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Structure and Function of DNA Photolyase

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ABSTRACT: Cyclobutane pyrimidine dimers (Pyr<>Pyr) are the major DNA photoproducts induced by the UV component of solar radiation. Photoreactivating enzyme (DNA photolyase) repairs DNA by utilizing the energy of visible light to break the cyclobutane ring of the dimer. Photolyases are monomeric proteins of 50–60 kDa with stoichiometric amounts of two noncovalent chromophore/cofactors. One of these cofactors is $FADH^-$, and the second chromophore is either methenyltetrahydrofolate (MTHF) or 8-hydroxy-5-deazariboflavin (8-HDF). The enzyme binds the DNA substrate in a light-independent reaction, the second chromophore of the bound enzyme absorbs a visible photon and, by dipole–dipole interaction, transfers energy to $FADH^-$ which, in turn, transfers an electron to Pyr<>Pyr in DNA; the $Pyr<>Pyr^-$ splits and back electron transfer restores the dipyrimidine and the functional form of flavin ready for a new cycle of catalysis.

(1) PHOTOREACTIVATION

The major photoproduct produced in DNA by the UV component of sunlight is the cyclobutane pyrimidine dimer (Pyr<>Pyr).[1] The Pyr<>Pyr kills cells by blocking replication and transcription, and on rare occasions when the DNA is replicated past the lesions it is mutated at the lesion site. Cells protect themselves against these effects by eliminating the photoproducts from their genome either by excision repair or by photoreactivation. Photoreactivation is the prevention of the effects (mutation, cancer, death) of far UV (200–300 nm) by concurrent or subsequent exposure to near UV–visible light (300–500 nm). This repair mechanism has now been found in members of all three kingdoms of life, but it is missing in many species, including man (Li *et al.*, 1993) in a seemingly unpredictable manner (Table 1).

The seminal work of Rupert elucidated the basic reaction mechanism of enzymatic photoreactivation (Rupert *et al.*, 1958; Rupert 1962a,b) as follows. An enzyme called photoreactivating enzyme (PRE; DNA photolyase; deoxyribocyclobutadipyrimidine pyrimidine-lyase, EC 4.1.99.3) binds to Pyr<>Pyr in DNA in a light-independent step, absorbs a near-UV–visible photon, splits the cyclobutane ring to restore the pyrimidines, and then dissociates from repaired DNA:

$$\text{PRE} + \text{Pyr<>Pyr} \rightarrow \text{PRE·Pyr<>Pyr} \rightarrow \text{PRE·Pyr–Pyr} \rightarrow \text{PRE} + \text{Pyr–Pyr}$$

Recent research, greatly aided by the cloning of the photolyase gene (Sancar, 1977; Sancar & Rupert, 1978; Sancar *et al.*, 1983) has confirmed this mechanism and has revealed an enzyme with an extraordinary chromophore/cofactor system and reaction mechanism.

Table 1: Distribution of Photolyase in the Biological World

	photolyase	
kingdom	present	absent
bacteria	*Escherichia coli* *Bacillus firmus*	*Haemophilus influenzae* *Bacillus subtilis*
eucarya	*Saccharomyces cerevisae* *Monodelphus domesticus*	*Schizosaccharomyces pombe* *Homo sapiens*
archaea	*Methanobacterium thermoautotrophicum*	*Methanococcus vannielii*

(2) STRUCTURE OF DNA PHOTOLYASES

Photolyases are monomeric proteins of $Mr = 55\,000$–$65\,000$ (Sancar, A., *et al.*, 1984a; Sancar, G., *et al.*, 1984) and contain stoichiometric amounts of two chromophores/cofactors (Jorns *et al.*, 1984). One of the cofactors is flavin adenine dinucleotide (Iwatsuki *et al.*, 1980; Sancar & Sancar, 1984; Eker *et al.*, 1988), and the other is either methenyltetrahydrofolate (Johnson *et al.*, 1988) or 8-hydroxy-5-deazariboflavin (Eker *et al.*, 1981). Accordingly, the enzymes have been divided into two groups, the folate class and the deazaflavin class (Sancar & Sancar, 1987, 1988). Enzymes from *Escherichia coli, Saccharomyces cerevisiae, Neurospora crassa,* and *Bacillus firmus* belong to the folate class, and those from *Anacystis nidulans, Streptomyces griseus, Scenedesmus acutus, and Methanobacterium thermoautotrophicum* belong to the deazaflavin class. No three-dimensional structural data are available on any of these enzymes yet. However, the *E. coli* (Park *et al.*, 1993) and the *A. nidulans* (Miki *et al.*, 1993) photolyases have recently been crystallized, and high-resolution X-ray diffraction data have been obtained.

(A) Apoenzyme. The amino acid sequences of about 10 photolyases are available [see Sancar (1990) and Yasuhira and Yasui (1992)]. Sequence comparison with other folate- and flavin-binding proteins failed to reveal any homology.

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[1] Abbreviations: Pyr<>Pyr, pyrimidine dimer T[*c,s*]T and T[*t,s*]T, *cis,syn*, and *trans*,syn-I cyclobutane dimers of TpT; SC, second chromophore; MTHF, methenyltetrahydrofolate; 8-HDF, 7,8-didemethyl-8-hydroxy-5-deazariboflavin; E-FAD, E-SC, and E-SC-FAD, photolyase apoenzyme bound to the indicated chromophores; ET, electron transfer; SET, single-electron transfer; ϕ_{et}, ϕ_{ET}, and ϕ_{spl}, quantum yields for energy transfer, electron transfer, and dimer splitting, respectively.

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This lack of homology might be rationalized as follows. Photolyases bind to both flavin and folate in their ground states as well as in their excited states. Since excited states are, to a degree, different chemical species compared to ground states, it is to be expected that enzymes which bind to both ground and excited states of the cofactors do not share sequence homologies with those which bind only to ground state cofactors. Indeed, the recent finding that the blue photoreceptor protein HY4 of *Arabidopsis thaliana* has homology to photolyases (Ahmad & Cashmore, 1993) supports this view.

In contrast to sequence comparisons with other proteins, sequence comparison among photolyases has been useful in predicting possible structural domains. The most striking feature in this regard is the carboxy-terminal 150 amino acids, in which 30% of residues are identical in all photolyases sequenced to date, including enzymes from both the folate and deazaflavin classes (Yasui *et al.*, 1988; Sancar, 1990). The N-terminal half is less conserved, and, in general, in this region, photolyases belonging to one class have higher sequence similarities to enzymes belonging in the same class than to enzymes in the other class. Thus, it was proposed that the amino-terminal half bound the second chromophore and the carboxy-terminal half contained the flavin and DNA binding sites (Sancar, 1990). This prediction was tested with the yeast DNA photolyase (Malhotra *et al.*, 1992): a fragment isolated by partial proteolysis and spanning residues 15–326 bound MTHF specifically whereas a fusion protein containing the carboxy-terminal 275 residues (of the 565 amino acid-long protein) bound FAD. Both the amino- and carboxy-terminal domains bound DNA nonspecifically. Specific binding to damaged DNA substrate requires the apoenzyme–FAD complex (Payne *et al.*, 1990). Finally, the single intron in the *N. crassa phr* gene is located at the junction of the two regions of conserved sequence (Yajima *et al.*, 1991). This suggests domainal evolution of *phr*, whereby a gene encoding a 25-kDa flavoprotein with affinity to DNA was fused to a folate- or a deazaflavin-binding enzyme of about the same size. The fusion provided additional contacts for the DNA and the chromophores, and it extended the range and efficiency of usable light by 40–80 nm and a factor of 3–4, respectively. The coevolution of the two domains resulted in the generation of modern day photolyases.

(B) Coenzymes. Photolyases contain either a flavin and a folate or a flavin and a deazaflavin as cofactors (Figure 1).

(1) FAD is the only flavin that has been found in photolyases; riboflavin or FMN does not substitute for FAD in reconstitution experiments (Payne *et al.*, 1990). The natural flavin analog, 5-deazaflavin in the dinucleotide form (5-deazaFAD), can replace FAD in *E. coli* photolyase, but the synthetic analog, 1-deazaFAD, cannot. Enzyme reconstituted with 5-deazaFAD binds to DNA with specificity identical to the FAD form, but it is catalytically incompetent (Payne *et al.*, 1990). The physiological form of FAD in photolyases is the dihydro form (Payne *et al.*, 1987) even though with the rare exception of yeast photolyase (Sancar *et al.*, 1987c) all photolyases purified under aerobic conditions contain the cofactor in blue neutral radical form (Jorns *et al.*, 1984; Eker *et al.*, 1990). The flavin chromophore is very tightly bound to the apoenzyme ($K_D < 10^{-11}$ M), and its binding affinity is not affected by the second chromophore.

(2) The second chromophore of the folate class is an unusual folate: 5,10-methenyltetrahydropteroylpolyglutamate (MTHF); the number of glutamates ranges from 3 to 6 (and rarely to 8), and the polyglutamate contains the novel (γ_3)(α_n) linkage in *E. coli* photolyase and in other folate class photolyases

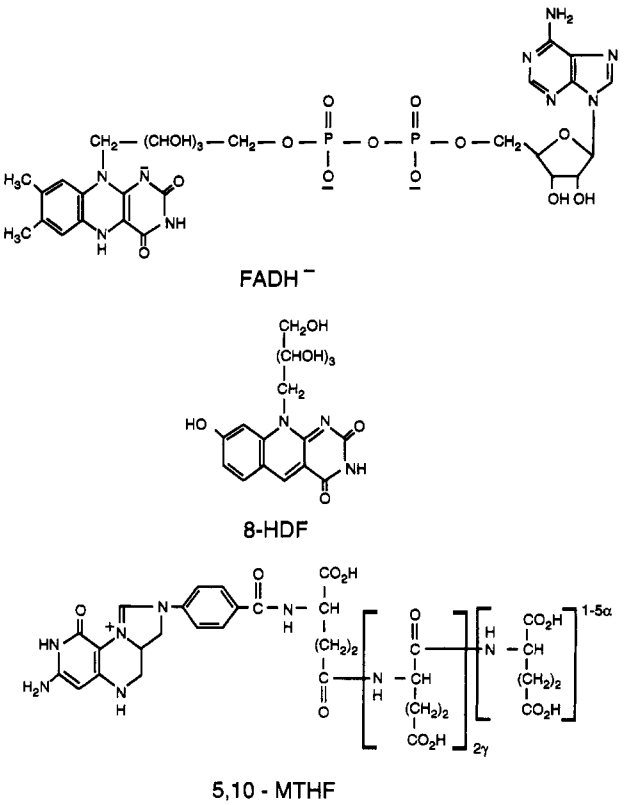

FIGURE 1: Structures of photolyase cofactors. The folate class contains contains $FADH^-$ plus 5,10-MTHF, and the deazaflavin class contains $FADH^-$ plus 8-HDF.

expressed in *E. coli* (Johnson *et al.*, 1988). The *E. coli* photolyase, when purified to homogeneity, contains substoichiometric amounts of MTHF, mainly because folates with 3–4 Glu residues dissociate readily from the enzyme (Hamm-Alvarez *et al.*, 1990a,b).

(3) The second chromophore of the deazaflavin class is 8-hydroxy-7,8-didemethyl-5-deazariboflavin, which is also called F_o (Eker *et al.*, 1981, 1988). This cofactor was first discovered in anaerobic methanogenic bacteria relatively recently [see Walsh (1986)] and was named F_{420} for its absorption peak at 420 nm. In fact F_{420} is an F_o derivative containing 4–8 γ-glutamates which are linked to the ribityl phosphate group of F_o through a lactyl group, reminiscent of the polyglutamate tail in folates. F_{420} is an obligatory two-electron redox cofactor, and it functions in this capacity in enzymes involved in methane and chlortetracycline biosynthesis [see Walsh (1986)]. In contrast to ground state chemistry, excited state 5-deazaflavin is a strong one-electron photoreductant and is often used in this capacity to reduce flavoproteins (Massey & Hemmerich, 1978). However, so far, it has not been found to act as a photoreductant in any biological system.

(C) Reconstitution. Apoenzyme (i.e., photolyase lacking both chromophores) has been prepared from both classes of photolyases (Payne *et al.*, 1990, Jorns *et al.*, 1990; Kim *et al.*, 1991). The apoenzyme does not bind to DNA substrate but binds to either or both chromophores rather rapidly and stoichiometrically. Thus the E-FAD, E-SC, and the E-FAD-SC forms of both classes have been reconstituted (Kim *et al.*, 1991; Malhotra *et al.*, 1992b).

(3) REACTION MECHANISM

Photolyase acts as a simple Michaelis–Menten enzyme with the notable exception that catalysis is initiated by light. The

reaction mechanism is as follows. The enzyme binds to Pyr<>Pyr in DNA independent of light, the second chromophore absorbs a 350–450-nm photon and transfers the excitation energy to the $FADH^-$ cofactor which in turn transfers an electron to Pyr<>Pyr; the 5–5 and 6–6 bonds of the cyclobutane ring are split to generate a Pyr and a Pyr^-, the latter donates an electron back to the flavin cofactor to regenerate $FADH^-$, and the enzyme dissociates from DNA. The conversion of Pyr<>Pyr to 2 Pyr does not result in a net gain or loss of electrons, so photolysis of Pyr<>Pyr is not a redox reaction.

(A) Substrate Recognition

Photolyase is a "structure-specific DNA binding protein" whose specificity is determined by the backbone structure of DNA at the binding site in contrast to the "sequence-specific DNA binding proteins" which rely on hydrogen-bond donors and acceptors in the grooves of the duplex (Husain *et al.*, 1987).

(1) Kinetics and Thermodynamics. The equilibrium dissociation constant (K_D) ranges from 10^{-9} M for *E. coli* photolyase to 10^{-11} M for the *M. thermoautotrophicum* enzyme [Sancar *et al.*, 1987a; Kiener *et al.*, 1989; see Sancar (1992)]. Photolyases, which make 1–2 ionic bonds with DNA, locate their target by three-dimensional diffusion in contrast to proteins such as the lac repressor which make 6–8 nonspecific phosphate contacts and locate their targets by unidimensional diffusion at rates surpassing the Smoluchovski limit. The association rate constants of all photolyases tested are in the range of 10^6–10^7 M^{-1} s^{-1} and thus are well within the limit of a diffusion-controlled reaction. In contrast to the association rate constants, the dissociation rate constants cover a wide range of 5×10^{-2} s^{-1} for *E. coli* photolyase (Sancar *et al.*, 1987a; Husain & Sancar, 1987) to 2×10^{-4} s^{-1} for the *M. thermoautotrophicum* enzyme (Kiener *et al.*, 1989). Thus, the differences between the equilibrium binding constants can be accounted for by the differences in the dissociation rate constants.

(2) Binding Determinants on the Enzyme. Sequence analyses suggest that most of DNA specificity determining groups are in the carboxy-terminal halves of photolyases (Sancar, 1990). Site-specific mutagenesis studies indicate that Trp277 in the carboxy-terminal half of *E. coli* photolyase makes a van der Waals contact with the dimer (Li & Sancar, 1990; Kim *et al.*, 1992a). In yeast photolyase (565 residues), based upon loss of DNA contacts in the R507A mutant, it has been proposed that R507 makes an ionic bond with the phosphate 5′to the dimer and by doing so anchors the enzyme at the dimer site allowing other enzyme–substrate interactions to develop (Baer & Sancar, 1993). In addition, in this enzyme W387 (the homolog of W277 in *E. coli* photolyase), K517 and K463 were identified to be in the binding site by the effect of mutations at these positions on phosphate and groove contacts of photolyase (Baer & Sancar, 1993). Finally, FAD, which is known to bind to the carboxy-terminal half (Malhotra *et al.*, 1992a), most likely is in direct contact with the Pyr<>Pyr because apoenzyme has no specific affinity for dimer-containing DNA (Payne *et al.*, 1990). In contrast to FAD, the second chromophore has no effect on substrate binding (Payne *et al.*, 1990; Malhotra *et al.*, 1992b).

(3) Binding Determinants on the Substrate. The natural substrate for photolyase is a Pyr<>Pyr in a duplex. The effects of primary, secondary, and tertiary structure of DNA on binding are considered below.

(a) Primary Structure. First, the length of DNA is important. Even though photolyase binds to thymine base dimer and dinucleotide thymine dimer (Kim & Sancar, 1991), a substrate of the form NpT<>TpNpNp is the minimal structure for high-affinity binding (Jorns *et al.*, 1985). Thus, the backbone appears to contribute most of the binding free energy (Kim & Sancar, 1991). Second, the enzyme makes intimate contact with the pentose part of the backbone because an -OH group at C2′(RNA) reduces the affinity by 10^5 (Kim & Sancar, 1991). In contrast, no contact is made with the neighboring bases because there is essentially no sequence effect on binding (Svoboda *et al.*, 1993). Finally, an important primary structure determinants on binding is the base composition of the dimer. The following hierarchy of affinities has been established: T<>T ≥ T<>U > U<>U > C<>C, with T<>T having 10-fold higher affinity than C<>C (Kim & Sancar, 1991).

(b) Secondary Structure. Photolyases from *E. coli, S. cerevisiae,* and *M. thermoautotrophicum* which differ greatly in their affinities for Pyr<>Pyr made essentially identical footprints on a T<>T containing duplex (Husain *et al.*, 1987; Baer & Sancar, 1989; Kiener *et al.*, 1989). The enzyme contacts the phosphate 5′ and the three phosphates 3′ to the T<>T on the damaged strand and the phosphate opposite the dimer across the minor groove on the complementary strand and occludes the major groove for about half a turn 3′ to the dimer. Nearly all of the contacts are with the damaged strand, and as a consequence *E. coli* DNA photolyase has the same affinity for UV-irradiated single- or double-stranded DNA (Sancar *et al.*, 1985). Because of this mode of binding, photolyase does not interfere but actually stimulates the excision repair enzyme, (A)BC excinuclease, which binds to the opposite face of DNA (Sancar *et al.*, 1984b; Sancar & Smith, 1989; Yamomoto *et al.*, 1983). Caffein inhibits photoreactivation by binding to DNA and interfering with the binding of photolyase (Selby & Sancar, 1990).

These findings have led to the following proposal regarding specificity determinants on the Pyr<>Pyr itself: photolyase contacts C2═O and C4═O (C4–NH_2) through specific H-bond donors. C2═O is common to all pyrimidines. C4═O is common to T and U, explaining the higher affinity for photodimers with these residues. In cytosine, at the 4 position a hydrogen-bond donor instead of an acceptor causes the loss of a hydrogen bond (~2 kcal mol^{-1}) or 10-fold lower affinity. Finally, T-containing dimers have marginally yet reproducibly higher affinities than U-containing dimers, suggesting that the C5–CH_3 group contributes to binding by van der Waals or hydrophobic interactions (Kim & Sancar, 1991).

(c) Stereochemistry and Tertiary Structure. Of the eight stereoisomers of T<>T (Cadet and Vigny, 1990) only the *cis,syn* isomer forms in a duplex and only the *cis,syn* and *trans,syn* isomers (at 7:1 ratio) form in single-stranded DNA and thus are biologically relevant. The T<*c,s*>T is the natural substrate. No specific binding of *E. coli* photolyase to T<*t,s*>T in DNA can be detected (Ben-Hur and Ben Ishai, 1968; Kim *et al.*, 1993a). However, at very high enzyme concentrations, the enzyme does repair this isomer (Kim *et al.*, 1993a). The tertiary structure has no effect on activity. Photolyase repairs relaxed and supercoiled DNA with equal efficiency and does not unwind or kink the DNA (Sancar et al., 1985).

(B) Catalysis

The reaction is a light-initiated $-(\pi_s^2 + \pi_s^2)$ cycloreversion of the cyclobutane ring joining the two pyrimidines. The

photoreactivating light (300–500 nm), however, is of insufficient energy to populate excited states (singlet or triplet) of Pyr<>Pyr which are known to yield pyrimidine monomers at high yield [see Wang (1976)]. Furthermore, no evidence exists for formation of a charge transfer complex with Pyr<>Pyr that would create low-lying orbitals that can be populated by direct excitation by photoreactivating light (Payne & Sancar, 1990; Kim & Sancar, 1991). Rather, the overwhelming evidence is that photoexcited photolyase transfers an electron to Pyr<>Pyr and the resulting anion radical splits into two pyrimidines. However, this is not a simple symmetry-allowed photocycloreversion (Hartman *et al.*, 1987).

(1) The Roles of the Chromophores. (a) Flavin Adenine Dinucleotide. The flavin chromophore is necessary and sufficient for catalysis. In purified photolyases the flavin is found in all three oxidation states, FAD_{ox}, FADH, and $FADH_2$($FADH^-$). The first two forms are purification artifacts. The E-FAD_{ox} form is catalytically inert (Payne *et al.*, 1990; Kim *et al.*, 1992c). The E-FADH° form is active; however, the quantum yield changes with the photoreactivation wavelength and it drops precipitously at >400 nm (Sancar *et al.*, 1987b). Photoreduction of FADH° is the casue of this unusual behavior. Excitation of E-FADH° leads to E-^{4}FADH° (Okamura *et al.*, 1989; Heelis *et al.*, 1992b) which abstracts an electron from Trp306 (Heelis *et al.*, 1987; Li *et al.*, 1991) to generate $FADH^-$ (Heelis & Sancar, 1986) which has a λ_{max} = 366 nm with no absorption at λ500 > nm. Thus, excitation with λ = 366 nm photoreduces the enzyme into the E-$FADH^-$ form which catalyzes several cycles of photosplitting before being reoxidized when the experiment is carried out under aerobic conditions (Payne *et al.*, 1987).

Recent evidence indicates that the two-electron-reduced flavin is not in the dihydro form ($FADH_2$) but rather in the anionic form ($FADH^-$). An EPR study with *E. coli* photolyase revealed that the excited FADH abstracted an electron from Trp 306 to generate a $Trp^{\cdot+}$, and by implication an $FADH^-$ (Kim *et al.*, 1993c). Studies with model systems support this assignment (Hartman & Rose, 1992; Yeh & Falvey, 1991).

(b) Second Chromophore. The sole function of the second chromophore is to absorb light and transmit the excitation energy to the catalytic cofactor, $FADH^-$. The roles of both the folate and the deazaflavin chromophores in photolyases differ in two aspects from their roles in other enzyme systems. First, they simply function as photoantennas in contrast to their roles in redox and one-carbon transfer reactions. Second, a catalytic cycle in photolyase leaves the second chromophore unchanged (Hamm-Alvarez *et al.*, 1989, 1990) in contrast to other enzyme systems where these chromophores are essentially cosubstrates.

(c) "The Third Chromophore". From studies with model systems, it is known that the indole ring of tryptophan can split Pyr<>Pyr by electron transfer (Hélène & Charlier, 1977; Young *et al.*, 1988, 1990; Cochran *et al.*, 1988; Kim *et al.*, 1990). Although this mechanism is of questionable significance in enzymatic photoreactivation, it does occur in photolyase. Kim *et al.* (1992a) have shown that Trp277 of *E. coli* photolyase, which plays an important role in substrate binding, can directly repair Pyr<>Pyr by electron transfer with high quantum yield (0.6).

(2) Energy and Electron Transfer. (a) Energy Transfer. Steady-state fluorescence studies have shown that the SC fluorescence is efficiently quenched by flavin in all three oxidation states (Jordan & Jorns, 1988; Kim & Sancar, 1991). The rate and yield of energy transfer were obtained by time-resolved spectroscopic methods for both classes of photolyases and are summarized in Table 3 (Kim *et al.*, 1991, 1992c). The deazaflavin class enzymes transfer energy more efficiently and consequently have an overall quantum yield higher than the folate class enzymes. The mechanism of energy transfer in both cases is by Förster dipole–dipole interaction [see Kim and Sancar (1993)].

Table 2: Physical and Functional Properties of Photolyases

enzyme	*E. coli*	*A. nidulans*
class	folate	deazaflavin
protein size (amino acids)	471	484
M_r	53 994	54 475
subunit	monomer	monomer
cofactors	$FADH^-$ + MTHF	$FADH^-$ + 8-HDF
absorption maxima (nm)		
E-FADH°-SC	384, 480, 580	438, 480, 588
E-$FADH^-$-SC	384	438
E-SC	384	438
E-$FADH^-$	366	355
fluorescence maxima (nm)		
E-FADH°-SC (weak)	465, 505	505, 470
E-$FADH^-$-SC	465	470, 505
E-SC	465	470
E-$FADH^-$	505	505
binding constant (K_D)	10^{-8}–10^{-9} M	10^{-8}–10^{-9} M
catalytic constant (k_{cat})	1.0 s^{-1}	1.0 s^{-1}
quantum yield of repair (ϕ_r)		
E-$FADH^-$-SC	0.5–0.6	0.9–1.0
E-$FADH^-$	0.8–0.9	0.9–1.0

Table 3: Photochemical Properties of Photolyases

enzyme	*E. coli*	*A. nidulans*
class	folate	deazaflavin
excited singlet state lifetime (ns)[a]		
E-SC*	0.35(F)–0.48(A)	2.0(F)
E-$FADH^-$-SC*	0.14(F)–0.18(A)	0.05(F)
E-FADH°-SC*	<0.03	<0.03
energy transfer (SC* → $FADH^-$)		
rate (s^{-1})	4.6×10^9	1.9×10^{10}
efficiency (%)	62	98
interchromophore distance (Å)	21	15
excited singlet state lifetime (ns)		
E-$FADH^{-*}$	1.5(F)–1.7(A)	1.8(F)
E-$FADH^{-*}$ + T<>T	0.16(F)–0.20(A)	0.14(F)
electron transfer ($FADH^{-*}$ → T<>T		
rate (s^{-1})	5.5×10^9	6.5×10^9
efficiency (%)	89	92

[a] (F) From time-resolved fluorescence; (A), From time-resolved absorbance.

(b) Electron Transfer. The $FADH^-$ excited singlet state formed directly by absorbing a photon, or more often by energy transfer from the second chromophore, initiates splitting of Pyr<>Pyr by electron transfer. Since excited state flavin is an efficient redox cofactor in all three oxidation states and since Pyr<>Pyr radical anions and cations are equally prone to cycloreversion (Pac *et al.*, 1982; Rokita & Walsh, 1984), the question arises as to why only the E-$FADH^-$(±SC) form of the enzyme is catalytically active. Heelis *et al.* (1992) have addressed this question by calculating the free energy changes for single electron transfer (SET) in either direction by relevant excited states of enzyme-bound flavin and Pyr<>Pyr. The results show that, on thermodynamic grounds, SET from $^1FADH^-$ to Pyr<>Pyr is the only realistic mechanism for photolyase ($\Delta G^{\circ\prime}$ = –30 kcal mol^{-1}). The rate and efficiency of SET from $^1FADH^-$ to Pyr<>Pyr have been investigated by time-resolved fluorescence, absorbance, and EPR spectroscopy.

(*i*) In the presence of substrate, the lifetime of flavin fluorescence in the E-$FADH^-$ form of *E. coli* and *A. nidulans* photolyases decreased drastically: from 1.4 to 0.16 ns in *E.*

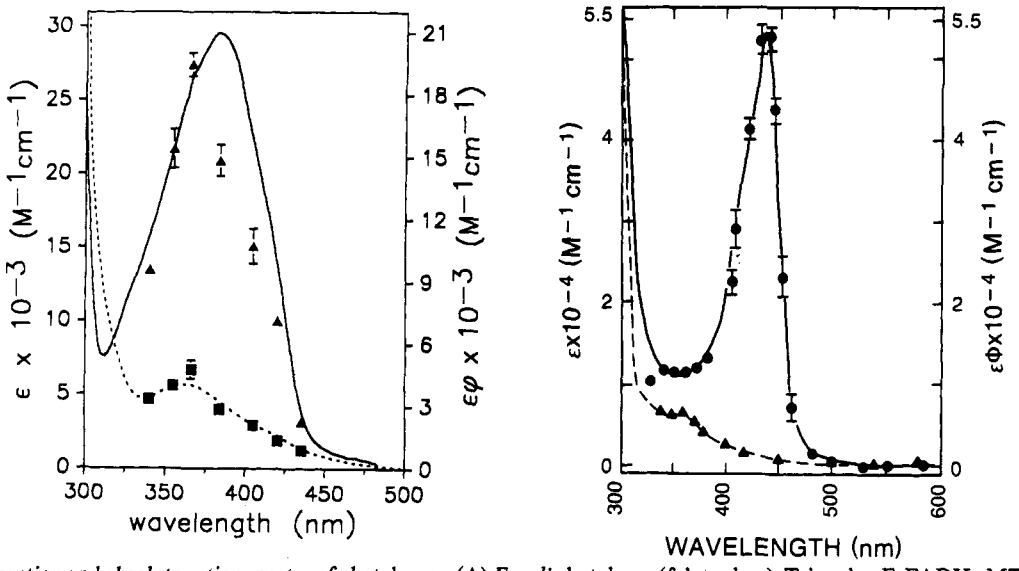

FIGURE 2: Absorption and absolute action spectra of photolyases. (**A**) *E. coli* photolyase (folate class). Triangles, E-FADH⁻-MTHF (holeenzyme); squares, E-FADH⁻ form of the enzyme. (**B**) *A. nidulans* photolyase (deazaflavin class). Circles, E-FADH⁻-8HDF (holoenzyme); triangles, E-FADH⁻ form of the enzyme.

coli photolyase (Kim *et al.*, 1991) and from 1.8 to 0.14 ns in *A. nidulans* photolyase (Kim *et al.*, 1992b). These values correspond to a rate of electron transfer of 5.5×10^9 s^{-1} at a quantum yield of 0.88 for the *E. coli* and a rate of 6.5×10^9 s^{-1} at a quantum yield of 0.92 for the *A. nidulans* photolyase (Table 3).

(*ii*) Using picosecond flash photolysis, Okamura *et al.* (1991) identified an excited state transient of *E. coli* E-FADH⁻ form photolyase with an absorption band in the 450–550-nm region (similar to the absorption of the excited singlet state of a deprotonated, reduced flavin; Heelis *et al.*, 1993b) and a lifetime of 1.7 ns as ^{1}FADH⁻. In the presence of U<>U the transient decayed rapidly with a $\tau = 0.2$ ns, in agreement with the fluorescence lifetime in the presence of T<>T substrate ($\tau = 0.16$ ns) (Table 3). The decay of the flavin singlet was followed by the appearance of a 400-nm species with $\tau \sim 2$ ns which was ascribed to the substrate radical.

(*iii*) More direct evidence for repair by electron transfer in *E. coli* enzyme was obtained by time-resolved EPR experiments with an E-FADH⁻ + T<>T system (Kim *et al.*, 1992c): when the enzyme–substrate mixture was exposed to 17-μs light flashes, an EPR signal was detected which decayed with the instrument time constant. Although it was not possible to assign the radical ($\tau > 10$ μs) to a particular species, these experiments provide further evidence that during repair of Pyr<>Pyr by photolyase a radical is produced.

(c) Action Spectra. The efficiency of splitting of Pyr<>Pyr⁻ generated by electron transfer in photolyase was determined by obtaining absolute action spectra for the E-FADH⁻ and E-SC-FADH⁻ forms of *E. coli* and *A. nidulans* enzymes (Figure 2). In both classes the absolute action spectrum of E-FADH⁻ perfectly matches the absorption spectrum, and the quantum yields of repair (0.90 for the deazaflavin class and 0.85 for the folate class enzymes) are, within experimental error, the same with the measured quantum yields of electron transfer for these enzymes. Thus the efficiency of splitting of T<>T⁻ in photolyase is 100%. The action spectrum of the E-SC-FADH⁻ form of the deazaflavin class perfectly matches the absorption spectrum, consistent with high efficiency energy transfer from the 8-HDF to FADH⁻ (Eker *et al.*, 1986; Malhotra *et al.*, 1992b). In contrast, the action spectrum of the *E. coli* holoenzyme shows a 5-nm blue-shift relative to the absorption spectrum because energy transfer from MTHF is less efficient than that of 8-HDF and as a consequene contribution of direct excitation of FADH⁻ becomes significant at lower wavelengths (Payne & Sancar, 1990).

(3) Mechanistic Considerations. Pyr<>Pyr can be reversed photochemically by direct absorption of short wavelength (e.g., 240 nm) UV light [see Wang (1976)]. This direct photoreversion occurs with a quantum yield of nearly 1.0 because it follows the Woodward–Hoffmann rules which state that in a concerted chemical reaction the orbital symmetries are retained in going from reactant to product. A corollary of the rules is that photochemically allowed reactions are thermally forbidden and vice versa. Strictly speaking, enzymatic photoreactivation is a thermal and not a photochemical reaction [see Hartman *et al.* (1987)]. The enzyme photochemically transfers an electron to the Pyr<>Pyr which thermally splits into a pyrimidine and a pyrimidine radical anion. Formation of a dimer radical anion does not change the fact that cycloreversion is still thermally forbidden because the ground state of the reactant would produce an electronically excited state of the product.

By applying molecular orbital theory to Pyr<>Pyr radical anion splitting, Hartman *et al.* (1987) have concluded that in the dimer radical anion the single electron may contribute electron density to a molecular orbital that has antibonding character across the cyclobutyl orbitals linking the two halves of the dimer. On the basis of Huckel MO energies of various potential transition states, the occupancy of such an orbital would be expected to significantly decrease the activation energy for splitting via a stepwise or nonsynchronous concerted pathway. Thus, although the reaction is still formally symmetry forbidden, it can proceed relatively efficiently because of lowering of the activation energy.

However, dimer anions generated by electron donation from tethered indoles (Young *et al.*, 1988; Van Camp *et al.*, 1987) are not cleaved that efficiently, indicating that the above arguments regarding the lowering of the activation energy of cleavage are insufficient to explain the cleavage of Pyr<>Pyr by E-^{1}FADH⁻ with a quantum yield of near unity in all photolyases. Again, work with model systems has provided a plausible answer. Kim *et al.* (1990) and Kim and Rose (1992) found that Pyr<>Pyr splitting by an indolyl group

tethered with a two-carbon linker was dramatically dependent on the dielectric constant of the solvent, reaching a maximum quantum yield of 0.41 in the least polar solvent tested, 1,4-dioxane-isopentane (5:95). The authors suggested that back electron transfer from the Pyr<>Pyr$^-$ to the donor may be in the Marcus inverted region [see Closs and Miller (1988)] and thus would be highly favorable thermodynamically and therefore slowed in nonpolar solvents. The flavin in *E. coli* photolyase is in an apolar environment (Jorns *et al.*, 1987; Payne et al., 1990), and thus back electron transfer between the flavin and dimer radical pair could be in the Marcus inverted region of strong driving force coupled with low reorganization energy of the solvent. As a consequence the rate of back electron transfer would be too slow relative to the cleavage of the cyclobutane ring and thus $\phi_{spl} \sim \phi_{ET}$.

The proposed cleavage mechanism for the dimer radical anion was tested by Begley and co-workers by measuring the secondary deuterium V/K isotope effects on the splitting of appropriately deuterated Pyr<>Pyr by photolyase or model photosensitizers. It was found that 5,5-dideuterated U<>U was cleaved by photolyase at a slightly slower rate (V/K = 1.082) compared to the 6,6-dideuterated U<>U (V/K = 1.071) consistent with strongly concerted cleavage of a dimer anion radical (Witmer *et al.*, 1989). By comparison, the cleavage of U<>U by SET from methoxyindole was more sensitive to 5,5-dideuterium substitution (V/K = 1.173) than the 6,6-substitution (V/K = 1.079), and the opposite was true for cleavage by SET from Pyr<>Pyr to anthraquinone: V/K = 1.030 for 5,5-dideutero- and V/K = 1.192 for 6,6-dideuterosubstitutions (McMordie & Begley, 1992). Thus, even though the secondary deuterium isotope effects on photolyase are different from both model systems, they are closer to the effects on the electron donor, consistent with cleavage of Pyr<>Pyr by photolyase via a dimer anion radical intermediate.

The proposed reaction scheme predicts that the splitting efficiency would be greatly influenced by the base composition of the Pyr<>Pyr. This is indeed the case (Setlow & Carrier, 1966; Kim & Sancar, 1991). Thus, the quantum yields of splitting for T<>T (0.9), T<>U (0.8), U<>U (0.6), and C<>C (0.05) by the E-FADH$^-$ form of *E. coli* photolyase are entirely consistent with the proposed mechanism (Kim & Sancar, 1991).

To summarize, the available evidence is consistent with the following scheme (Heelis *et al.*, 1993a; Kim & Sancar, 1993) for splitting of Pyr<>Pyr by photolyase (Figure 3). The second chromophore absorbs a photon (**1**) and transfers the excitation energy to the flavin by dipole–dipole interaction with quantum efficiency of 63%–100% depending on the enzyme (**2**); the excited singlet state *FADH$^-$ then transfers an electron to Pyr<>Pyr (**3**). The cyclobutane ring of Pyr<>Pyr$^-$ undergoes a concerted but nonsynchronous cleavage. The resulting pyrimidine anion is then oxidized by the FADH° to restore the dipyrimidine and the functional form of flavin (**4**) ready for a new cycle of catalysis.

(4) PROSPECTS

Two of the most significant developments in structural biology over the past decade have been the elucidation of the structure of the photosynthetic reaction center, RC (Deisenhofer & Michel, 1991), and of structures of a number of specific DNA-binding proteins [see Steitz (1990)]. Photolyase combines the key functional elements of both systems. Like other specific DNA-binding proteins, it binds to its target site (Pyr<>Pyr) with high specificity and affinity. Once bound, it performs catalysis by a mechanism reminiscent of the RC:

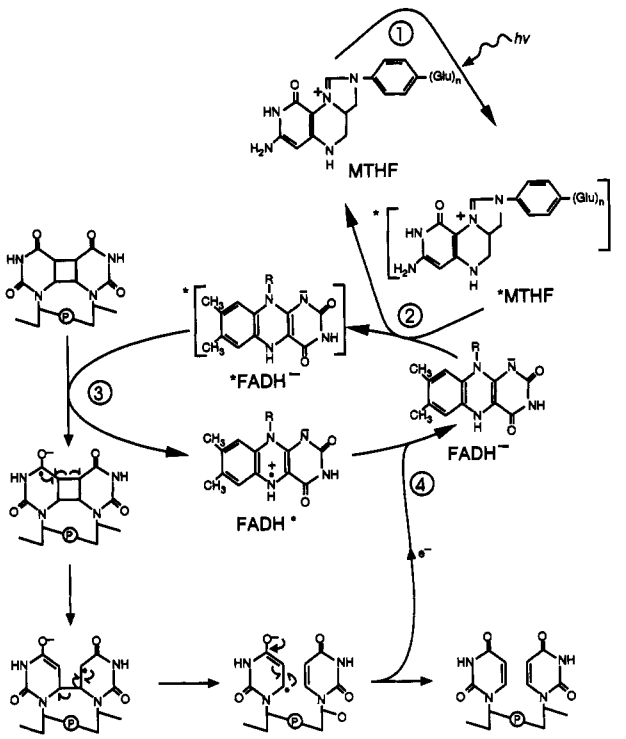

FIGURE 3: Reaction mechanism of photolyase. (**1**) Absorptin of a photon by the second chromophore (in this scheme, MTHF). (**2**) Energy transfer. (**3**) Electron transfer and cycloreversion. (**4**) Back electron transfer and regeneration of catalytically active flavin.

a photoantenna molecule (second chromophore) absorbs a photon and transfers energy to a special catalytic cofactor (flavin) which then transfers an electron to DNA. All these functional domains are pakced within a 60-kDa protein. Thus, photolyase, because of its relative simplicity, offers an alternative system to study the structural aspects that are important for three fundamental phenomena in biology, DNA binding, energy transduction, and electron transfer.

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