DNA Photolyase is a flavoprotein that uses light to repair cyclobutylpyrimidine dimers in DNA. From considerations of the crystal structure of the protein, it has been hypothesized that the dimer lesion is flipped out of the DNA double helix into the substrate binding pocket. We have used a fluorescent adenine analog, 2-aminopurine (2-Ap), as a probe of local double helical structure upon binding of the substrate to the protein. Our results show that the local structure around the thymidine lesion changes dramatically upon binding to Photolyase. This is consistent with base flipping of the lesion into the protein binding cavity with concomitant destacking of the opposing complementary 2-Ap nucleotide.

Cyclobutylpyrimidine dimers (CPDs) are lesions formed in DNA between adjacent pyrimidines upon absorption of UV light. These lesions cause replicational errors and can lead to cell death or cancer if left unrepaired (1, 2). One repair protein, DNA photolyase, incorporates a non-covalently bound FAD and requires blue light for repair (3). There is ample evidence that repair of the CPD proceeds by electron transfer from a photoexcited fully reduced FADH$^-$ to the CPD, which subsequently monomerizes within 2 ns (3). Studies by Payne et al. (4) have demonstrated that the oxidized enzyme can bind CPD-containing DNA but cannot efficiently repair the CPD lesion. As we show below, this differential behavior is extremely useful in understanding the substrate binding mode of photolyase.

The crystal structures of the Escherichia coli and Aspergillus nidulans holoenzymes were solved by Kim et al. (5) and Tamada and co-workers (6), respectively. These crystal structures revealed important structural elements that were both familiar and surprising. It was noted that the cavity has approximately the correct dimensions to enclose the CPD if the thymidines were folded in a coplanar geometry, leading to the prediction that photolyase would bind the CPD by “flipping out” the lesion from the double helical DNA (5). The FAD$^-$ cofactor was found to lie at the bottom of this cavity, consistent with the ability of the cofactor to resist oxidation by molecular O$_2$. The surface of the protein above the cavity incorporates a strip of positively charged amino acid residues that are thought to help orient the negatively charged phosphate backbone of the damaged DNA strand.

Unfortunately, no crystal structure of the enzyme-substrate complex is available, but a recent crystal structure of Thermus thermophilus complexed with thymine was published by Komori and co-workers (7), which shows the thymine residing in the putative substrate cavity. While thymine is not a substrate, it can be thought of as a partial product of the repair reaction. This is the strongest evidence regarding the substrate binding mode of photolyase for CPDs. However, it does not clarify whether one or both of the thymine bases of the CPD is bound in the cavity.

Other experimental evidence for the mode of substrate binding is available. Most notable are the ethylation and footprinting studies of Husain et al. (8). They showed that the ethylation of the phosphates 3’ and 5’ to the CPD inhibit the binding of the lesion strand to photolyase. Ethylation of phosphate groups on the complementary strand had no effect on binding. Footprinting experiments showed that only 6–7 bases around the CPD were protected by the protein. This small footprint is significant in that it suggests that photolyase binds substrate in a structure-specific, as opposed to a sequence-specific manner.

Binding studies using specific nucleotide sequences have shown that distortions of the backbone caused by the CPD aid in the recognition of the lesion by PL (9). Alanine substitution mutagenesis studies by Berg and Sancar (10) also support this hypothesis. The question has sparked several computational studies as well, most of which support the base flipping hypothesis (11, 12). Thermodynamic studies suggest that there is little energy cost for flipping the CPD into solution (13).

There are several DNA-binding proteins that appear to manipulate DNA by flipping out bases from the helical structure (14). For example, DNA methyltransferase is thought to base flip an adenine into the enzyme for methylation (15, 16). Another repair protein, T4 endonuclease V, has been shown to flip an adenine opposing the CPD lesion out of the helix (17). Several of these studies have used a fluorescent adenine analog, 2-aminopurine (2-Ap), as a reporter of helical structure. 2-Ap can be incorporated into a DNA oligomer using automated synthesis and has a strong absorption band centered at 303 nm (ε = 6000 M$^{-1}$ cm$^{-1}$ for the nucleotide) (18). High quantum yield emission peaks at about 370 nm for the nucleoside in aqueous solution (19). When 2-Ap is incorporated into oligomeric ss-DNA, the emission is significantly quenched and blue-shifted (20). When a 2-Ap containing ss-DNA oligomer is annealed to its complement, the 2-Ap fluorescence is further quenched by base stacking (16). Thus, the quantum yield and emission maximum of 2-Ap are effective reporters of base stacking and solvent exposure (polarity), respectively.

We have utilized these properties to probe base flipping in photolyase by incorporating 2-Ap opposite a thymidine dimer.
Any structural changes that occur in the region of the lesion would be detected as changes in quantum yield and perhaps emission maximum. 2-Ap absorbs in the same spectral window as reduced photolyase. This will complicate the analysis of the experiment, because the 2-Ap emission will come from oligonucleotides in various stages of repair. The rate of repair of CPDs by DNA photolyase is in the nanosecond range (although the turnover rate is much slower (21)). To circumvent this difficulty the oxidized enzyme (PL<sub>ox</sub>) is used, which can bind the CPD lesion without repairing it. The results using oxidized enzyme show that base flipping is operative in DNA photolyase. Experiments using the reduced enzyme demonstrate that the 2-Ap-containing CPD duplex is a substrate for photolyase.

**EXPERIMENTAL PROCEDURES**

**Photolyase Protein—**E. coli DNA Photolyase was overexpressed from JM109 cells transformed with the pMS969 plasmid containing the phr gene (the plasmid was a generous gift of Professor Aziz Sancar, University of North Carolina, Chapel Hill, NC). The purification of the protein was based on the method of Payne et al. (4) and modified as described in detail in a previous paper (22). Briefly, E. coli photolyase was isolated in the blue semiquinone form. The apoprotein was made by stripping the FAD and folate chromophores using low pH buffers containing saturated KBr (23). The apoprotein was reconstituted with oxidized FAD and purified on a phenyl-Sepharose column. The purity of the reconstituted protein was determined by the ratio of absorbances at λ = 280 nm (protein) and 450 nm (FAD). The ratio of A<sub>250</sub>/A<sub>450</sub> was typically around 20–25. Pure protein would have a ratio of about 12 based on the known extinction coefficients of the apoprotein and FAD-reconstituted protein (24), indicating that the protein purity was about 60%. Further purification was deemed unnecessary as apophotolyase will not bind substrate (25).

**DNA Oligonucleotides: Irradiation and Purification—**Two HPLC-purified 11-mer oligonucleotides were purchased from Integrated DNA Technologies, Inc. The 2-Ap-containing oligonucleotide with the sequence 5'-CTCCTAACCTG-3' (‘2-Ap,’ where A = 2-Ap) and its complement, 3'-GAGGTGAACAG-5' (‘2-T’) were resuspended in HPLC-grade water and used without further purification, though their purity was checked by reverse phase HPLC on a C<sub>18</sub> column (data not shown).

The 2-T oligonucleotide was irradiated with UV-A light to produce bisulfate. 1.0 mM T-T 11-mer and 1 mM acetophenone in 10 mM Tris containing 50 mM sodium acetate was reacted with water to produce bisulfate. 1.0 mM T-T oligonucleotide was collected and desiccated to dryness, and without a CPD (56 and 48 °C) (27).

**RESULTS**

**Preparation and Purification of Target Oligonucleotides—**The duplexes used in this study were as follows,

T-T: 5'-GCAAGTTGGAG-3’

2-Ap: 3'-CGTCCAACCTC-5’

and

T <T> T: 5'-GCAAGTTGGAG-3’

2-Ap: 3'-CGTCCAACCTC-5’

where the underlined A indicates the position of the 2-amino purine base in the 2-AP containing ss-DNA, T-T stands for the complementary strand, and T <T> T indicates the cyclobutylthymidine dimer-containing oligonucleotide (the position of the lesion is indicated by TT). UV-A lamps were used to generate the CPD lesion in the TT-containing 11-mer oligonucleotide, and HPLC purification of the photoproduct was performed as described by Banerjee et al. (26). It should be noted that the complementary strand sequence is a subset of that used by Husain et al. (8) in their ethylation studies.

**Activity of the Reconstituted Photolyase—**To verify that the HPLC-purified DNA photoproduct contained the cis-syn dimer, a 1 μmol solution of this oligomer was mixed with 0.50 μmol “blue” photolyase (the holoenzyme containing both folate and flavin chromophores) in buffer T. The mixture was deoxygenated and then reduced with 12 μM sodium dithionate. Irradiation of this mixture with 365 nm light from a UV hand lamp (∼500 μW/cm<sup>2</sup>) resulted in photoreactivation of the dimer lesion as measured by the recovery of absorbance at 265 nm (data not shown) (23, 25). The absorbance change at this wavelength corresponds to the reappearance of the C5 = C6 and C5’ = C6’ double bonds in the thymidine nucleotides, which were lost upon dimerization. The negative absorbance at 1.230 nm is due to the greater extinction coefficient of the CPD relative to the repaired thymines (27).

**Determination of T<sub>m</sub> for the 2-Ap Duplexes—**It is important to ascertain whether changes in the fluorescence quantum yield of the 2-Ap probe are due to conformational changes of the helix produced by protein binding or a partial melting of the duplex due to perturbations introduced by the CPD and/or 2-Ap base. Fig. 1 shows the melting curves obtained for the 2-Ap<T>T and 2-Ap<T>T duplexes by monitoring the 2-Ap fluorescence as a function of temperature. Melting points were derived by finding the temperature corresponding to the maximum of the derivative of the melting curve. The melting point of the 2-Ap/T-T duplex is T<sub>m</sub> = 48 °C, while the 2-Ap<T>T duplex has a T<sub>m</sub> = 42 °C (±2 °C). The 6 °C difference between the melting points agree well with that obtained for a decamer duplex with and without a CPD (56 and 48 °C) (28), although the absolute...
would be small. Other experiments performed at 15°C (29). However, the melting points for the duplexes used in this study are reported for the peak fluorescence intensity and the integrated intensity of the fluorescence spectrum between 350/400 nm. The samples for the protein-duplex studies were prepared by guest on July 24, 2018http://www.jbc.org/Downloaded from

![Graph](image)

**Fig. 1.** DNA melting curves obtained by measuring the fluorescence intensity versus temperature. The 2-Ap/T-T duplex shows a melting point of about $T_m = 48 \degree C$. The 2-Ap/T<>T duplex melts at a lower temperature, $T_m = 42 \degree C$.

$T_m$ is about 8 \degree C lower than for the decamer without the 2-Ap. We ascribe this difference to the aminopurine itself, which has been shown to lead to lower melting points, as much as 10 \degree C (29). However, the melting points for the duplexes used in this study are well above the temperature at which the quenching experiments were done (23 \degree C) where any melting artifacts would be small. Other experiments performed at 15 \degree C in our laboratory have given similar results (data not shown).

**Emission Spectra of 2-Ap Oligomers and Duplexes**—The emission spectra of ss- and ds-DNA oligomers were measured with excitation at 317 nm. The buffered samples consisted of (a) 0.50 \muM 2-Ap oligonucleotide, (b) 0.50 \muM 2-Ap oligonucleotide annealed to 0.50 \muM T-T oligo, and (c) 2-Ap containing oligonucleotide annealed to 0.50 \muM T<>T oligonucleotide. The spectra are shown in Fig. 2a. Each spectrum is the average of three separate experiments and preparations.

Quantification of the changes in fluorescence in these scans is reported for the peak fluorescence intensity and the integrated intensity of the fluorescence spectrum between 350–400 nm. This range was chosen to reduce the potential influence of Rayleigh scattering of the excitation wavelength into the emission spectrum. These quantities are ratioed to the 2-Ap oligonucleotide value in each case to get the relative quenching. The values are shown in Table I. Good agreement between peak and area values were obtained.

The single-stranded 2-Ap 11-mer in buffer had the highest fluorescence yield, with an emission maximum of 368 ± 2 nm. Addition of one equivalent of its complementary T-T oligonucleotide followed by heating to 62 \degree C with slow cooling (2-Ap/T-T) quenched the 2-Ap fluorescence by a factor of about 7 ($I_{2,Ap,T-T}$ = 0.15 ± 0.03). The emission maximum was unchanged within experimental error. We take this as evidence for double-helix formation and Watson-Crick base pairing between the 2-Ap and the opposing thymidine on the complementary strand, as 2-Ap is known to undergo significant quenching due to base stacking facilitated by favorable Watson-Crick base pairing (15–17, 20, 30).

When one equivalent of the T<>T complementary oligonucleotide was annealed with the 2-Ap oligonucleotide, fluorescence emission was quenched by a factor of about 4 ($I_{2,Ap,T<>T}/I_{2,Ap} = 0.25 ± 0.03$) when compared with the ss-2-Ap 11-mer. The slight increase in fluorescence yield compared with 2-Ap/T-T can be ascribed to a reduction in base stacking at the 2-Ap site due to the perturbation introduced by the CPD on the opposing strand. This interpretation is consistent with the results from a solution state NMR structure of a CPD-containing dodecamer DNA duplex (28) in which the 3'-side of the CPD is perturbed relative normal B-DNA structure. In that study, a decided propeller twist of the T-..A planes across the helix was observed, resulting in a loss of base stacking of the 5'-adenine opposing the CPD. The hydrogen bond length between these two bases is also slightly longer than that for other base pairs in the dodecamer. Nordlund and co-workers (29) have explored the changes in duplex structure and rigidity due to the incorporation of a 2-Ap base in a decamer duplex. They found that 2-Ap is less rigidly constrained and more prone to spontaneous excursions out of the duplex, leading to less base stacking. Both a loss of base stacking and base pairing are consistent with the increase in fluorescence yield in our experiments.

Interestingly, the 2-Ap/T<>T duplex exhibited a 6 nm red shifted emission maximum compared with the 2-Ap 11-mer. In terms of solvatochromism, a red shift is expected if a chromophore goes from a less polar to a more polar solvent, as would be the case if 2-Ap suffered more solvent exposure (31). An additional requirement for the red shift is that the permanent dipole moment of the excited state is greater than the ground state dipole moment, which has been verified experimentally (32). However, this red shift is not apparent in the emission spectrum of the single-stranded 2-Ap oligonucleotide, which suggests that the 2-Ap base opposing the thymidine dimer has neither flipped out into solvent nor retained its normal interactions in the double helix. One possible explanation is that the 2-Ap undergoes enough of a rotation that its difference dipole moment relative to the helix electric field produces the observed red shift, although this is only a speculation.

**Emission Spectra of 2-Ap Oligomers and Duplexes with Oxidized Photolyase**—The samples for the protein-duplex studies consisted of (d) 0.50 \muM 2-Ap, 0.55 \muM PLox, (e) 0.50 \muM 2-Ap, 0.50 \muM T-T, 0.55 \muM PLox and (f) 0.50 \muM 2-Ap, 0.50 \muM T<>T, 0.55 \muM PLox. A control sample of 0.55 \muM PLox was also scanned and subtracted from samples d–f to remove buffer and protein fluorescence and Raman scattering (scan not shown). The results are shown in Fig. 2b and the quantitative analysis summarized in Table II. Addition of photolyase to the ss-2-Ap oligonucleotide gave essentially no change in the fluorescence yield or wavelength. This shows that there is little interaction between the 2-Ap oligonucleotide and PLox, which is consistent with the low association constant for a single stranded oligo without a CPD lesion ($K_a = 10^3 \text{ M}^{-1}$) (10).

The fluorescence yield of the 2-Ap/T-T/PLox sample is somewhat higher than without protein ($I_{2,Ap,T-T}/I_{2,Ap,PL} = 0.19 ± 0.06 \text{ versus } 0.15$), and the emission maximum is the same within experimental error. Photolyase is known to bind undamaged duplex DNA with higher affinity than ss-DNA (9). For the normal duplex 11-mer ($K_a = 10^4 \text{ M}^{-1}$) only about 0.003 \muM will bind (nonspecifically) to photolyase. The crystal structure shows that the substrate binding cavity is in the middle of a positively charged strip of amino acid residues (5). These residues undoubtedly attract and bind the negatively charged phosphate backbone of the duplex whether or not it has a CPD (9). The increased fluorescence may be the result of small structural changes to the helix bound to the protein surface.

When reconstituted oxidized photolyase was added to the 2-Ap/T<>T duplex, the fluorescence increased by a factor of about 5 relative to the 2-Ap/T-T/PLox sample and is very close to the emission yield observed for the single-stranded 2-Ap/
Cyclobutylpyrimidine Dimer Base Flipping By DNA Photolyase

PLox sample. The emission maximum of the enzyme-bound duplex shifted back to 368 ± 2 nm from the 374 nm peak for 2-Ap/T-T in buffer alone. Using an association constant of $K_A = 10^9$ M$^{-1}$ for a DNA duplex containing a CPD (10), the concentration of protein-substrate complex will be about 0.49 μM (or about 98%) of the duplex is bound. Such a large change in fluorescence yield indicates that a severe distortion of the local helical structure has occurred around the 2-Ap base.

The band shape of the emission spectra for the duplexes with PLox was somewhat different from that of the duplexes alone. Part of the fluorescence increase occurs below and above the 2-Ap emission range (roughly 340–460 nm). It is possible that this may be due to energy transfer from the excited 2Ap base to the FAD cofactor, which has significant absorption below 490 nm. However, oxidized photolyase alone show very little fluorescence when excited directly with 317 nm light (data not shown), and its fluorescence emission is peaked at about 520 nm. It is more likely that the broad fluorescence signal is due to a distribution of conformations experienced by 2-Ap as the duplexes bind non-specifically to the photolyase protein.

Reduced PL Repair Assay—Using the oxidized enzyme for these experiments has the advantage that base flipping can be observed without significant repair of the CPD during the fluorescence scans. However, to verify that the 2-Ap/T-T duplex is a true substrate for photolyase, we performed fluorescence experiments after reduction of the photolyase by dithionite.

A sample consisting of 0.50 μM 2-Ap, 0.50 μM T<>T, 0.55 μM PLox was transferred to the 10-mm fluorescence quartz cuvette and subsequently purged with argon to achieve anoxic conditions. Four equivalents (in a 2-μl volume) of sodium dithionite was added to reduce the photolyase. The sample was allowed to sit for 1 h to allow any excess dithionite to react to form bisulfate. All preparative steps were performed under yellow lights, and all subsequent measurements were done in the dark so that no unwanted photorepair occurred.

The cuvette containing the reduced enzyme-substrate was exposed to 365 nm light over 30 min, and fluorescence emission spectra were taken at 5-min intervals. The results can be seen in Fig. 3, where $I(t) - I(0)$ is plotted versus emission wavelength. As repair proceeds the 2-Ap fluorescence is quenched. This is consistent with repair of the CPD lesion and subsequent dissociation of the protein-substrate complex. No shift in the emission maximum was observed. The 2-Ap fluorescence decreases to about $3.7 \times 10^5$ at $I_{\text{max}}$ which is roughly equal to the difference between the fluorescence yield of II(2-Ap/T-T/PLox) and I(2-Ap/T-T/PLox) (=3.4 × 10^5). Reduction of the photolyase protein with dithionite in the presence of the 2-Ap/T-T duplex showed no effect on the 2-Ap fluorescence as long as the sample was not exposed to blue light (data not shown). Dithionite treatment of a 2-Ap/T<>T sample had no effect on its fluorescence yield (data not shown).

\[ \text{TABLE I} \]

| Sample | $\lambda_{\text{max}}$ | $I_{\text{max}}$ | $I_{\text{max}}$ | Area (350–400 nm) | Area/area(2-Ap) (350–400 nm) |
|--------|-----------------|----------------|----------------|-----------------|----------------|
| 2-Ap   | 368 ± 2         | 5.6 (± 0.6) × 10^6 | 1              | 1.2 (± 0.1) × 10^7 | 1              |
| 2-Ap/T-T| 368 ± 2         | 8.3 (± 2.4) × 10^6 | 0.15 ± 0.03    | 1.6 (± 0.6) × 10^7 | 0.15 ± 0.04  |
| 2-Ap/T<>T| 374 ± 2         | 1.36 (± 0.05) × 10^7 | 0.25 ± 0.03    | 3.1 (± 0.1) × 10^6 | 0.26 ± 0.02  |

\[ \text{TABLE II} \]

| Sample | $\lambda_{\text{max}}$ | $I_{\text{max}}$ | $I_{\text{max}}$ | Area (350–400 nm) | Area/area(2-Ap) (350–400 nm) |
|--------|-----------------|----------------|----------------|-----------------|----------------|
| 2-Ap/PLox | 368 ± 2         | 5.2 (± 0.4) × 10^5 | 1              | 1.1 (± 0.9) × 10^7 | 1              |
| 2-Ap/T-T/PLox | 370 ± 2         | 1.1 (± 0.1) × 10^5 | 0.19 ± 0.06    | 2.4 (± 0.5) × 10^6 | 0.21 ± 0.03  |
| 2-Ap/T<>T/PLox | 368 ± 2         | 4.5 (± 0.4) × 10^5 | 1.06 ± 0.07    | 9.7 (± 0.8) × 10^6 | 0.89 ± 0.11  |
PLred complex. If base flipping occurs in photolyase then it would some-
E. coli by destroying base stacking interactions. The protein, exposing the complementary 2-Ap base to solvent and 
CPD has been flipped out of the helix into the cavity of the 
dherence that local melting has occurred and suggests that the 
conformational change to the duplex around the 2-Ap probe 
observed loss of fluorescence (quenching).

![Figure 3: Fluorescence-based repair assay of the 2-Ap/T<>T/PLred complex.](http://www.jbc.org/)

**FIG. 3.** Fluorescence-based repair assay of the 2-Ap/T<>T/ 
PLred complex. The FAD cofactor was reduced to FADH₂ by dithion- 
ite. The spectra are corrected by subtracting the pre-exposure fluores-
cence. The exposure time to 365 nm reactivating light is indicated on 
the figure. After 25 min repair is nearly complete, leading to the 
observed loss of fluorescence (quenching).

**DISCUSSION**

These experiments show that binding of the CPD substrate 
by *E. coli* DNA photolyase is accompanied by a substantial 
conformational change to the duplex around the 2-Ap probe 
base. This local disruption of helical structure is strong evidence 
that local melting has occurred and suggests that the 
CPD has been flipped out of the helix into the cavity of the 
protein, exposing the complementary 2-Ap base to solvent and 
destroying base stacking interactions. Other studies have suggested that base stacking will be 
disrupted by photolyase at the 5'-A opposite the 3'-T of a CPD. 
The work of Husain et al. (8) is particularly noteworthy in that 
it shows that the N3 of the complementary adenine opposing 
the 5'-T of the CPD can be methylated. The accessibility of this 
adene points to the kind of duplex disorder observed in our 
fluorescence experiments. Unfortunately, no mention is made 
of whether the adenine opposing the 3'T shows similar acces-
sibility, as this adenine has been replaced by 2-Ap in our 
experiments.

The mutagenesis and ethylation experiments of Berg and 
Sancar (10) were used by these authors to justify a model where the 
CPD is flipped into the photolyase cavity and stabilized by 
hydrophobic and electrostatic interactions. The authors predict 
that the 3'T of the CPD interacts more strongly with the 
protein binding pocket. As in the work of Zhao et al. (35) they 
measured a 2-fold increase on equilibrium binding affinity for a 
2-Ap-containing duplex versus the complementary duplex. 
They measured the dynamics of the base flipping reaction 
using fluorescence stopped-flow methods, obtaining a flipping 
rate of about 21 s⁻¹. It will be interesting to see whether the 
rate of flipping a CPD or (6-4) lesion differ from single nucleo-
 tide rate.

Finally, base flipping has been observed for T4 endonuclease 
V, which repairs CPDs in a light-independent manner (17). In 
this protein it is thought that the 3'-A base opposing a CPD is 
flipped into the endonuclease protein cavity, exactly the oppo-
site behavior hypothesized for photolyase. McCullough and 
co-workers (17) observed no change in 2-Ap fluorescence when 
the 2-Ap opposed the 3'-T of the CPD, in sharp contrast to our 
results. We have preliminary data that show a similar fluores-
cence increase using a 3'-2-Ap probe as compared with the 5'- 
2-Ap case presented herein (data not shown). Quantitative anal-
ysis of the 3'-2-Ap results will provide more detailed information 
on how photolyase flips its substrate into the protein cavity, 
where it is repaired with nearly unit quantum efficiency.

Taken together, these data show that photolyase distorts the 
CPD-containing DNA duplex to such an extent as to disrupt 
base stacking around the lesion, strongly supporting the base 
flipping model originally proposed by Park et al. (5). The 
intriguing photolyase-thymine cocrystal structure introduces an 
ambiguity as to whether one or both thymines in the CPD 
actually enters the cavity. In another study, we have shown 
that both thymines are necessary to produce an apparent elec-
 trometric shift in the PLred-CPD absorption spectrum (22). 
Thus we expect that the entire CPD is accommodated by the 
protein cavity. Further experiments are planned to extend our 
knowledge of the extent to which the duplex is deformed to 
repair the CPD. The 2-Ap approach used in this study can be 
what mimic an abasic site for the opposing 2-Ap base. Our 
results show a factor of 5 increase in fluorescence when the 
target duplex is bound by photolyase. This suggests that the 
deformation of the duplex by base flipping is considerably 
greater than that generated by an abasic lesion, supporting the 
base flipping model.

The computational work of Sanders and Wiest (12) agrees 
well with our results. They performed molecular dynamics 
simulations of the protein-substrate complex, which predicts 
that a base-flipped CPD, is stabilized by the protein binding 
site over the duration of the simulation. Specifically, their 
simulation shows that the 3'T will experience disruption of 
base pairing and stacking, whereas the 5'T is much less 
perturbed.

Zhao et al. (35) used mismatched bases opposing the (6-4) 
DNA lesion as a target duplex to probe whether (6-4) pho-
lyase base flips this substrate for repair. Since a mismatched 
sequence would give greater conformational flexibility to the 
lesion, it was expected that the binding affinity for this target 
would be higher than for the completely complementary se-
queness if base flipping were operative. They observed that 
the mismatch duplex had a factor of two higher affinity than the 
complementary duplex. Given the high homology between (6-4) 
and CPD photolyase (36), it is reasonable to presume that both 
(6-4) and CPD photolyases use base flipping for substrate 
binding.

Allan et al. (37) have also used mismatched base pairs to 
confirm that base flipping occurs in EcoRI DNA methyltrans-
ferase. This DNA-modifying enzyme transfers a methyl group 
from S-adenosyl-L-methionine to adenine N⁶. By replacing 
the target adenine with 2-Ap they showed that prior to the meth-
ylation step the target adenine is flipped out into a hydrophobic 
pocket of the protein. As in the work of Zhao et al. (35) they 
measured a 2-fold increase on equilibrium binding affinity for a 
2-Ap-containing duplex versus the complementary duplex. 
They measured the dynamics of the base flipping reaction 
using fluorescence stopped-flow methods, obtaining a flipping 
rate of about 21 s⁻¹. It will be interesting to see whether the 
rate of flipping a CPD or (6-4) lesion differ from single nucleo-
tide rate.

In conclusion, we have shown that base flipping 
occurs in photolyase and that it is 
the dominant mechanism for CPD repair. 
Our results support the base flipping 
model originally proposed by Park et al. (5) 
and provide further evidence for its 
importance in DNA repair. Further 
experiments are needed to 
fully understand the 
mechanism of base flipping 
in DNA photolyase.
extended into the time domain by stopped-flow and time-resolved fluorescence techniques. Such experiments are under way in our laboratory.

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