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Energy and electron transfer processes in flavoprotein-mediated DNA repair

Paul F. Heelis [a], Rosemarie F. Hartman [b], Seth D. Rose [b]

[a] *North East Wales Institute, Mold Road, Wrexham, Clwyd LL11 2AW, UK*

[b] *Department of Chemistry and Biochemistry, Arizona State University, Tempe, AZ 85287-1604, USA*

Abstract

The flavin photoenzyme, DNA photolyase, utilizes a photon to repair the main damage to DNA (pyrimidine dimers) caused by UV light. DNA photolyases are monomeric flavoproteins that bind to the *cis-syn*-pyrimidine dimer in DNA in a light-independent step, forming a stable enzyme–substrate complex. This complex absorbs a photon, leading to electron transfer to the pyrimidine dimer, producing an unstable cyclobutane radical anion and thereby initiating bond cleavage in the dimer. The mechanism and energetics involved in the action of this remarkable enzyme are reviewed.

Keywords: Energy transfer; Electron transfer; Flavoprotein-mediated DNA repair

1. Introduction

DNA photolyase is an enzyme that repairs the principal damage induced by UV light in DNA, i.e. cyclobutane-type pyrimidine dimers [1,2] (Figs. 1 and 2). It accomplishes this task by recognizing and binding to such a dimer, transferring an electron to it, and thereby splitting the dimer and hence restoring the DNA functionality. Neither DNA-recognizing nor electron-transferring enzymes are unique or even unusual. What distinguishes DNA photolyase from virtually all other enzymes is that it is powered by electromagnetic radiation. Furthermore, it has a unique system for utilizing light, with one pigment absorbing and transferring the energy over 15 Å to a redox-active pigment which is capable of dimer splitting. This characteristic dependence on light offers a great advantage for the study of the enzyme's mechanism. Following the addition of a substrate, no chemical change takes place until a photon is absorbed by one of

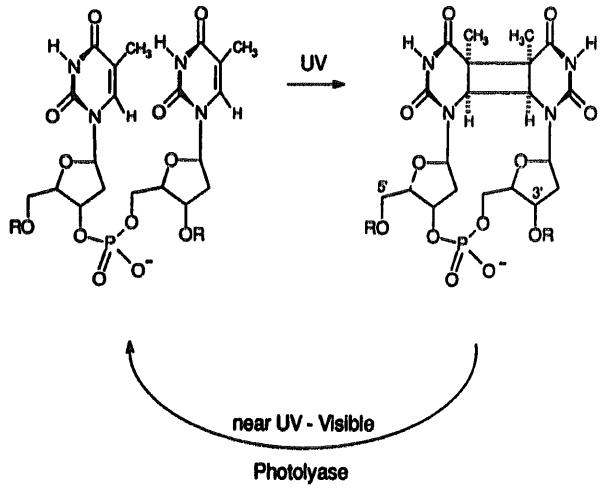

Fig. 1. Thymine dimer formation by stacked thymine nucleotides in the same strand of double-strand DNA induced by UV light (UVB, 280–315 nm). The reverse reaction, i.e. repair, is catalysed by DNA photolyase, which uses near-UV and visible light.

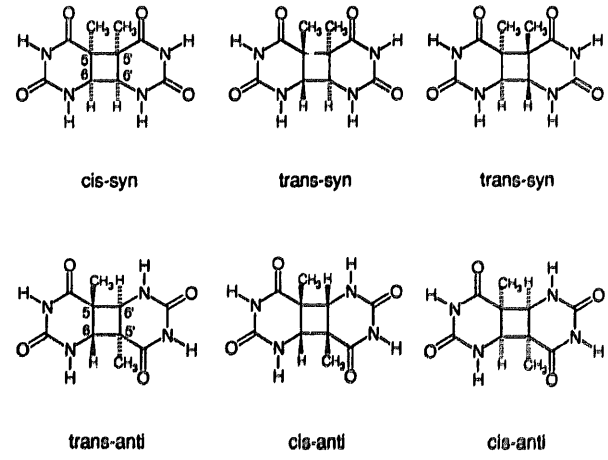

Fig. 2. The structures of the isomeric thymine dimers.

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the enzyme-bound chromophores. Unlike all other enzymes, mechanistic studies are not limited by factors such as mixing times or substrate turnover rates.

2. DNA damage by UV light

The absorption of light by DNA results in the localization of the energy at thymine nucleotides in the DNA strand. This is because the initially produced excited singlet states undergo spin inversion to form triplet states, followed by energy migration to thymine, which acts as an energy "sink" because thymine has the lowest triplet energy of the common bases. When the thymine triplet is formed adjacent to another thymine (or possibly a cytosine) in the same strand of the DNA double helix, a photochemical reaction between the two pyrimidines may take place. This photocycloaddition by adjacent pyrimidines is a concerted pericyclic reaction, i.e. a $\pi^2 + \pi^2$ photoaddition. Accordingly, the photoaddition of two pyrimidines is orbital symmetry allowed and, once formed, the pyrimidine dimer cannot revert to two pyrimidines by a non-photochemical process (i.e. a thermal reaction). Although the reverse photochemical reaction is symmetry allowed, dimers do not absorb near-UV light significantly and thus the dimers accumulate in DNA.

3. Photolyases are flavoproteins

DNA photolyases are monomeric proteins with a molecular weight of approximately 55 000–65 000. Their action is well described by classic enzyme catalysis kinetics (Michaelis–Menten), with the important exception that catalysis is light initiated. Photolyases are widespread in nature and, for example, have been reported in many bacteria, blue–green algae, fungi, higher plants and all major groups of vertebrates, with the possible exception of placental mammals. Of particular importance in regard to skin cancer is whether photolyase is present in humans, but the matter is still controversial [3,4].

Only a few photolyases have been fully characterized to date, but all contain two chromophores [5]. One is always 1,5-dihydroflavin adenine dinucleotide ($FADH_2$, probably in its anionic form $FADH^-$), and its presence is essential for DNA repair. The other may be either methenyltetrahydrofolate (MTHF) or 8-hydroxy-5-deazariboflavin (8-HDF) (Fig. 3). Accordingly, the enzymes have been classified into two groups: the folate class, which exhibits maximum catalytic activity at 360–390 nm, and includes enzymes from *Escherichia coli* and *Saccharomyces cerevisiae*, and the deazaflavin class, which has an action spectrum (i.e. wavelength dependence of catalytic activity) with a maximum at 430–460 nm, and includes photolyases from *Anacystis nidulans*, *Streptomyces griseus*, *Scenedesmus acutus*, *Halobacterium halobium* and *Methanobacterium thermoautotrophicum*.

4. Dimer recognition by photolyase

The crystal structure of *E. coli* DNA photolyase [6] has been solved to 2.3 Å. The overall structure consists of two domains, an $\alpha\beta$ domain and an α domain. The α domain

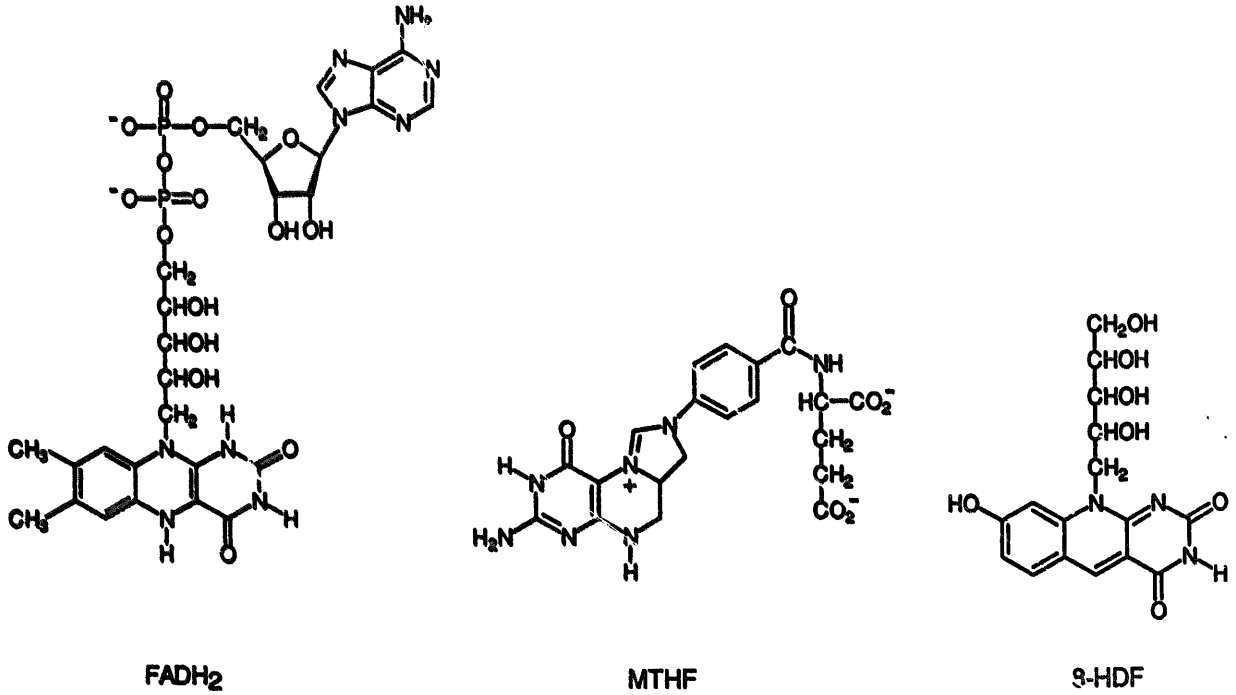


Fig. 3. The structures of the electron-donating cofactor $FADH_2$ and the two light-gathering antenna cofactors MTHF and 8-HDF.

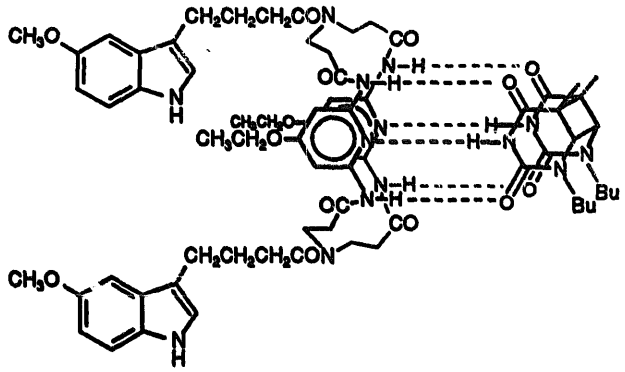


Fig. 4. A macrocycle that recognizes pyrimidine dimers by the characteristic hydrogen bonding pattern and acts as a photocatalyst of dimer splitting.

forms a flat surface. In the centre of this surface, a hole large enough to accommodate a thymine dimer leads to a cavity in which $FADH^-$ is bound. A patch of surface around the hole exhibits a positive electrostatic potential. On the basis of these observations, it was proposed that DNA containing a thymine dimer is bound to this surface with the dimer occupying the hole. DNA photolyase binds exclusively to *cis-syn*-cyclobutane pyrimidine dimers ($K_{assoc} = 2.6 \times 10^8$ M^{-1} for the *E. coli* enzyme). This represents a 10^5-fold selectivity for DNA containing a thymine dimer compared with a monomer ($K_{assoc} = 3.5 \times 10^3$ M^{-1} for dimer-free DNA) and is a remarkable testament to the enzyme's ability to recognize unique structural features of the substrate [7]. It is not only the pyrimidine dimer itself that determines the binding of the enzyme, but the adjacent bases of the same strand and even those of the opposite strand which contribute to the overall interaction. Comparison of K_{assoc} for binding to dimer-containing poly(dT) vs. the dinucleotide, TpT dimer, indicates that 50% of the binding energy comes from interactions with flanking nucleotides. *E. coli* DNA photolyase binds with equal affinity to thymine photodimers in either single- or double-strand DNA. This is possibly due to dimer-induced conformational changes in single-strand DNA that may parallel those observed in duplex B-DNA, the presumed natural substrate for the enzyme. In addition, studies of the ionic strength dependence have allowed the ionic interactions between enzyme and substrate to be probed. In this way, it has been shown that the binding site on DNA behaves as if it has a charge of -2. However, there is evidence that this value actually reflects the net effect of four phosphate groups (three on the 3′ side and one on the 5′ side in the dimer-containing strand).

A simple macrocyclic model system based on the molecular recognition of a dimer by its characteristic hydrogen bonding pattern has been devised and prepared as shown in Fig. 4. The macrocycle has covalently tethered chromophores which photosensitize dimer splitting. The macrocycle complexes to a dimer ($K_{assoc} \sim 10^4$ M^{-1}), and the subsequent absorption of light by the tethered methoxyindole chromophore produces the indole excited state. This excited indole then presumably donates an electron to the non-covalently complexed dimer. After the dimer radical anion splits, an electron is returned to the donor and the macrocycle dissociates from the "repaired" dimer (i.e. the monomeric pyrimidines). The macrocycle has a quantum yield of 0.11 in acetonitrile solution under saturating conditions (i.e. when all the molecules of the macrocycle are bound to dimers). The macrocycle then binds to another dimer and the cycle is repeated. Thus the macrocycle acts as a photocatalyst.

5. Photochemical efficiency of dimer repair by photolyase

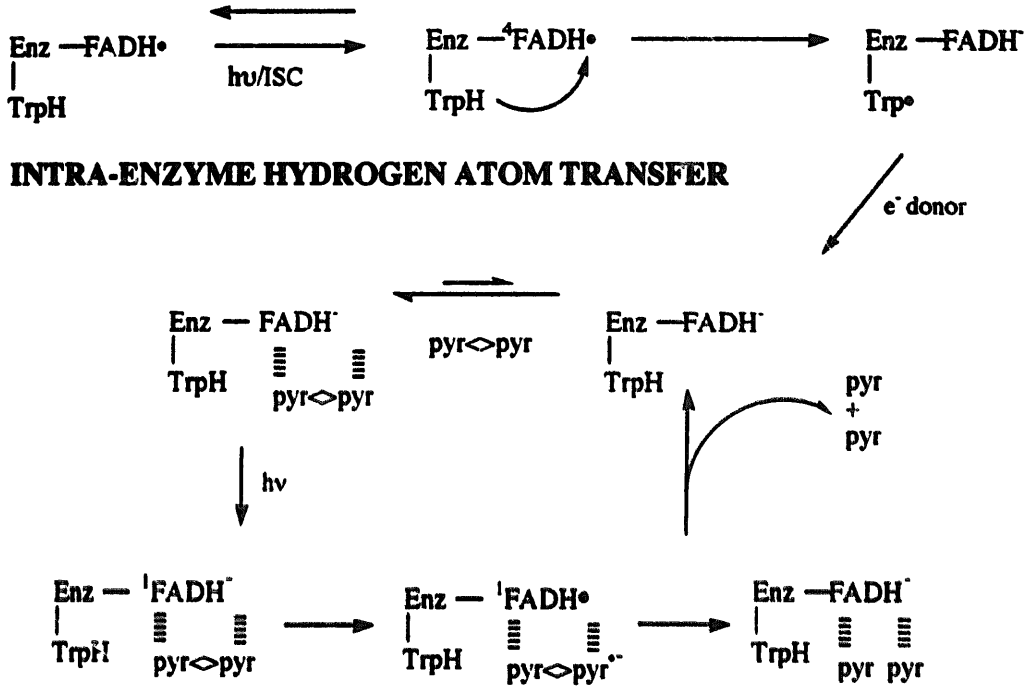
Photolyases exhibit a value for the splitting quantum yield (Φ) of 0.5–0.9, depending on the source, i.e. an efficiency of 50%–90%, if the light is absorbed directly by the flavin [5]. The value is somewhat lower if the light is absorbed by the folate or deazaflavin chromophore, as some energy is lost through the energy transfer process, as discussed further below. In the absence of photolyase, experimental values of Φ around 10^{-3}–10^{-4} are observed for simple model systems involving mixed solutions of flavins and pyrimidine dimers, although higher values have been found for other photosensitizers [8].

6. Dimer splitting by photolyase is not by excitation of the dimer

The formation of the enzyme–substrate complex precedes photon absorption. Subsequently, photoexcitation of the $FADH^-$ chromophore produces an excited state of π–π^* character, with a lifetime of 1.6 ns and an energy of 240 kJ mol^{-1} above the ground state. The overall repair reaction is simply the reverse of the $\pi^2 + \pi^2$ photocycloaddition. Just as the conservation of orbital symmetry designates that the concerted photocycloaddition is allowed, the reverse (enzymatic) process cannot occur thermally by a concerted process. At first sight this may be confusing, as surely light is also again involved in the photoreversion, but the essential point is that the reaction does not proceed from the excited state of the dimer, but rather from the ground state. This follows from the relative energies of the excited chromophores involved: $^1FADH^-$, 240 kJ mol^{-1}; ^1Fol, 307 kJ mol^{-1}; ^{1}dimer, approximately 500 kJ mol^{-1}.

7. Intra-enzyme electron transfer

DNA photolyase from *E. coli* is isolated with the flavin in its free radical form (Enz-$FADH^\cdot$) although in vivo it is fully reduced (Enz-$FADH^-$). Studies of DNA photolyase from *E. coli* have employed nanosecond and picosecond laser flash photolysis and steady state and time-resolved fluorescence [9]. The suggested reaction sequence in vitro, leading from photon absorption through to dimer splitting, is summarized



Scheme 1.

in Scheme 1. Following excitation, the presence of a primary intermediate (40 ps after excitation), assigned to the π–π^* excited doublet state, has been detected. This intermediate rapidly undergoes ($\tau = 100$ ps) intersystem crossing ($\Phi_{ISC} \gg 0.1$) to give the n–π^* excited quartet state. Intra-enzyme hydrogen atom or electron transfer from tryptophan residue 306 then occurs to give the fully reduced form. Finally, substrate binding is followed by light absorption, dimer splitting and the subsequent dissociation of the pyrimidine monomers.

8. Electronic energy transfer from folate to reduced flavin

Photolyase contains two chromophores, flavin and folate (or deazaflavin). Hence an obvious question is why are there two chromophores present when one (flavin) can achieve dimer splitting? It is easy to understand from an evolutionary sense that a flavin was "chosen" due to their ubiquitous presence as redox-active components of numerous enzymes. In addition, a reduced rather than oxidized flavin was preferred due to its greater ability to donate electrons. However, reduced flavin suffers from a serious deficiency from the standpoint of a photocatalyst: it simply does not absorb light very well! Photolyase adopts a simple solution to this problem: one chromophore (folate) is used for light absorption, and the other (flavin) for chemistry. This is supported by the absorptivities of the two chromophores: folate exhibits $\epsilon = 25\,000\ M^{-1}\ cm^{-1}$ vs. $\epsilon = 5000\ M^{-1}\ cm^{-1}$ for $FADH^-$.

Hence we propose that long-range or Forster energy transfer occurs via dipole–dipole coupling between folate and flavin. Clear evidence for this is that the enzyme with only folate present (i.e. no flavin) displays a folate singlet lifetime of 354 ps, which is drastically reduced by the re-introduction of flavin to less than 30 ps (flavin radical form) or 130 ps (reduced flavin). This dramatic quenching corresponds to rate constants of energy transfer for $^1Fol \rightarrow FADH^•$ and $^1Fol \rightarrow FADH^-$ of 3×10^{10} and $3.6 \times 10^9\ s^{-1}$ respectively [10].

The rate of energy transfer depends on a number of factors, including the extent of overlap of the emission spectrum of the donor with the absorption spectrum of the acceptor, the relative orientation of the donor and acceptor transition dipoles and the distance between the chromophores.

Theoretical expressions for long-range coulombic energy transfer as developed by Forster were applied using the above rate constants. The critical distance (nm), defined as the distance at which 50% of the donor energy is transferred to the acceptor, is given by the following equation

$$R_0 = (K^2 \Phi_d n^{-4} J)^{1/6} \times 9.79 \times 10^4$$

where K is an orientational factor (taken as two-thirds, but see later), Φ_d is the quantum yield of folate emission in the absence of flavin (0.32), n is the refractive index of the medium and J is the spectral overlap integral (overlap between the folate emission spectrum and the absorption spectrum of the acceptor flavin).

Table 1
Energy transfer parameters for folate → flavin

Parameter	Fol → FADH$^{\cdot}$	Fol → FADH^{-}
E (%)	92	62
R_0 (Å)	31.8	23.5
$J \times 10^{14}$ (cm^3 M^{-1})	2.06	0.34
R (Å)	21.1	21.7

E, efficiency of energy transfer; R_0, Forster critical distance; J, overlap integral; R, calculated distance.

Furthermore, for R, the actual distance between the donor and acceptor, the efficiency of energy transfer (calculated from the fluorescence lifetimes) is given by

$$\text{Efficiency} = R_0^6/(R_0^6 + R^6$$
$$= \sum F_d(\lambda)\epsilon(\lambda)\lambda^4\Delta\lambda / \sum F_d(\lambda)\Delta\lambda$$

where $f_d(\lambda)$ is the corrected fluorescence intensity of the donor and $\epsilon(\lambda)$ is the extinction coefficient of the acceptor. The results obtained (Table 1) lead to the following conclusions: folate to FADH$^{\cdot}$, $R_0 = 3.18$ nm, $R = 2.11$ nm; folate to $FADH_2$, $R_0 = 2.35$ nm, $R = 2.17$ nm.

One of the main problems in any such study is the determination of the orientation factor K, which is difficult. In fact, it is not the orientation of the molecular framework of the donor and acceptor that is needed, but the orientation of the transition dipoles, which is even more difficult to evaluate. Generally, a random orientation of chromophores is assumed ($K = 2/3$). Only recently has the crystal structure of the enzyme been determined, and this showed that the flavin–folate distance was in fact 15 Å. This apparent discrepancy between the X-ray crystallography data and the energy transfer calculation can be explained by the fact that the orientation of the transition dipoles is not random. Furthermore, it appears that photolyase has not adopted the best orientation of the chromophores for energy transfer. This is rather puzzling as it is expected that the enzyme would have evolved such that an ideal orientation would be adopted.

9. Electron transfer from excited flavin to dimer

From the foregoing discussion, we can effectively exclude energy transfer from the enzyme to DNA, and we are therefore left with electron transfer processes as possible mechanisms of dimer splitting. An indication that electron transfer is involved came experimentally from time-resolved electron spin resonance (ESR) studies involving photoexcitation of the enzyme–substrate complex [11]. Although no information on the structure(s) of the radical(s) was obtained, its mere observation was highly significant in confirming that electron transfer had taken place. More significant are recent observations of a spin polarized electron paramagnetic resonance (EPR) signal following excitation of the enzyme–substrate complex at 4 K. This is highly suggestive of a radical pair being involved. Having established that electron transfer takes place, the obvious question is in which direction, from reduced flavin to dimer or the reverse?

Photon absorption by FADH^{-} essentially promotes an electron from the highest occupied molecular orbital (HOMO), which is a π bonding orbital, to the lowest unoccupied molecular orbital (LUMO), which is an antibonding or π^* orbital (Fig. 5). Thus ^{1}FADH^{-} is a much better electron donor than the ground state, and an electron can be

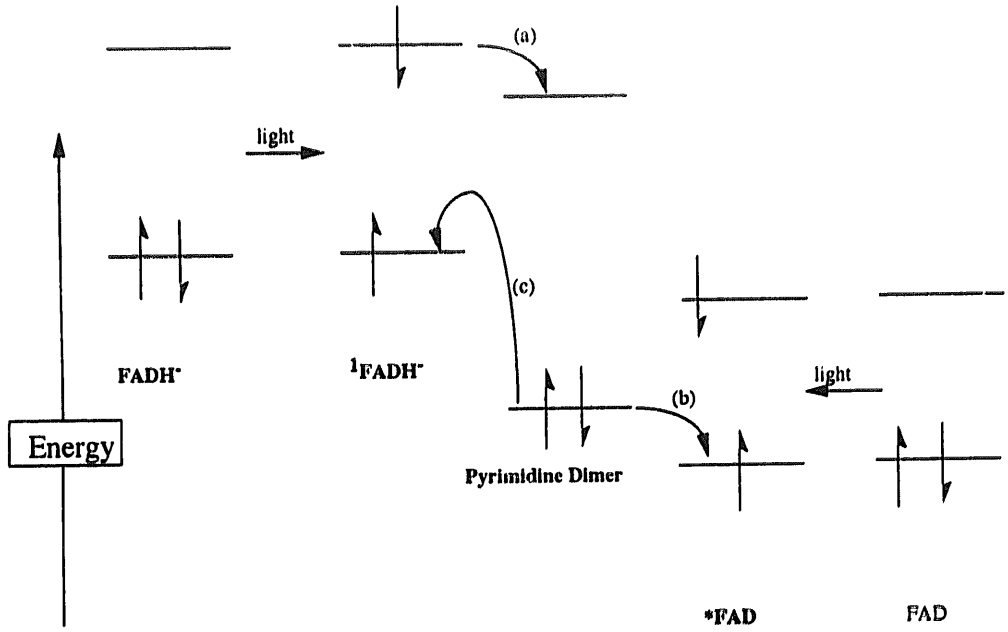


Fig. 5. Orbital energies of the reduced (FADH^{-}) and oxidized (FAD) chromophores in their ground and excited states. Electron transfer from flavin to dimer (a) and dimer to flavin (b).

Table 2
Thermodynamic parameters related to pyrimidine dimer splitting

Excited state	Electron transfer direction	ΔG (kJ mol^{-1})
Enz-^{2}FADH$^-$	←pyr⟨⟩pyr	62 ± 8
Enz-^{4}FADH$^-$	←pyr⟨⟩pyr	73 ± 8
Enz-^{2}FADH$^-$	→pyr⟨⟩pyr	−35 ± 35
Enz-^{1}FADH$_2$	←pyr⟨⟩pyr	180 ± 40
Enz-^{1}FADH$_2$	→pyr⟨⟩pyr	−125 ± 30

transferred from the singly occupied π^* orbital of ^{1}FADH$^-$ to the LUMO of the dimer. The alternative process, electron transfer from the dimer to flavin, may be thought to be unlikely as, intuitively, reduced flavins would be expected to be electron donors rather than acceptors. However, just as photoexcitation increases the exergonicity of electron loss, the electron affinity of FADH$^-$ will be increased markedly on excitation. Electron gain by ^{1}FADH$^-$ would result in the formation of three-electron reduced flavin species (FADH$^{\cdot 2-}$), i.e. a kind of super-reduced flavin. Just such a species has been formed in pulse radiolysis studies [12]. Furthermore, molecular orbital calculations and experimental studies predict that both the Pyr⟨⟩Pyr cation radical and anion radical are prone to decay by ring splitting to constituent monomers [13].

One approach to the problem of the direction of electron transfer is to calculate the energetics of the various possible processes, and the results are given in Table 2. The energetics of the two alternatives exclude electron transfer from the dimer to ^{1}FADH$^-$ because the reduction potential of ^{1}FADH$^-$ is too negative by at least 1.5 V to abstract an electron from a pyrimidine dimer. Hence excited reduced flavin can transfer an electron to, but not from, pyrimidine dimers.

10. Unique energetics of the enzymatic reaction

The rate of dimer cleavage is important in determining the efficiency of the enzymatic reaction (and, of course, for the non-enzymatic model systems). Splitting must be faster than back electron transfer for the reaction to proceed. The rate [14] of splitting of the *cis-syn*-thymine dimer by electron donation from *N,N*-dimethylaniline singlet state is approximately 10^6 s^{-1}. It is clear, however, that enzymatic splitting must be much faster, as such a slow rate of splitting would not be able to compete with back electron transfer. From the free energy of the back reaction, the rate is expected to be similar to the forward rate, around 10^9–10^{10} s^{-1}. Hence the splitting rate must be faster. One way for the enzyme to accomplish an increase in the splitting rate is to increase the strain in the reactant by making it resemble a transition state (and therefore make ΔG more negative and reduce $\Delta G^{\ddagger}$). Theoretical calculations have shown that factors which twist the cyclobutane ring, such as methyl substitution, markedly increase the exergonicity of the reaction. For example, the enthalpies of splitting of various pyrimidine dimers (ΔH_{spl}) were calculated using the MNDO/PM3 hamiltonian and are listed in Table 3. As can be seen, splitting of the *cis-syn*-uracil dimer is considerably more exothermic than the splitting of cyclobutane itself. This undoubtedly reflects the great degree of strain involved in this configuration due to the interaction of the pyrimidine rings. Although the trans-syn stereoisomer of uracil is 18.7 kJ mol^{-1} more stable than the cis-syn isomer, because of the reduced interactions of the pyrimidine rings, there is still a difference of approximately 131.4 kJ mol^{-1} compared with cyclobutane. As expected, the introduction of a methyl group at C(5) and C(5′), as in the thymine dimer, increases the exothermicity of splitting for both the cis-syn and trans-syn isomers.

The differences in ΔH_{spl} between cis-syn and trans-syn stereoisomers correlates quite well with the experimental observation that X-ray irradiation at 77 K of *cis-syn*-*N*(1),*N*(3)-dimethyluracil dimer results in splitting which is too fast to measure by ESR detection (i.e. radiolysis forms the monomer radical anions directly) [15]. In marked contrast, the trans-syn isomer gives a novel isotropic 19 G ESR doublet, assigned to an asymmetrical dimer radical anion, which is stable for several minutes. In fact, if a small increase in strain enhances the splitting rate, this has important biological implications as the cis-syn dimer in DNA has been shown to be puckered due to deformation of the double helix.

An alternative method of increasing the splitting efficiency is to slow the back electron transfer (i.e. the transfer of an electron from the dimer radical anion to FADH$^{\cdot}$ before splitting can occur). In a non-polar environment, however, this highly exergonic reaction may be in the Marcus inverted region, i.e. the large driving force slows the rate of back electron transfer (see Fig. 6). It may be that photolyases use a low polarity active site to slow the back electron transfer and thereby increase the competitive splitting reaction.

Results of photosensitized dimer splitting as a function of solvent are shown in Table 4. The nature of the active site of photolyases is believed to be relatively non-polar on the basis of a study of the UV–visible absorption spectrum of the fully

Table 3
Enthalpy of splitting of pyrimidine cyclobutane-type dimers

Compound	ΔH_{spl} (kJ mol^{-1})	
	RHF calc. for neutral molecule	UHF calc. for anion radical
cis-syn-Uracil dimer	4.0	−16.4
cis-syn-Thymine dimer	−27.1	−36.1
trans-syn-Uracil dimer	22.7	−2.9
trans-syn-Thymine dimer	−12.1	−26.1
cis-syn-*N*(1),*N*(1′)-Trimethylene-bridged uracil dimer	−26.0	–
Cyclobutane	154.1	–

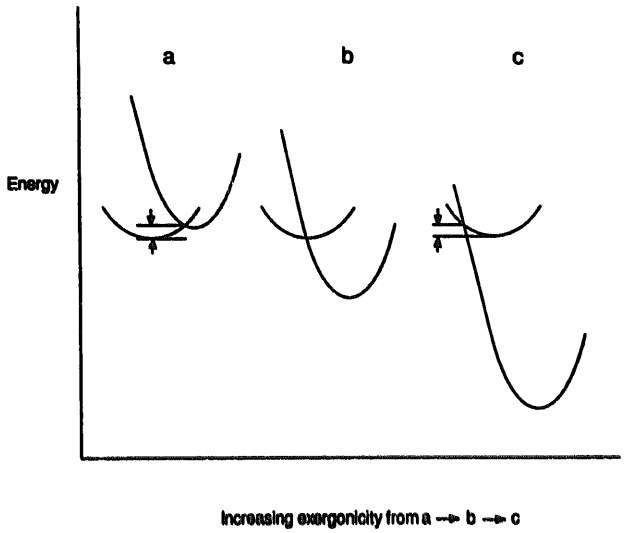


Fig. 6. The effect of increasing exergonicity on electron transfer. In (a), the process is slowed by the activation barrier (arrows). The process is faster in (b), which is "activationless". A further increase in exergonicity, as in (c), shows the reappearance of a barrier (arrows) and hence there is a slowing of the process.

oxidized (yellow) form of the enzyme, which exhibits vibrational fine structure typical of an environment with a dielectric constant around 10. Hence most of the FAD molecules must be buried in the protein and not be exposed to the aqueous phase in agreement with the X-ray crystal structure. This may well help to explain the success of the enzyme in facilitating splitting relative to the competing back electron transfer.

The electron transfer from $^1FADH^-$ to the dimer may proceed over relatively large distances. To date no crystal structure for the enzyme–substrate complex is available and, even if it were, it might not represent the situation in solution. However, we know that the rate constant for electron transfer is approximately $5 \times 10^9\ s^{-1}$ from the difference between the rate constant of decay of $^1FADH^-$ in the enzyme–substrate complex and the enzyme alone. The rate of electron transfer depends on the distance between the donor and acceptor, as well as the nature of the intervening medium (amino acid side-chains, peptide bonds, hydrogen bonding, aromatic groups, conformations of groups, etc.). Nevertheless, we can estimate the distance to be less than 10 Å.

Of particular interest is that it has been shown [9,16] that the lifetime of $^1FADH^-$ is 0.1 ns when free in aqueous solution, but 1.6 ns when enzyme bound. This is of some significance given that the rate of the forward electron transfer is only approximately $10^9\ s^{-1}$. It follows that photolyase would in fact fail in its task if it were not for the enhanced singlet lifetime when enzyme bound, as the electron transfer would not be competitive. It is known from earlier studies that reduced flavin is probably bent along the N(1)–N(5) axis due to antiaromaticity of a planar, central dihydropyrazine ring [17]. The enzyme-bound chromophore may be more fluorescent due to restriction in flexing along this axis.

11. Energetics of the overall repair process

As well as the calculation of the thermodynamics of the primary step, the overall thermodynamics of the enzyme-catalysed process are outlined in Fig. 7. The first step, flavin excitation, as we have seen, has $\Delta G = 240\ kJ\ mol^{-1}$. Next, electron transfer to the dimer occurs, to give $FADH^{\cdot}$ and $Pyr\langle\rangle Pyr^{\cdot -}$ ($\Delta G = -125\ kJ\ mol^{-1}$). More problematical is the estimation of the free energy of dimer anion splitting. One experimental determination gave $\Delta H = -110\ kJ\ mol^{-1}$ for splitting of a highly strained bridged dimer [18]. However, as we have seen (Table 3), the precise configuration is thought to influence markedly the energetics of splitting. Hence the best estimate of ΔG for anion splitting is -50 to $-80\ kJ\ mol^{-1}$ for a pyrimidine dimer in a B-DNA helix. Finally, the return of the electron (essential for the completion of the catalytic cycle) has a ΔG value of $-120\ kJ\ mol^{-1}$. It should be noted that the overall change is certainly exergonic, and hence the action of photolyase is photocatalytic rather than storing chemical energy in the product.

12. Mechanism of dimer splitting

In one sense the question of the mechanism of dimer splitting can be resolved in isolation if we assume that, once an electron has been added to a dimer, splitting will take place with the same mechanism (if not necessarily the same rate) whether enzyme bound or not, and hence we can apply the results of model studies. The relative susceptibility of pyrimidine dimers is certainly enhanced by electron addition, as clearly shown experimentally in radiolysis experiments. It is known that γ radiolysis of pyrimidine dimers leads to cleavage [19], according to

$$e^-(aq) + Pyr\langle\rangle Pyr \rightarrow Pyr\langle\rangle Pyr^{\cdot -} \rightarrow Pyr + Pyr^{\cdot -}$$

The difference in one-electron reduction potentials between the dimer and monomer has been estimated as

Table 4
Solvent dependence of splitting of *cis-syn*-1,3-dimethyluracil dimer covalently linked to an indole photosensitizer

Solvent	Φ_{sp}
Water	0.06
DMSO	0.15
Acetonitrile	0.19
Methanol	0.23
Ethanol	0.24
1-Propanol	0.25
2-Propanol	0.25
1-Butanol	0.26
1-Pentanol	0.27
1-Hexanol	0.30
Diethyl ether	0.37
Benzene	0.39
1,4-Dioxane	0.40

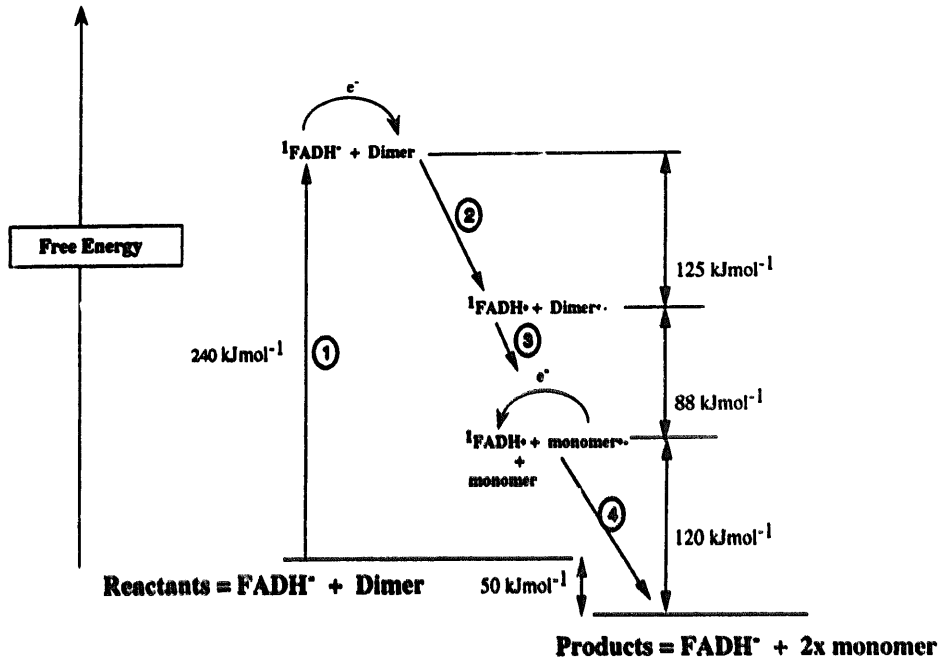


Fig. 7. The thermodynamics of the major processes involved in DNA repair: 1, excitation; 2, electron transfer from excited $FADH^-$ to the pyrimidine dimer; 3, dimer splitting; 4, back electron transfer from the monomer anion to the FADH• radical; 5, splitting of the neutral dimer (does not occur at a measurable rate).

0.3 V (dimer potential is more negative) by studies of the rates of electron transfer between dimer or monomer and a series of excited electron acceptors. This gives an estimate of the free energy of splitting of the radical anion of the dimer of 38 kJ mol^{-1} more exergonic than that of the neutral compound.

Following the primary electron addition and splitting, a chain reaction occurs in simple solutions as follows

$$Pyr^{\bullet -} + Pyr\langle\rangle Pyr \rightarrow Pyr + Pyr\langle\rangle Pyr^{\bullet -}$$

We have used pulse radiolysis to follow the growth of $Pyr\langle\rangle Pyr^{\bullet -}$ over 250 μs. The chain length can be as high as 50. However, it is very unlikely that the same reaction takes place in the enzyme, as release of the monomer anion (in order to react with a free dimer) would have to compete with the return of an electron from $Pyr^{\bullet -}$ to $FADH^\bullet$.

The mechanism of dimer splitting following electron addition has been studied by measurement of secondary deuterium isotope effects and low temperature ESR spectroscopy. Deuterium substitution has been carried out at C(5) and C(6) positions of a 2'-deoxyuridine photodimer. Following electron addition to the dimer from 5-methoxyindole excited singlet state, isotope effects of 1.17 and 1.08 were detected for the C(5) and C(6) deuterated compounds respectively [20]. In contrast, the photolyase-catalysed reaction shows almost equal effects of deuteration at C(5) or C(6). This suggests that the energetics of transition state formation and breakdown are significantly different in this case. This may reflect an alteration in conformation of the substrate on binding to the enzyme.

A fundamental question about dimer splitting concerns why the addition of an electron should facilitate the splitting reaction. It can be shown that the addition of an electron to the dimer does not render the splitting reaction orbital symmetry allowed. This can be seen by reference to Fig. 8, which is an orbital symmetry correlation diagram which shows how orbital symmetry in a sense relates the σ orbitals in the reactant with the π orbitals in the product. As the figure shows, the thermal cycloreversion (illustrated for cyclobutane radi-

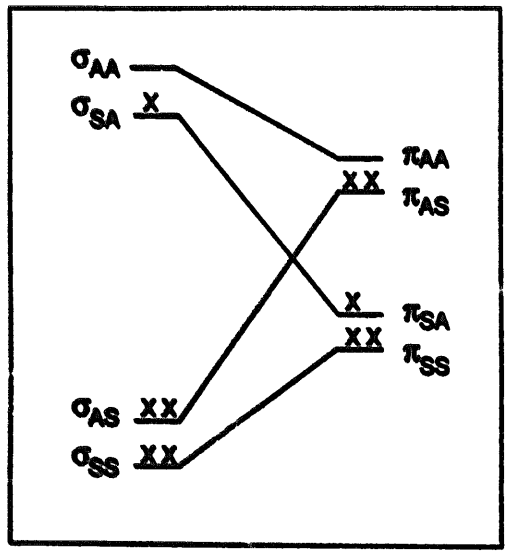


Fig. 8. An orbital symmetry correlation diagram for the splitting of the cyclobutane radical anion into ethene and ethene radical anion, shown as a model for pyrimidine dimer radical anion splitting. The thermal process is orbital symmetry forbidden.

cal anion → ethene + ethene radical anion) is forbidden because the products of splitting would be produced in an electronically excited state (π_{AS} is fully occupied while the lower energy π_{SA} is only singly occupied). Apparently then, photolyases do not add an electron to the dimer to evade the proscription against concerted thermal splitting of the dimer. Instead, the extra electron lowers the activation barrier to splitting.

Another question is whether the reaction is concerted or stepwise. Simple HMO theory was used to examine the energetics of various possible pathways for dimer radical anion splitting [13]. A detailed analysis revealed that addition of an electron to the dimer reduced the energy barrier to splitting. This occurred only for the stepwise mechanism in which the C(5)–C(5′) bond of the dimer split before the C(6)–C(6′) bond and for the non-synchronous concerted pathway in which C(5)–C(5′) bond breaking was significantly accelerated relative to C(6)–C(6′) bond breaking. These effects were exerted by the energy of the orbital that contained the "extra" electron, which was lower for the transition state (compared with the reactant) when the process was stepwise or non-synchronous concerted. For the synchronous concerted pathway, the energy difference between the reactant radical anion and the transition state was higher. Furthermore, our experiments on picosecond flash photolysis of the enzyme–substrate complex identified at least one and probably two spectroscopically distinct intermediates following photoexcitation [21]. This is highly significant, as only a stepwise mechanism would involve intermediates between the dimer anion and the product monomer and monomer anion radical. An example of a possible reaction scheme is given in Fig. 9.

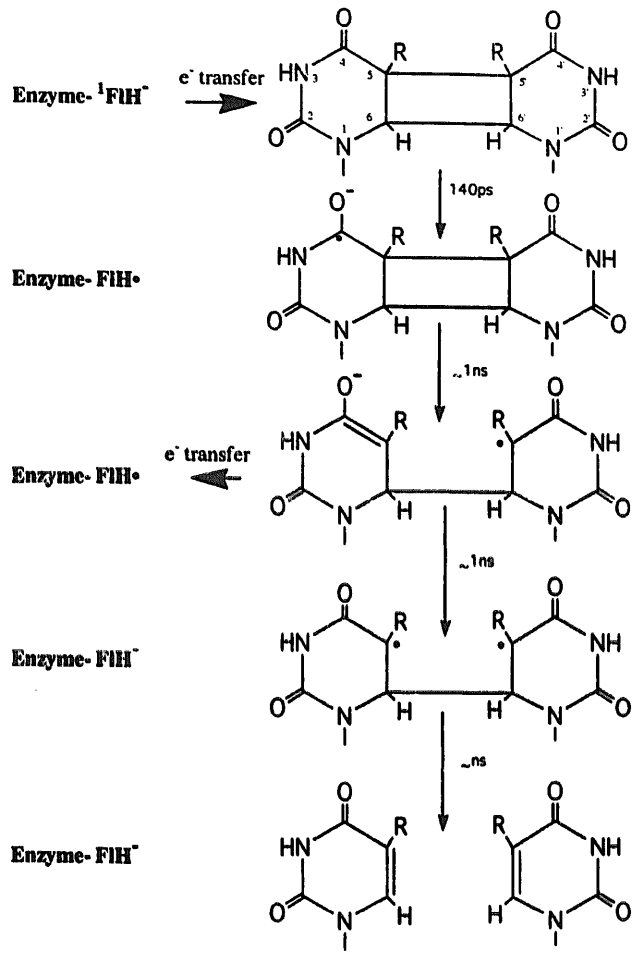


Fig. 9. Proposed reaction mechanism for pyrimidine dimer splitting by photolyase.

13. Conclusions

The role of DNA photolyase in pyrimidine dimer splitting can be summarized as follows:

(1) to recognize the dimer and bind it sufficiently close to FLH^-, so that within the lifetime of $^1FLH^-$ electron transfer can take place;
(2) to add an electron to the dimer; electron transfer from $^1FLH^-$ to the dimer occurs at a rate of $5 \times 10^9\ s^{-1}$ over approximately 10 Å, i.e. the one-electron reduced dimer is more prone to splitting;
(3) to increase the lifetime of $^1FLH^-$, i.e. $\tau = 0.1$ ns free in solution and $\tau = 1.7$ ns when enzyme bound, perhaps by holding the flavin in a more planar conformation by reducing N(1)–N(5) flexing;
(4) to decrease the rate of back electron transfer by lowered polarity at the active site, i.e. back electron transfer moves into the Marcus inverted region;
(5) to increase the strain in the cyclobutane ring, as this may increase the rate of dimer splitting.

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