Photoenzymic Repair of UV-Damaged DNA: A Chemist's Perspective

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1 Introduction

1.1 The Fundamental Reaction

DNA photolyase is an enzyme that reverses the principal damage caused to DNA by UV light, *i.e.*, it acts on cyclobutane-type pyrimidine dimers formed by adjacent pyrimidine bases[1,2] (Figure 1). It accomplishes this task by recognizing and binding to such a dimer, transferring an electron to it, and thereby splitting the dimer, hence restoring the DNA functionality. Neither DNA-recognizing or electron-transferring enzymes are unique or even unusual. What distinguishes DNA photolyase from virtually all other enzymes is that it is 'photon powered'. Further, it has a unique system for gathering light with one pigment and transferring the energy over 15—17 Å to a redox-active pigment that is capable of dimer splitting. Thus, the great advantage to the chemist hoping to understand the enzyme's mechanism is that following the addition of the reactant (*i.e.* substrate=pyrimidine dimers), the reaction is essentially 'put on hold' until the vital ingredient, 'the photon', is added. Unlike all other enzymes, mechanistic studies are not limited by factors such as mixing times or substrate turnover rates (reaction rates). Instead, DNA photolyase is amenable to study on any time scale, limited only by the instrumentation available.

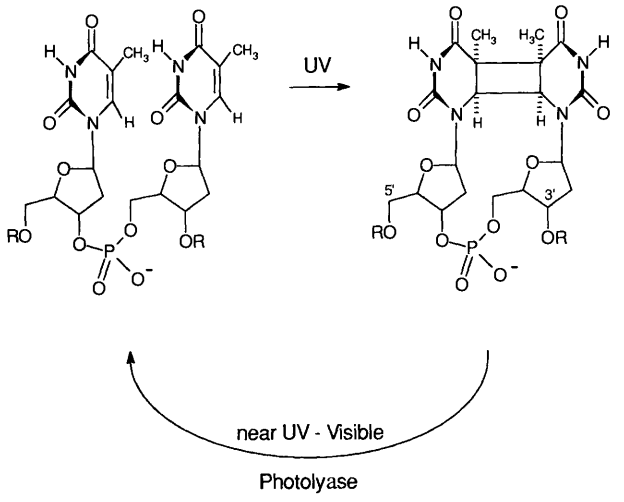

Figure 1 Thymine dimer formation by stacked thymine nucleotides in the same strand of double strand DNA induced by ultraviolet light (UV-B, 280—315 nm). The reverse reaction, *i.e.*, repair, is catalysed by DNA photolyase, which uses near-UV and visible light.

1.2 A Little Biochemical Background

DNA photolyases are monomeric proteins of $M_r \sim 55000$—65000. 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 the light of concerns regarding 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 light-absorbing cofactors (*i.e.*, chromophores).[5] One is always 1,5-dihydroflavin adenine dinucleotide ($FADH_2$, possibly in its anionic form, *i.e.* $FADH^-$), and the other can be either methenyltetrahydrofolate (MTHF) or 8-hydroxy-5-deazariboflavin (Figure 2). Accordingly, the enzymes have been classified into two groups: the folate class, which has an action spectrum (*i.e.*, the wavelength dependence of their catalytic activity) of 360–390 nm, and includes enzymes from *Escherichia coli* and *Saccharomyces cerevisiae*, and the deazaflavin class, which has an action spectrum maximum at

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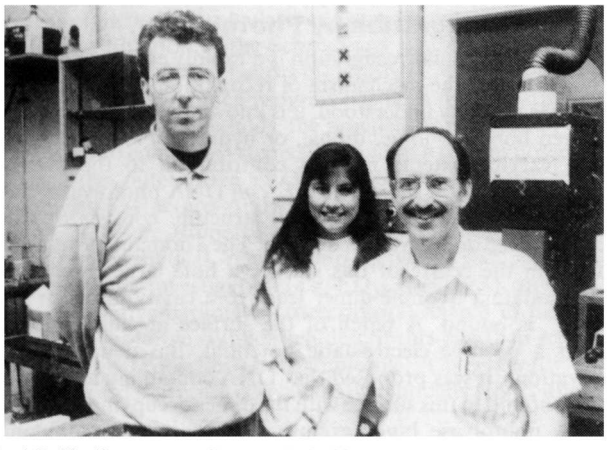

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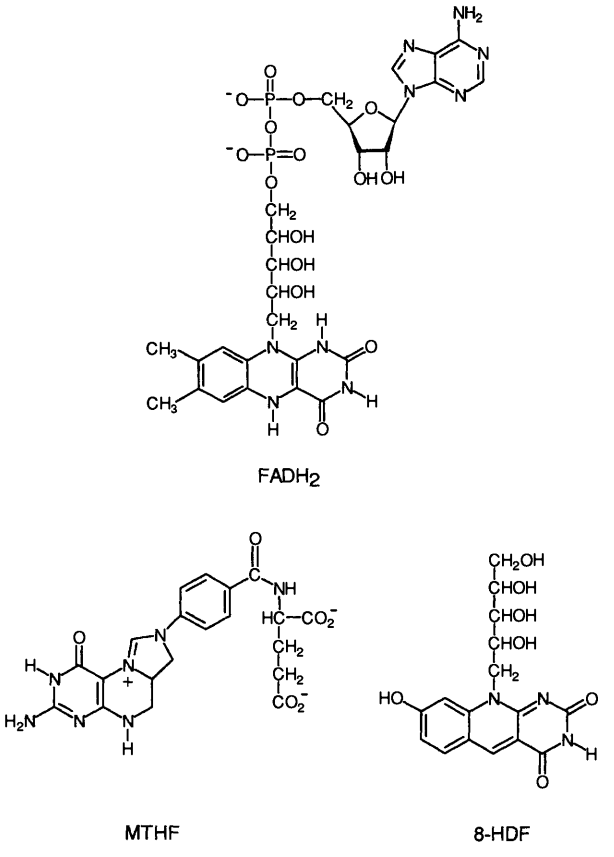


Figure 2 The structures of the electron donating cofactor $FADH_2$ and the two light-gathering antenna cofactors MTHF and 8-HDF.

430—460 nm, and includes photolyases from *Anacystis nidulans*, *Streptomyces griseus*, *Scenedesmus acutus*, *Halobacterium halobium*, and *Methanobacterium thermoautotrophicum*. Recently, however, photolyase[6] from *Neurospora crassa*, which contains $FADH^-$ and MTHF, was found to exhibit an action spectrum maximum at 396 nm. Additionally, a medium wavelength type DNA photolyase[7] has been isolated from *Bacillus firmus*. This enzyme has been shown to contain folate as the second chromophore and displays an absorption and action spectrum peak at 410 nm.

Most other photolyases await characterization. Nonetheless, photoreactivation action spectra, either *in vivo* or in crude, cell-free *in vitro* systems, generally reveal maxima either at 360—396 nm or in the 430—460 nm range, which suggests that they also belong either to the folate or the deazaflavin class.

2 DNA Damage by UV Light

First we must understand the nature of the damage to DNA. Absorption of light by DNA results in 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, which pass their energy to thymine, because thymine has the lowest triplet energy of the common bases. The thymine triplet state can return to the ground state without any reaction. Occasionally, however, it may be formed adjacent to another thymine (or possibly a cytosine) in the same strand of the DNA double helix and a photochemical reaction between the two pyrimidines can then take place.

This photochemical reaction is a pericyclic reaction, *i.e.*, a concerted reaction involving a cyclic system of interacting orbitals. Concerted means that bond breaking and bond formation occur simultaneously. The bond breaking and bond formation can, of course, involve several pairs of atoms. The reaction between adjacent pyrimidine bases in DNA results in the conversion of two pi bonds (one in each pyrimidine) into two sigma bonds, a process that is analogous to the conversion of two molecules of ethene into one molecule of cyclobutane. This is described as a $[\pi^2 + \pi^2]$ photocycloaddition. In their pioneering and elegant work, Woodward and Hoffmann showed that such reactions are governed by orbital symmetry considerations. Accordingly, the photoaddition of two pyrimidines is orbital symmetry allowed, and once formed, the cyclobutane product (*i.e.*, the pyrimidine dimer) cannot revert to two pyrimidines by a nonphotochemical process (*i.e.*, a thermal reaction of the ground state). Although the reverse photochemical reaction is symmetry allowed, dimers to not significantly absorb near-UV light, as they do not possess the conjugated π systems of the original pyrimidines, and thus, the dimers accumulate in DNA.

The structures of the isomeric dimers of thymine are shown in Figure 3. The dimers designated *cis-syn* and *trans-syn* are stereoisomers. Because they are not mirror images, they are diastereomers. The *cis-syn* isomer has a mirror plane of symmetry and thus is not chiral, but is a *meso* compound (*i.e.*, does not exhibit enantiomerism). In contrast, the *trans-syn* dimer exists as two mirror-image isomers that are, of course, chiral (*i.e.*, enantiomers). Thus, the *cis-syn* dimer is a diastereomer of each of the enantiomeric *trans-syn* dimers.

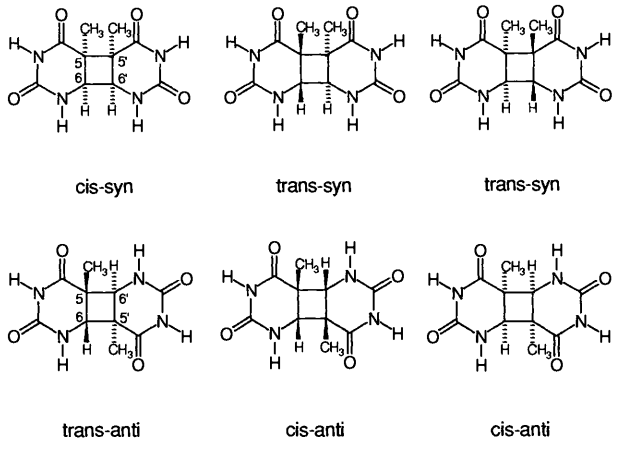


Figure 3 The structures of the isomeric thymine dimers.

In contrast, the *cis-anti* and *trans-anti* dimers do not have the same connectivity as the *cis-syn* and *trans-syn* dimers, so the *syn* and *anti* sets of isomers are not stereoisomers but rather constitutional isomers (specifically, regioisomers). The *trans-anti* dimer has a centre of inversion and thus is not chiral. The *cis-anti* dimer exists as a pair of enantiomers, each of which is a diastereomer of the *trans-anti* dimer.

3 Dimer Recognition by Photolyase

The field of molecular recognition is a rapidly emerging area of study. Although the mechanism of recognition of substrate by enzymes is not fully understood, it is known that factors such as hydrogen bonding, ionic, dipole, or hydrophobic interactions can to varying extents make a contribution to the overall binding. The crystal structure of *E. coli* DNA photolyase[8] has been solved to 2.3 Å. The overall structure consists of two domains, an $\alpha\beta$ domain and α domain. The α domain 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 the $FADH^-$ is bound. A patch of the surface around the hole exhibits a positive electrostatic potential. Based upon 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 contain-

ing a thymine dimer compared with a monomer (K_{assoc} = 3 5 × 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 [9]

It is known that 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 contribute to the overall interaction Comparison of K_{assoc} for binding to dimer-containing poly(deoxythymidine) (K_{assoc} = 5 × 10^7 M^{-1}) *versus* the dinucleotide (1 8 × 10^4 M^{-1}), indicates that 50% of the binding energy comes from interactions with flanking nucleotides *E coli* DNA photolyase binds with equal affinity to thymidine photodimers in either single or double strand DNA This is possibly because the dimer-induced conformational changes observed in duplex B-DNA are present in single strand DNA as well [10 11]

Binding of substrate is generally insensitive to the sequence around the dimer, yet very sensitive to the base composition of the dimer itself, with relative affinities Thymine–Thymine dimer > Uracil–Thymine dimer > Uracil–Uracil dimer ≫ Cytosine–Cytosine dimer In addition, it was found that *E coli* DNA photolyase binds to and repairs uracil dimers in RNA [12]

Experiments that investigate features of the substrate and the enzyme that are important in recognition have been carried out Ethylnitrosourea treatment of substrate DNA to esterify various phosphate oxygen positions along the sugar–phosphate backbone relative to the dimer was followed by addition of photolyase In this way those phosphate esters that disrupted binding to the enzyme either by preventing essential ionic interactions or by steric hindrance could be identified For yeast photolyase, ethylation of the phosphate immediately 5′ to the dimer weakly inhibited binding, whereas ethylation of any of the three phosphates 3′ to the dimer strongly inhibited binding The fourth phosphate 3′ to the dimer was weakly involved in binding

To determine the role nucleotides surrounding the dimer play in binding, dimer-containing DNA was allowed to react with dimethyl sulfate, and then photolyase was added The patterns of guanine N(7)-methylation in the enzyme-bound *versus* free DNA showed that guanine nucleotides immediately 5′ to the dimer and those up to three nucleotides 3′ to the dimer were important in binding to the enzyme Methylation may interfere with either formation of a key hydrogen bond, or, more likely, since binding is not affected by nucleotide sequence, by steric hindrance [9]

In addition, studies of the ionic strength dependence 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 figure 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)

Comparison of the amino acid sequence of the enzyme isolated from evolutionarily diverse organisms shows regions of highly similar sequences (homology) from one species to another Regions that show high homology only to other enzymes within the folate class are thought to be involved in folate binding, and the same is true for high homology regions within the flavin class It was also reasoned that regions of highly conserved sequences within all photolyases must contain the substrate binding domain The carboxy-terminal half contains the FAD binding site and is generally thought to be involved in DNA binding [13]

A single tryptophan residue located within a region highly homologous in all other photolyases was found to be important in DNA binding Mutant proteins in which trp^{277} was replaced with selected amino acids were prepared and characterized Photolyase in which trp^{277} was replaced by phenylalanine showed similar substrate affinity (K_{assoc} = 1 7 × 10^9 *versus* 2 2 × 10^9 M^{-1} for trp^{277}), which indicates that tryptophan hydrogen bonding capability was not essential for binding Mutants in which trp^{277} was replaced with one of five other amino acids showed 300- to 1000-fold lower substrate affinity, yet the mutant proteins were photochemically and catalytically competent It was concluded that trp^{277} is part of the DNA binding site and contributes to specificity either by van der Waals or stacking interactions [14]

Yeast photolyase was subjected to reductive methylation by formaldehyde/$NaBH_3CN$ both in the presence and in the absence of substrate Methylation of the lysine side chains of the enzyme in the absence of the substrate destroyed the DNA-binding ability In contrast, methylation carried out with substrate bound to the enzyme active site yielded enzyme in which DNA binding ability was maintained A double-label experiment enabled the particular lysine in the active site to be identified [15] as lys^{517}

Studies of enzymes from *E coli* and *S cerevisiae* show interaction with 6—8 base pairs (nucleotides) of the dimer-containing strand of DNA and 3—4 nucleotides on the opposite, undamaged strand It was suggested that photolyases approach the helix from the backbone of the dimer-containing strand and protrude into the major groove, where they interact with the cyclobutane ring and one or more bases, and also into the minor groove near the intradimer phosphodiester bond [16]

Surprisingly, this seemingly complex situation can easily be simulated 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 Figure 4 The macrocycle has covalently tethered chromophores that photosensitized dimer splitting The macrocycle complexes to a dimer (K_{assoc} ~ 10^4 M^{-1}), and subsequent absorption of light by the tethered methoxyindole chromophore produces the indole excited state This excited indole then presumably donates an electron to the noncovalently 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 (see Section 4 1) of 0 11 in acetonitrile solution under saturating conditions (*i e*, when all the molecules of macrocycle are bound to dimers) The macrocycle then binds to another dimer and the cycle is repeated Thus, the macrocycle acts as a photocatalyst

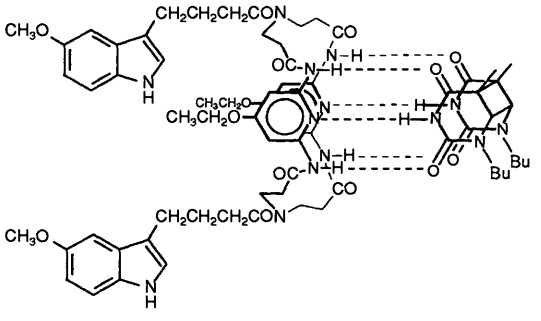


Figure 4 A macrocycle that recognizes pyrimidine dimers by the characteristic hydrogen bonding pattern and acts as a photocatalyst of dimer splitting

4 Dimer Splitting by Photolyase

4.1 How Efficient is Photolyase?

Photochemists measure the efficiency of photochemical reactions by the quantum yield (ϕ), which is defined as ϕ = (the number of product molecules formed) divided by (the number of photons absorbed) The value of ϕ is normally found to be between 0 and 1, unless a chain reaction is involved Photolyases exhibit a value for ϕ of 0 5—0 9, *i e*, an efficiency of 50—90%, if the light is absorbed directly by the flavin [5 6] This compares with experimental values around 10^{-3}—10^{-4} for simple model systems involving mixed solutions of flavins and pyrimidine dimers, although higher values have been found for other photosensitizers [17]

4.2 The Role of Excitation

No reaction occurs in the enzyme–substrate complex until photon absorption occurs The first step, *i e* , photoexcitation of the $FADH^-$ chromophore, produces an excited state of $\pi\pi^*$ character (*i e* involves excitation of an electron from a π bonding $\rightarrow\pi$ antibonding orbital), with a lifetime of 1 6 ns and an energy of 240 kJ mol ^{1} above the ground state The latter energy was determined from the well-resolved vibronic structure in the low temperature fluorescence emission spectrum of $FADH^-$

The fundamental reaction is simply to reverse the [2 + 2] cycloaddition 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 is not proceeding from the excited state of the dimer, but rather from the ground state This is because the light energy stays with the flavin chromophore and cannot be passed 'uphill' to the dimer This follows from the relative energies of the excited chromophores involved, which are $^1FADH^-$, 240 kJ mol^{-1}, ^{1}folate, 307 kJ mol^{-1}, and ^{1}dimer, ~ 500 kJ mol^{-1} Consequently, the conservation of orbital symmetry rules for a thermal reaction apply

4.3 How Photolyase Splits Dimers: Electron Transfer

From the foregoing we can effectively exclude energy transfer from the enzyme to the DNA, and we are therefore left with electron transfer processes as a possible mechanism 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 [18] In ESR spectroscopy, radicals give characteristic signals or spectra that reflect the nature and even the identity of the radical species present A signal was observed after averaging thousands of flashes from a laser Although no information on the structure(s) of the radical(s) was obtained, its mere observation is highly significant in confirming electron transfer had taken place

Flavoenzymes are widespread in nature and usually occur with their flavin in a fully oxidized form Although it is not unknown for enzymes to have a reduced flavin as a redox-active chromophore, why has photolyase evolved to function in this way? The answer appears to be that excited oxidized flavin is so electrophilic that it reacts preferentially with neighbouring amino acids rather than with the pyrimidine dimer In contrast, although excited reduced flavin is a strong reducing agent, the neighouring amino acids are not good electron acceptors Other reasons for the use of a reduced instead of an oxidized flavin by photolyases were gleaned from model studies Oxidizing photosensitizers (*e g* quinones) are efficient when not covalently linked to a dimer, *i e* , when the resulting radicals (quinone $^-$ and dimer $^+$) can diffuse apart When covalently linked, as a model for the enzyme–substrate complex, quinones are inefficient at splitting dimers (presumably because of efficient back electron transfer) and actually sensitize their formation!

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 orbital, to the lowest unoccupied molecular orbital (LUMO), an antibonding or π^* orbital (Figure 5) Thus $^1FADH^-$ is a much better electron donor than the ground state, and an electron can be transferred from the singly occupied π^* orbital of $^1FADH^-$ to the LUMO of the dimer (equation 1) The alternative process, electron transfer from the dimer to the flavin (equation 2), might be thought unlikely, as intuitively, reduced flavins would be expected to be electron donors rather than acceptors

$$*FADH + Pyr <> Pyr \rightarrow FADH + Pyr <> Pyr \quad (1)$$

$$*FADH + Pyr <> Pyr \rightarrow FADH^{2} + Pyr <> Pyr^{+} \quad (2)$$

However, just as photoexcitation increased the exergonicity of electron loss, the electron affinity of $FADH^-$ would be increased markedly (again by 240 kJ mol^{-1}) upon excitation, as the electron gained would now fall into the lower energy singly occupied π bonding molecular orbital (SOMO) rather than the π^* orbital (LUMO) Such a process of electron gain by $^1FADH^-$ would result in the formation of three-electron reduced flavin species ($FADH^{2-}$), *i e* , a kind of super-reduced flavin Just such a species has been formed in pulse radiolysis studies[19] (a rapid pulse of fast electrons followed by optical detection of transient intermediates), and it had spectral characteristics very similar to those detected after photoexcitation of the enzyme–substrate complex

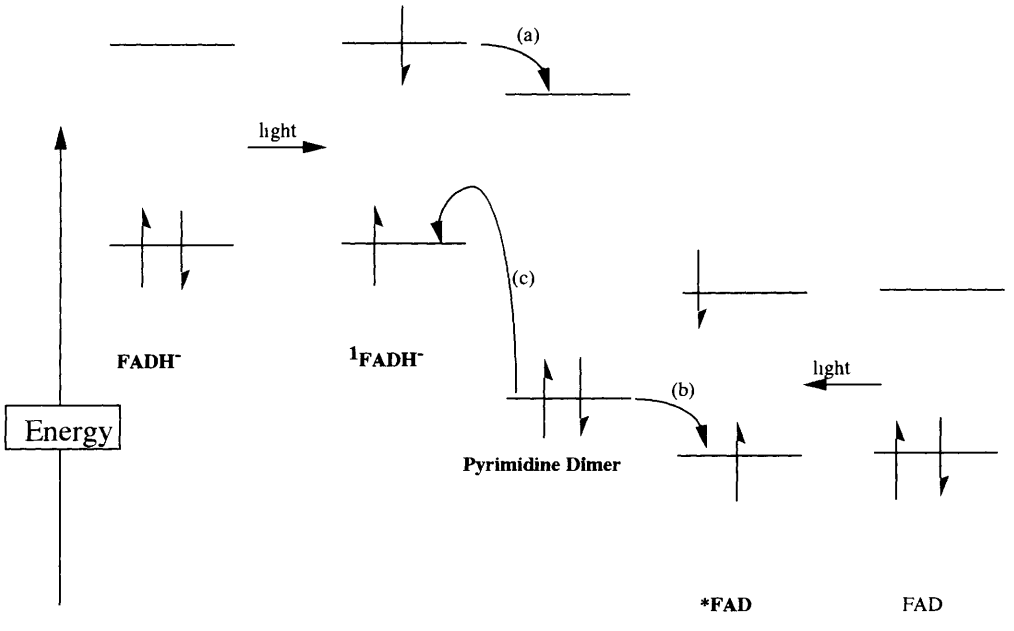


Figure 5 Orbital energies of the reduced ($FADH^-$) and oxidized (FAD) chromophores in their ground and excited states Electron transfer from the flavin→dimer (a) and dimer→flavin (b)

Further, 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 This is due to a kinetic acceleration of splitting that results from addition of an electron to or removal of an electron from a dimer [20] It is as though the transition state for splitting is not as destabilized as the ground state is by electron transfer, so the energy difference between dimer radical ion and transition state (*i e* , the activation barrier) is lower than for the neutral species One approach to the problem of the direction of electron transfer is to try to calculate the energetics of the various possible processes

The free energy change for a photoinduced electron transfer reaction is given in equation 3

$$\Delta G \text{ (kJ mol}^{-1}) = 96\,5\,[E^0(D^{\cdot+}/D) - E^0(A/A^{\cdot-}) - 0\,026] - E_{0\,0} \quad (3)$$

where $E^0(D^{\cdot+}/D)$ and $E^0(A/A^{\cdot-})$ represent the reduction potentials of the donor and acceptor (A), and $E_{0\,0}$ is the energy of the excited state of the flavin chromophore (calculated from spectroscopic information) Hence, the free energy changes for an electron transfer reaction in either direction between DNA photolyase and pyrimidine dimers can be calculated For example, $\Delta G = -125$ kJ mol^{-1} for electron transfer to the dimer, but $\Delta G = +180$ kJ mol^{-1} for electron transfer from the dimer These considerations lead us to exclude the electron transfer from dimer to ^{1}FADH$^-$ because the reduction potential of ^{1}FADH$^-$ is too negative by at least 1 8 V Hence excited reduced flavin is able to transfer an electron to, but not from, pyrimidine dimers

4.4 Overall Thermodynamics

As well as the calculation of the thermodynamics of the primary step, the overall thermodynamics of the enzyme-catalysed process are outlined in Figure 6 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 < > Pyr$^{\cdot-}$ ($\Delta G = -125$ kJ mol^{-1}) More problematical is the estimation of the free energy of splitting of the dimer anion radical One experimental determination gave $\Delta H = -110$ kJ mol^{-1} for splitting of a highly strained (neutral compound rather than anion radical) bridged dimer [21] However, it is known from semi-empirical molecular orbital calculations that the precise configuration markedly influences the energetics of splitting A more likely value of ΔG for anion splitting of a less strained neutral dimer is -50 kJ mol^{-1} The splitting of the anion radical is expected to be 38 kJ mol^{-1} more exothermic (see later) than the neutral compound (*i e* -88 kJ mol^{-1}) Finally the return of the electron (essential for the completion of the catalytic cycle) has a $\Delta G = -120$ kJ mol^{-1} (calculated from equation 3)

The sharp-eyed reader may well suspect a violation of Hess's law, as the sequence reactants→products in Figure 6 yields a value ΔG of -50 or -93 kJ mol^{-1}, depending on the route taken However, this merely reflects the uncertainties in the free energy changes assigned to each step It should be noted that the overall change is certainly exergonic, and hence the action of photolyase is photocatalytic rather than to store chemical energy in the product This is quite normal for enzymes, as otherwise they would lose energy on each cycle, and from where could this energy come? However, for a photoenzyme this need not be the case as energy is supplied on each cycle by the photon

4.5 Mechanism of Dimer Splitting

In one sense the question of the mechanism of dimer splitting can be resolved in isolation if we assume (perhaps dangerously) 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

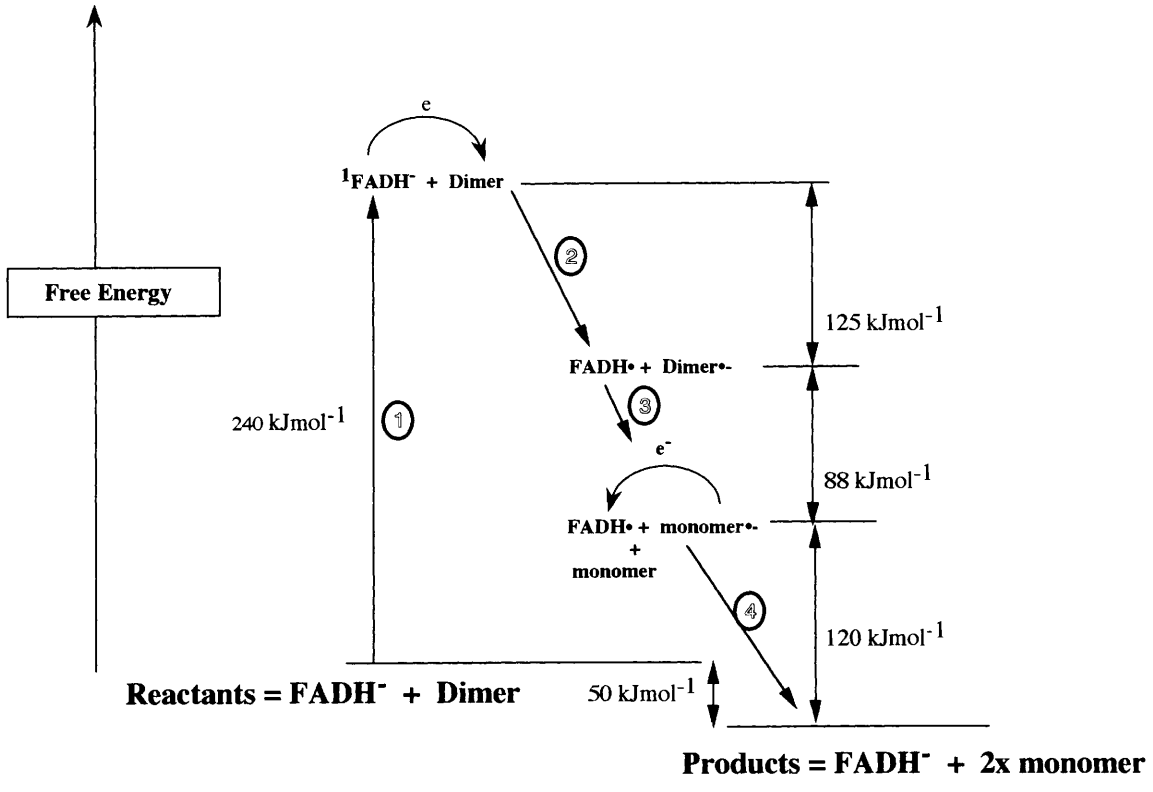


Figure 6 The thermodynamics of the major processes involved in DNA repair (1) Excitation (2) Electron transfer from excited FADH$^-$ to the pyrimidine dimer (3) Dimer splitting (4) Back electron transfer from the monomer anion to the FADH radical (5) Splitting of the neutral dimer (does not occur at a measurable rate)

shown experimentally in radiolysis experiments It is known that γ-radiolysis of pyrimidine dimers leads to cleavage,[22] according to equation 4

$$e\ (aq) + Pyr <> Pyr \rightarrow Pyr <> Pyr \rightarrow Pyr + Pyr \quad (4)$$

The difference in one-electron reduction potentials between the dimer and monomer has been estimated as 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 dimer radical anion as 38 kJ mol $^{-1}$ more exergonic than the neutral dimer

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

$$Pyr + Pyr <> Pyr \rightarrow Pyr + Pyr <> Pyr \quad (5)$$

Pulse radiolysis has been used to follow the growth of Pyr < > Pyr over 250 microseconds The chain length can be as high as 50 However, it is very unlikely that a chain reaction takes place in the enzyme catalysed reaction, 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 to FADH

The mechanism of dimer splitting following electron addition has been studied by the 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 [23] In contrast, the photolyase-catalysed reaction shows almost equal effects of deuteration at C(5) or C(6) This suggests that the energetics of the transition state formation and breakdown are significantly different in this case This may reflect an alteration in conformation of the substrate upon binding to the enzyme

When electron addition to Pyr < > Pyr (γ-radiolysis at 77 K) was monitored by ESR spectroscopy, the hypothetical dimer anion intermediate was not observed Instead, the monomer anion radical spectra were observed, which are characterized by well-defined anisotropic doublets with isotropic g-values close to that of the 'free spin' The doublets arise from hyperfine coupling to the C–H proton at the C(6) position of the pyrimidine ring The spectra are identical with those of the radical anions derived directly from the corresponding monomers and generated in a similar matrix Hence even at this low temperature, splitting is too rapid for the detection of the dimer radical

In marked contrast, the *trans-syn* isomer of 1,3-dimethyluracil dimer gave a novel isotropic 19 G ESR doublet with a g-value also close to that of a 'free spin' This can be assigned to an asymmetric dimer radical anion in which the large hyperfine coupling stems from σ–π overlap involving the β-proton, *i e* , at C(5)

4.6 Dimer Splitting, Concerted or Stepwise?

A fundamental question about dimer splitting concerned why the addition of an electron should facilitate the splitting reaction It can be shown that addition of an electron to the dimer does not render the splitting reaction orbital symmetry allowed This can be seen by reference to Figure 7, which is an orbital symmetry correlation diagram that 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 radical 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

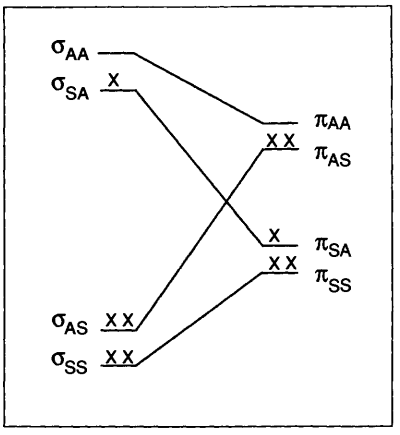


Figure 7 An orbital symmetry correlation diagram for the splitting of cyclobutane radical anion into ethene and ethene radical anion shown as a model for pyrimidine dimer radical anion splitting The thermal process is orbital symmetry forbidden

thermal splitting of the dimer Instead, the extra electron lowers the activation barrier to splitting, as described in Section 4 3

Another question is whether the reaction is concerted or stepwise In a concerted process, there are no intermediates along the reaction pathway from reactant to product, only a single transition state The breaking and formation of bonds do not have to occur at precisely the same rates, which allows for nonsynchronous concerted *versus* synchronous concerted processes to be described The essential distinction between stepwise and concerted processes is that in the former, the reactant goes to product through discreet intermediates with finite lifetimes (as a consequence of a potential energy minimum in the energy profile for the reaction), in contrast to the latter which go through a single transition state (potential energy maximum in the energy profile)

Simple HMO theory was used to examine the energetics of various possible pathways for dimer radical anion splitting 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′) and for the nonsynchronous 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 to reactant) when the process was stepwise or nonsynchronous concerted For the synchronous concerted pathway, the energy difference between reactant radical anion and transition state was higher Further, picosecond flash photolysis of the enzyme–substrate complex has identified at least one and probably two spectroscopically distinct intermediates following photoexcitation This is highly significant as only a stepwise mechanism would involve intermediates between the dimer anion and the product monomer and monomer anion radical

4.7 Dimer Splitting: The Mechanistic Details

Here we will speculate on the chemical structures of these intermediates Pyrimidine dimers (*e g* UpU dinucleotide dimer) reduces the lifetime of ^{1}FADH from 1 4 to 0 16 ns, representing a quenching rate constant of 7×10^9 s $^{-1}$ This rate presumably corresponds to the rate of primary electron transfer[24] from ^{1}FADH In Figure 8, a possible reaction mechanism for dimer splitting by photolyase is given An electron is transferred from ^{1}FADH to the dimer and then back again from the monomer after splitting to complete the catalytic cycle

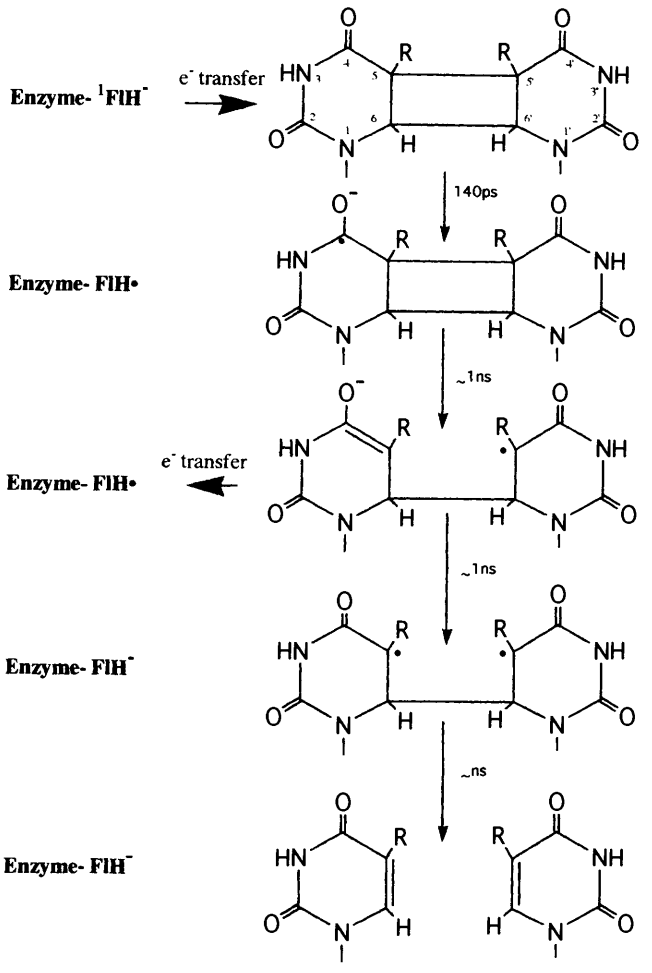


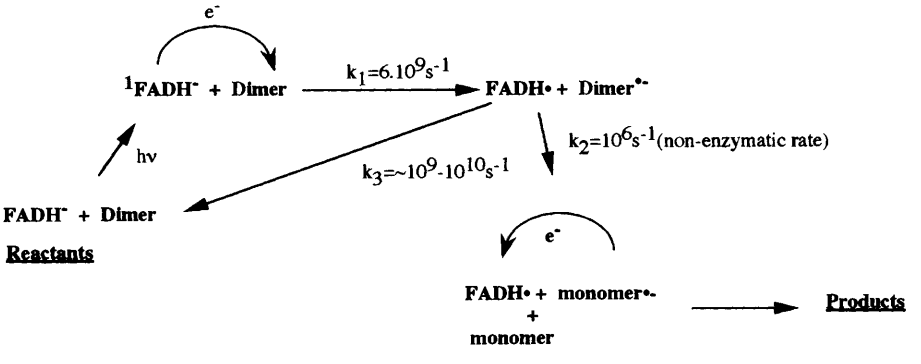
Figure 8 Proposed reaction mechanism for pyrimidine dimer splitting by photolyase.

4.8 Unique Energetics of the Enzymic 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). The competing processes important in determining the efficiency of dimer splitting are shown in Scheme 1. The rate[25] of splitting of *cis-syn*-thymine dimer following electron donation from the dimethylaniline singlet state is $\sim 10^6$ s^{-1} (k_2 Scheme 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 dimer anion (k_3 Scheme 1, *i.e.* back transfer without splitting). Although the rate of the latter reaction is not known, it is expected to be similar to the forward rate, around 10^9 s^{-1} (estimated from the free energy change of the back reaction). 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 the transition state (and therefore make ΔG more negative and reduce $\Delta G^{\ddagger}$). Theoretical calculations have shown that twisting the cyclobutane ring markedly increases the exergonicity of the reaction. This can occur as a consequence of steric hindrance, *e.g.* the introduction of the methyl group at C(5) in thymine makes splitting of this dimer more favourable than splitting of the corresponding unmethylated uracil dimer. Clearly incorporation of a dimer in a DNA helix would accentuate such effects. Further, binding of DNA to the active site could have a similarly pronounced effect on the energetics if binding involves a further increase in strain.

An alternative method of increasing splitting efficiency is to slow the back electron transfer (*i.e.*, the transfer of an electron from dimer radical anion to FADH$^{\cdot}$ before splitting can occur). As noted earlier from the ΔG for back-electron transfer (-120 kJ mol^{-1}), a rate constant of $\sim 10^9$ s^{-1} might be expected. In a nonpolar environment, however, this highly exergonic reaction may be in the so-called Marcus inverted region, *i.e.*, the large driving force slows the rate of back electron transfer. A decrease in rate for a process with a large driving force is, at first sight, counter-intuitive. The basis of this phenomenon is illustrated schematically in Figure 9. The driving force for the back electron transfer process increases as the potential energy curve for product drops, from (a) to (b) to (c). The activation barrier is shown by arrows in (a). The barrier has vanished in (b), where the driving force equals the energy required by the system to assume a configuration conducive to movement of the electron (*i.e.*, the reorganization energy), and the maximum rate is observed (*i.e.*, the process is 'activationless'). In (c) the further increase in driving force has caused a barrier to reappear (arrows). This is the Marcus inverted region, wherein increased driving force causes a slowing of the process. It requires less of a driving force to enter the inverted region in low polarity environments, where the reorganization energy is small. In covalently linked dimer-sensitizer systems, splitting efficiency of the dimer radical anion within the charge-separated species (*i.e.*, Pyr < > Pyr$^{\cdot -}$—Sensitiser$^{\cdot +}$) increased significantly as solvent polarity decreased, which was rationalized on the basis of a slowing of the back electron transfer in the low polarity solvents. This allowed the competitive dimer radical anion splitting to occur to a greater extent.

It may be that photolyases use a low polarity active site to slow the back electron transfer and thereby to increase the competitive splitting reaction. The nature of the active site of photolyases is believed to be relatively non-polar on the basis of a study of the UV–VIS absorption spectrum of the fully oxidized (yellow) form of the enzyme, which exhibits vibrational fine structure typical of an environment of dielectric constant around 10. Hence, most of the FADH$^-$ molecules must be buried in the protein and would not be exposed to the aqueous phase. This may well help to explain the success of the enzyme in facilitating splitting relative to the competing back electron



Scheme 1

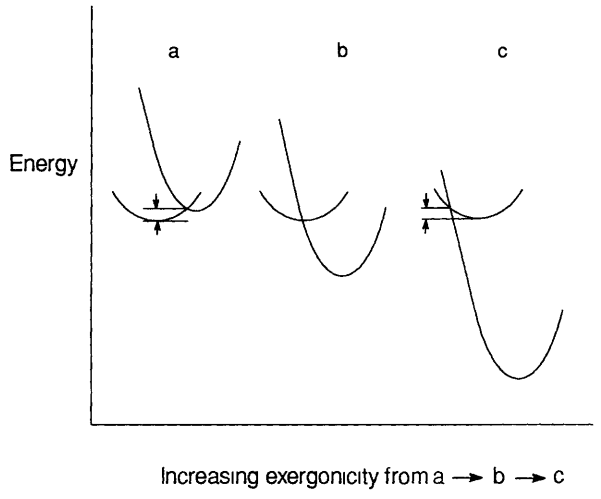


Figure 9 The effect of increasing exergonicity on electron transfer In (a), the process is slowed by the activation barrier (arrows) The process is faster in (b), which is 'activationless' Further increase in exergonicity, as in (c), shows the reappearance of a barrier (arrows) and hence there is a slowing of the process

transfer This would mean that photolyases had evolved in such a way as to favour the forward electron transfer but retard the back electron transfer

The electron transfer from ^{1}FADH 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 We know, however, that the rate constant for electron transfer is $\sim 6 \times 10^9$ s^{-1} from the difference in the rate constant of decay of ^{1}FADH$^-$ in the enzyme–substrate complex and the enzyme alone The relationship between the rate constant of electron transfer and the distance between the chromophores is complex In addition the nature of the intervening medium (amino acid side chains, peptide bonds, hydrogen bonding, aromatic groups, conformations of groups, *etc*) affects the rate Nevertheless, we can estimate the distance between the cyclobutane ring of the dimer and the flavin as < 10Å

4.9 Electronic Energy Transfer

Photolyase contains two chromophores, flavin and folate (or deazaflavin), and hence a quite obvious question is, why are two present when one (the flavin) can achieve dimer splitting? It is easy to understand from an evolutionary sense that a flavin was 'chosen' – these chromophores are ubiquitous as redox-active components of numerous enzymes In addition, a reduced rather than oxidized flavin was preferred owing 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 (the folate) is used for light absorption, and the other (flavin) is used for chemistry The ability of a compound to absorb light is measured in terms of its extinction coefficient (ϵ), for folate $\epsilon = 25000$, *versus* $\epsilon = 5000$ M^{-1} cm^{-1} for FADH$^-$ Further, the maximum of absorption of folate (390 nm) occurs at a longer wavelength than for FADH$^-$ (350 nm) An examination of the solar spectrum and also the dependence of light penetration into cells due to scattering shows that folate far excels flavin as a light absorber How then can energy be trapped by the flavin? Electronic energy can in fact be transferred from a donor (folate) to an acceptor (flavin) without the intermediate appearance of a photon This transfer occurs *via* dipole–dipole coupling between the donor and acceptor The rate of energy transfer depends upon 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 chromophores This is made clear by the following equation

$$k_{\text{energy transfer}} \propto k^2 J / R^6 \qquad (6)$$

where k^2 takes into account the orientation between the interacting dipoles, J is the spectral overlap referred to above, and R is the distance separating the dipoles Transfer is effective over distances up to 50 Å The intervening medium, *i e* , solvent or macromolecule, has little effect This long range transfer is called Forster energy transfer after the scientist who formulated most of the theoretical basis

Earlier studies had in fact measured the rate of transfer as 5×10^9 s^{-1}, and this rate was used to calculate a flavin–folate distance of 22 Å (centre to centre) One of the main difficulties in any such study is the orientation factor (k^2), which is clearly difficult to know In fact it is not the orientation of the molecular framework of the donor and acceptor, but the orientation of the transition dipoles that is needed, which is even more difficult to evaluate Generally, a random orientation of chromophores is assumed Only recently has the crystal structure of the enzyme been determined, and this showed that the flavin–folate distance is 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 Further, it appears that photolyase has not adopted the best orientation of the chromophores for energy transfer This is rather puzzling as it might be expected that a more nearly ideal orientation would have evolved

5 Summary

Photolyases provide an opportunity to gain insights into the way in which natural systems take advantage of the energy source consisting of solar radiation They also offer many challenges to physical and synthetic organic chemists, who employ the vast range of modern spectroscopic techniques to elucidate an intriguing photobiochemical reaction that has an important role in the maintenance of accuracy in the genetic information of cells

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