 1 - I_{\text{red}}/I_{\text{calc}})\).  
\( b \) The distances were calculated from the intensity reduction ratio, using Equation 3 in the text.  
\( c \) Of the two resonances, we did not determine which is from the 5' or 3' side.

**DISCUSSION**

The present study using FAD-induced PRE shows that the distance between FAD and CPD in the *T. thermophilus* CPD photolyase-DNA complex is \( \sim 16 \) Å. In this study, the correlation between the FAD-induced intensity reduction and the distances between the observed atoms and FAD was investigated by the resonances from tryptophan residues. The intensity reduction ratios and the distances fit well with the Solomon-Bloembergen equation (Fig. 2). These results suggest that the intensity reduction observed in the radical semiquinone form was derived predominantly from FAD-induced PRE. This analysis is based on the assumption that the oxidation states of FAD do not significantly affect the conformation of the CPD photolyase-DNA complex. We concluded that this assumption is valid for the CPD photolyase-DNA complex for the following reasons. First, in the 31P NMR analysis of the CPD photolyase-DNA complex, the chemical shifts of the phosphates of CPD-containing DNA were not affected by the oxidation state (Fig. 5). The chemical shift values of the 31P NMR resonances are dependent on the backbone conformation of the DNA (26), and thus the result shows that the backbone conformation of CPD-containing DNA in the complex is not perturbed by the oxidation state. Second, the chemical shifts of the tryptophan residues in the 1H,15N HSQC spectra were not significantly affected by the oxidation state (Fig. 1A). This result indicates that the overall conformation of the enzyme is not significantly influenced by the oxidation state. Third, the chemical shifts of the resonances from the CPD in the complex were not affected by the oxidation state (Fig. 3). In addition to the conformation of the CPD, the aromatic groups in the CPD-binding site affect the chemical shifts of the resonances from CPD (20). Therefore, the results suggest that the conformation of the CPD-binding site is not significantly perturbed by the oxidation state. Finally, previous studies demonstrated that the affinity to CPD-containing DNA was not influenced by the FAD oxidation state (6, 27).

The FAD-CPD distance in the CPD photolyase-DNA complex has been predicted in previous studies. Several groups have proposed CPD photolyase-DNA complex models, based on molecular dynamics simulations (28–30). However, the relative orientations of FAD and CPD in the models did not agree with each other. Studies of the CPD photolyase-DNA complex using electron paramagnetic resonance (31), UV-visible absorption spectroscopy (32), and resonance Raman spectroscopy (33) were recently reported. The studies were based on observations of the FAD signals and comparisons between the signals in the substrate-free form and those in the complex form. The FAD signals are equally perturbed by both the direct enzyme-DNA interactions and the DNA-induced conformational change of
W353A mutants all exhibited significantly less repair activity than that of the wild type (20). The R201A, K240A, W247A, and W353A mutants of these residues were measured (Table III). In the previous NMR study established in the previous NMR study (20) are shown in the spectra.

The enzyme. On the other hand, the present study was based on the observations of DNA signals and the comparison between the signals in the reduced and radical semiquinone forms, respectively. Assignments of the resonances established in the previous NMR study (20) are shown in the spectra.

For further insight into the CPD-repair mechanism, we constructed a model of the photolyase-DNA complex based on the NMR analysis results, as follows: (i) the CPD base is 16 Å away from FAD; (ii) the CPD bases are closer to FAD than the deoxyribose; and (iii) the 5’ and 3’ sides of the DNA bind to clusters I and II of the enzyme, respectively (20) (Fig. 6).

The model revealed two characteristics. First, the O4 of the 5’ side of CPD is close to Arg-201 and Lys-240 (Fig. 6A). During the CPD repair reaction, a CPD anion intermediate is formed after the electron transfer from FAD to CPD (34, 35). The O4 of the 5’ side of the intermediate is supposed to be negatively charged (36). Thus, Arg-201 and/or Lys-240 might form electrostatic interactions with the CPD anion intermediate, thus prolonging the lifetime of the CPD anion. Second, several aromatic groups exist between FAD and the CPD. Trp-247, Trp-353, separate the donors and acceptors, leading to a decrease in the back electron transfer rate (37). The prolonged lifetime of the CPD anion intermediate reduced the back electron transfer rates, leading to the high efficiency of the reactions (37). Thus, Arg-201, Lys-240, Trp-247, and Trp-353 may play important roles in the CPD-repair reaction.

To examine the role of Arg-201, Lys-240, Trp-247, and Trp-353 in the CPD-repair reaction, the CPD-repair activities of the mutants of these residues were measured (Table III). In the previous study, it was confirmed that the global folds and the structures around the FAD for all mutants were not affected by the introduction of the mutations, by their 1H NMR spectra, and UV-visible absorption spectra, respectively, and all mutants except for W353A in Table III exhibited similar substrate-binding activities, and they were remarkably lower than that of the wild type (20). The R201A, K240A, W247A, and W353A mutants all exhibited significantly less repair activity than the wild type, whereas the mutants of other residues within the DNA-binding region, R141A and R376A, retained activity equivalent to that of the wild type. These results suggest that Arg-201, Lys-240, Trp-247, and Trp-353 play an important role in both the substrate-binding and the CPD-repair reaction.

CPD photolyase has been proposed to induce the CPD flipping in dsDNA (20, 38). If the CPD bases are located inside the DNA helix in the complex, then the CPD should be more than 20 Å away from the FAD, and FAD should not induce PRE. Thus, the FAD-induced PRE in the CPD photolyase-DNA complex (Fig. 4) supports the CPD-flipping recognition mecha-

![FIG. 5. Effect of the FAD oxidation states on the backbone conformation of DNA in complex with CPD photolyase. A and B show 31P NMR spectra of d(TAT(CPD)TATG) in complex with excess amounts of CPD photolyase in the reduced and radical semiquinone forms, respectively. Assignments of the resonances established in the previous NMR study (20) are shown in the spectra.](image)

![FIG. 6. CPD photolyase-CPD complex model based on the NMR analysis results. The structure of CPD is from the NMR structure of a CPD-containing ds12mer (PDB code: ITTD) (26) and that of the enzyme is from the crystal structure of T. thermophilus CPD photolyase in the substrate-free form (PDB code: 1IQR) (10). They were docked based on the NMR analysis results as described in the text. A and B are viewed from the opposite side. In both of the models, the FAD is yellow, and the CPD is magenta. In A, Arg-201 and Lys-240, which are supposed to stabilize the CPD anion intermediate, are cyan. The O4 of the 5’ side of CPD, which is supposed to be negatively charged in the CPD anion intermediate, is shown by a circle. In B, Trp-247 and Trp-353, which are supposed to mediate the electron transfer from FAD to CPD, are green. Putative electron transfer pathways are shown by arrows. The molecular diagrams were generated with WebLab Viewer Pro (Molecular Simulations, Inc.).](image)

| TABLE III | CPD-repair activity of the CPD photolyase mutants of the putative catalytically important residues |
|-----------|-------------------------------------------|
|           | ε0° | μ^-1 cm^-2 × 10^-5 |
| Wild type | 1.1 | 0.04 |
| W247A     | 0.8 | 0.005 |
| W353A     | 0.2 | 0.14 |
| R201A     | 0.8 | 0.16 |
| K240A     | 0.8 | 0.07 |
| R141A     | 1.1 | 0.07 |
| R376A     | 1.1 | 0.2 |

*Photoreactivation cross-section (ε0) at 370 nm was determined as previously described (24). Each value represents the average ± S.D. of two experiments.*
nism. dsDNA containing $^{13}$C-labeled CPD will provide more information on the orientations of FAD and the CPD lesion in the enzyme-dsDNA complex and further evidence of the CPD flipping.

The study of model compounds, in which the isalloxazine ring is designed to be close to the CPD base, have been performed, aiming toward the development of artificial photolyase enzymes that repair CPD in vivo and bring relief to patients suffering from xeroderma pigmentosum or trichothiodystrophy (39, 40). However, the efficiency of the model compounds was less than one-tenth of that of the CPD photolyase. This study has shown that CPD photolyase may improve the efficiency of the reaction by inhibition of back electron transfer and stabilization of the CPD-anion intermediate. Improvement of the model compounds based on these results may increase their catalytic efficiency.

The PRE effect provides the distance information in various systems (12–19). In the present study, we demonstrate that PRE is useful also for analyzing the interactions between FAD- and the CPD lesion in enzymes that repair CPD

Acknowledgments—We thank Dr. M. Osawa and M. Sakakura for their stimulating advice.

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NMR Study of Repair Mechanism of DNA Photolyase by FAD-induced Paramagnetic Relaxation Enhancement

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J. Biol. Chem. 2004, 279:52574-52579.
doi: 10.1074/jbc.M409942200 originally published online October 1, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M409942200

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