Photoenzymes: A Novel Class of Biological Catalysts

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Introduction

Sunlight is essential for sustaining life on earth. In addition to photosynthesis, photochemical reactions are involved in vision, in the biosynthesis of vitamin D, and in the control of plant development.[1] Enzymes that require light energy represent photosynthesis in its simplest biological form. At this time, only two photoenzymes have been identified. In the first system, bacteria that have been killed by irradiation with ultraviolet light can be revitalized by exposure to visible light. This remarkable phenomenon, called photoreactivation, is mediated by the enzyme DNA photolyase and plays a crucial role in protecting bacteria from the lethal and mutagenic effects of the UV component of sunlight. In the second process, yellow, dark-grown plants become green when transferred to the light. This greening reaction, catalyzed by the enzyme protochlorophyllide reductase, is a key step both in the biosynthesis of chlorophyll and in the morphogenesis of the chloroplast. This Account will summarize the current state of mechanistic studies on these two systems.

DNA Photochemistry and Photoreactivation

The nucleic acids constitute and dominant UV-absorbing chromophore in the cell, and a large number of different photoreactions have been characterized. These include hydration of the bases, cross-linking to proteins, strand cleavage, and the formation of **2**–**6**[2] (Figure 1). These photoproducts are toxic, and all cells contain efficient repair systems that are essential for the cells to survive exposure to the UV component of sunlight. Typically, the repair involves either excision of the toxic lesion followed by fill-in of the resulting gap in the nucleic acid with DNA polymerase or direct *in situ* repair.[3]

The biologically relevant photoreactions of DNA involving adjacent thymines are summarized in Figure 1. A formal [2 + 2] cycloaddition reaction between the carbon–carbon double bonds of adjacent thymines gives the cyclobutane photodimer **2**. An alternative [2 + 2] cycloaddition between the carbon–carbon double bond of one thymine and the C4 carbonyl double bond of an adjacent thymine can also occur, to give **4**. This moiety is unstable and undergoes fragmentation to **5**, which may undergo further photoreaction to give the Dewar pyrimidone **6**.[4] In contrast to the photochemistry of DNA in solution, irradiation of DNA in spores does not yield any of the cyclobutane photodimer **2**. Instead, the novel adduct **3**, resulting from a formal addition of one of the thymine methyl CH bonds across the carbon–carbon double bond of the adjacent thymine, is the major photolesion.[5]

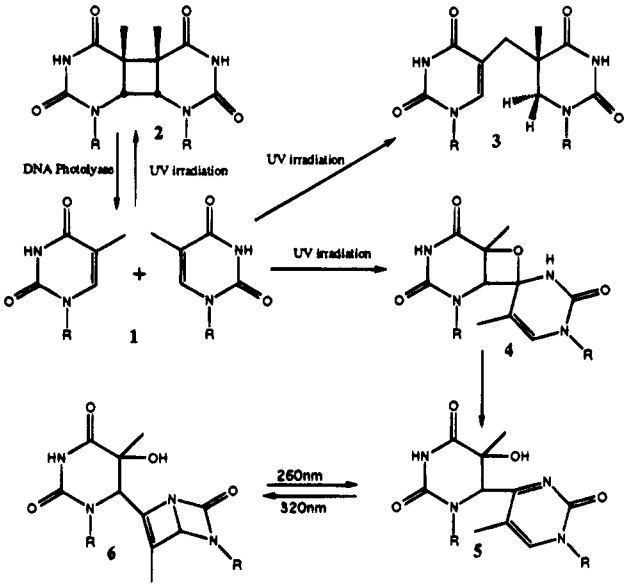

Figure 1. DNA modifications resulting from photoreactions between two adjacent thymines.

Formation of the cyclobutane photodimer **2** is the highest quantum yield lethal damage caused to cells by UV irradiation.[6] During photoreactivation, this toxic lesion is recognized by the enzyme DNA photolyase, which uses the energy of a visible light photon to effect the cleavage of the dimer.[7]

Properties of the Pyrimidine Photodimer

While the fragmentation of the photodimer is an exothermic reaction ($\Delta H°_r = -110 \pm 5.2$ kJ mol^{-1}),[8] the photodimer is kinetically stable. It can be treated with strong acid and heated to >200 °C without decomposition.[9]

Tadhg P. Begley was born in Ireland and received his B.Sc. degree from University College Cork in 1977. He carried out his Ph.D. studies at Caltech under the direction of Peter Dervan and postdoctoral research with Wolfgang Oppolzer at the University of Geneva and Christopher Walsh at MIT. In 1986, he joined the faculty of the Chemistry Department at Cornell University. His research interests are broadly based in biological chemistry and include mechanistic enzymology, biosynthesis, combinatorial libraries, lipid chemistry, and nucleic acid chemistry.

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(2) (a) Morrison, H., Ed. *Bioorganic Photochemistry*; John Wiley and Sons: New York, 1990; Vol. 1. (b) Wang, S. Y., Ed. *Photochemistry and Photobiology of Nucleic Acids*; Academic Press: New York, 1976; Vols. 1 and 2.
(3) (a) Friedberg, E. C. *DNA Repair*; W. H. Freeman and Co.: New York, 1985. (b) Taylor, J. S. *Acc. Chem. Res.* **1994**, *27*, 76–82.
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(6) Harm, H. In *Photochemistry and Photobiology of Nucleic Acids*; Wang, S. Y., Ed.; Academic Press: New York, 1976; Vol. 2, pp 219–263.
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(8) Diogo, H.; Dias, A. R.; Dhalla, A.; Minas da Piedade, M.; Begley, T. P. *J. Org. Chem.* **1991**, *56*, 7340–7341.
(9) Fahr, E. *Angew. Chem., Int. Ed. Engl.* **1969**, *8*, 578–593.

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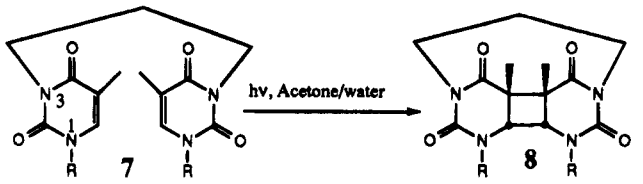


Figure 2. Synthesis of a typical photodimer model system.

The Achilles heel of the molecule can be exposed by one-electron oxidation or reduction.[10] The pyrimidine photodimer radical cation and anion are both highly reactive species and undergo facile monomerization reactions. These have therefore been proposed as possible intermediates during photoreactivation.[11]

Synthesis of Pyrimidine Photodimer Model Systems

Much of the detailed mechanistic information on the monomerization of the pyrimidine photodimer has come from model studies on the fragmentation of the pyrimidine photodimer radical anion and cation. These model systems can be readily synthesized by the irradiation of N1 or N3-linked bis(pyrimidines) (Figure 2).[12] Historically, model studies on enzymatic reactions have proven to be of particular scientific value when the uncatalyzed version of the reaction is poorly understood. Notable examples are the cytochrome P_{450},[13] the vitamin K,[14] the vitamin B_{12},[15] and the photosynthesis reaction center[16] model systems.

Mechanistic Studies on the Fragmentation of the Pyrimidine Photodimer Radical Cation

The photodimer radical cation can be readily generated by electron transfer from the photodimer to a photoexcited oxidizing sensitizer (flavin,[17] quinones,[18] and metal ions[19]). Two possible mechanisms, involving either a stepwise[20] or a concerted[21] fragmentation, can be considered (Figure 3).[22] The earliest experimental evidence in support of radical intermediates

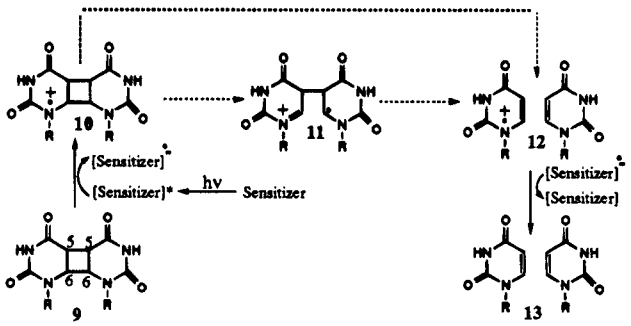
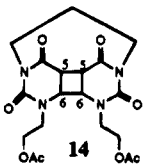


Figure 3. Mechanistic proposals for the fragmentation of the pyrimidine photodimer radical cation.



Isotope effects	
6,6-d_2	1.19±0.02
5,5-d_2	1.03±0.02
5,5,6,6-d_4	1.25±0.02

Figure 4. Isotope effects on the fragmentation of the photodimer radical cation.

came from the observation of a substrate and product photo-CIDNP signal.[23] Isotope effects and radical trapping experiments have been used to determine the timing of the bond cleavage events.

The rate of a reaction involving a hybridization change can be retarded or accelerated, often in a predictable manner, by replacing hydrogen with deuterium. These deuterium isotope effects have been extensively used in mechanistic studies to determine the rate-limiting step or the first irreversible step in a reaction sequence and to probe transition state structure.[24] The photodimer radical cation fragmentation reaction involves the conversion of four sp^3 centers to four sp^2 centers (Figure 3). This reaction should therefore show a normal (i.e., $k_H > k_D$) secondary deuterium isotope effect. For a stepwise fragmentation, the competitive isotope effect[25] should be localized at the 6,6 positions. For a concerted fragmentation, the isotope effect should be registered at all four centers.

The isotope effects on the fragmentation of the radical cation of **14** are given in Figure 4. The relatively small isotope effect at the 5,5 positions compared to the 6,6 positions suggests that the fragmentation occurs by a stepwise mechanism.[26]

This conclusion can be further tested using a trapping experiment. A carbon–iodine bond β to a radical center undergoes very fast homolysis ($k > 5 \times 10^9$

(10) For a review on the activation of molecules by one-electron oxidation or reduction, see: Chanon, M.; Rajzmann, M.; Chanon, F. *Tetrahedron* **1990**, *46*, 6193–6299.

(11) Lamola, A. A. *Mol. Photochem.* **1972**, *4*, 107–133.

(12) (a) Leonard, N. J.; Cundall, R. L. *J. Am. Chem. Soc.* **1974**, *96*, 5904–5910. (b) Golankiewicz, K.; Skalski, B. *Pol. J. Chem.* **1978**, *52*, 1365–1373. (c) Burdi, D.; Hoyt, S.; Begley, T. P. *Tetrahedron Lett.* **1992**, *33*, 2133–2136.

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(21) Several examples of concerted alkene radical cation cyclobutanations have been proposed. Bauld, N. L. *Tetrahedron* **1989**, *45*, 5307–5363.

(22) AM1 calculations on the fragmentation of the photodimer radical cation predict a stepwise mechanism. Bauld, N.; Begley, T. P. Unpublished results.

(23) (a) Roth, H. D.; Lamola, A. A. *J. Am. Chem. Soc.* **1972**, *94*, 1013–1014. (b) Young, T.; Nieman, R.; Rose, S. *Photochem. Photobiol.* **1990**, *52*, 661–668. (c) Hartman, R. F.; Rose, S. D.; Pouwels, P. J.; Kaptein, R. *Photochem. Photobiol.* **1992**, *56*, 305–310.

(24) (a) Melander, L.; Saunders, W. H. *Reaction rates of isotopic molecules*; Wiley Interscience: New York, 1980; p 95–102. (b) Dolbier, W. R., Jr. In *Isotopes in Organic Chemistry*; Buncel, E., Lee, C. C., Eds.; Elsevier Press: Amsterdam, 1975; Vol. 1, p 27–60. (c) Carpenter, B. K. *Determination of Organic Reaction Mechanisms*; John Wiley and Sons: New York, 1984; p 83–111.

(25) All of the isotope effects described here were carried out under competitive conditions in which a mixture of the protio and the deuterio substrates was subjected to the reaction conditions. Such isotope effects are sensitive to hybridization changes occurring up to and including the first irreversible step in the reaction sequence. Hybridization changes after the first irreversible step, even if originating from the rate-determining step in the reaction, are not registered.

(26) McMordie, R. A.; Begley, T. P. *J. Am. Chem. Soc.* **1992**, *114*, 1886–1887.

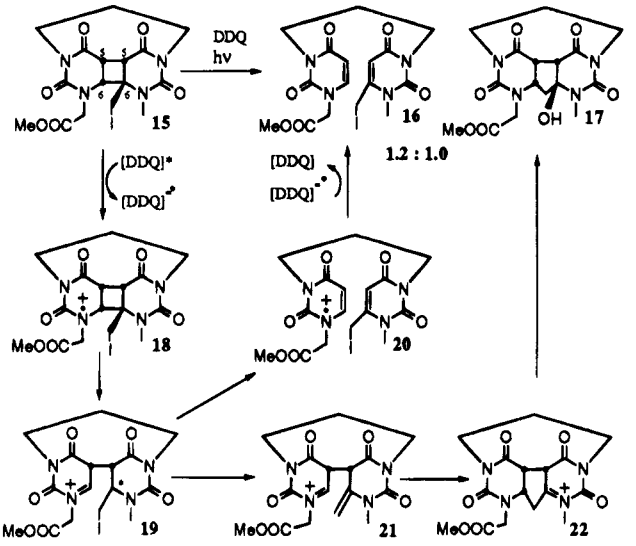


Figure 5. The trapping of the one-bond-cleaved intermediate during the fragmentation of the photodimer radical cation.

s^{-1}).[27] Therefore, it should be possible to trap **11** using a 6-iodomethyl-substituted photodimer.

When photodimer **15** was irradiated in the presence of dichlorodicyanobenzoquinone (DDQ) as sensitizer, two reaction products, **16** and **17**, were cleanly formed. A mechanistic proposal for the formation of these two products is outlined in Figure 5. Cleavage of the 5–5 bond of **19** followed by reduction with the sensitizer radical anion would give **16**. Cleavage of the carbon–iodine bond of **19** would give **21**. Acyl iminium ion cyclization followed by addition of water would give **17**.[28]

The successful trapping of the one-bond-cleaved intermediate **19** combined with the isotope effects provides strong experimental support for a stepwise fragmentation mechanism of the photodimer radical cation.[29]

Mechanistic Studies on the Fragmentation of the Pyrimidine Photodimer Radical Anion

The photodimer radical anion can be formed by irradiating the photodimer in the presence of several different photoreducing sensitizers (indole,[30] catalytic antibodies,[31] reduced flavin,[32] dimethylaniline[33]) and also undergoes facile cleavage ($k_{cl} = 1.8 \times 10^6$ s^{-1}).[34] A photo-CIDNP experiment on the indole-sensitized fragmentation provides evidence for radical ion inter-

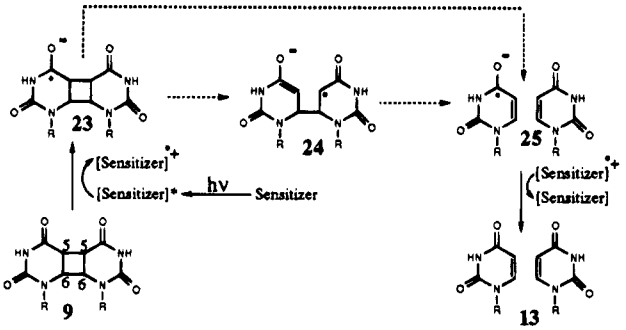
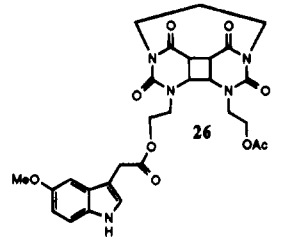


Figure 6. Mechanistic proposals for the fragmentation of the pyrimidine photodimer radical anion.



Isotope effects	
5,5-d_2	1.17±0.01
6,6-d_2	1.08±0.01
5,5,6,6-d_2	1.31±0.01

Figure 7. Isotope effects on the fragmentation of the photodimer radical anion.

mediates.[35] In principle, the photodimer radical anion may fragment by either a concerted[36,37] or a stepwise mechanism (Figure 6).[38] Isotope effects and a trapping experiment have also proved useful in differentiating between these two mechanisms.

If the fragmentation of the photodimer radical anion occurs by a stepwise pathway, the isotope effect should be localized at the 5,5 positions; if the fragmentation is concerted, the isotope effect should be observed at all four centers. The isotope effects on the fragmentation of **26** are shown in Figure 7 and are consistent with a nonsynchronous concerted fragmentation mechanism.[39]

A trapping experiment, analogous to that outlined in Figure 5 for the radical cation, was not possible for the radical anion fragmentation reaction because of the reductive lability of the carbon–iodine bond.[40] An alternative trapping strategy, based on the very fast

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(37) While there are many examples of facile cleavage of cyclobutane radical anion derivatives, in no case has the timing of the bond cleavage been experimentally examined. (a) Robbins, R. J.; Falvey, D. E. *J. Org. Chem.* **1993**, *58*, 3616–3618. (b) Horner, M.; Hunig, S. *Liebigs Ann. Chem.* **1982**, *8*, 1409–1422. (c) Stevenson, G. R.; Sedgwick, J. B.; Miller, R. *J. Phys. Chem.* **1982**, *86*, 2441–2442. (d) Gerson, F.; Weidmann, B. *Helv. Chim. Acta* **1966**, *49*, 1837–1844. (e) Takamuku, S.; Kigawa, H.; Miki, S.; Sakurai, H. *Chem. Lett.* **1979**, 797–800. (f) Dekker, J.; Martins, F.; Kruger, J.; Gossen, A. J. *Tetrahedron Lett.* **1974**, *42*, 3721–3724. (g) Russel, G. A.; Dodd, J. R.; Ku, T.; Tanger, C.; Chung, C. S. *J. Am. Chem. Soc.* **1974**, *96*, 7255–7265. (h) vonGizycki, U. *Justus Liebigs Ann. Chem.* **1971**, *753*, 1–5. (i) Mullen, K.; Huber, W. *Helv. Chim. Acta* **1978**, *61*, 1310–1326.

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(39) McMordie, R. A.; Begley, T. P. *J. Am. Chem. Soc.* **1992**, *114*, 1886–1887.

(40) Irradiation of indole in the presence of butyl iodide, under the conditions used for the photodimer cleavage reaction, resulted in extensive cleavage of the carbon–iodide bond. Altmann, E.; Begley, T. P. Unpublished results.

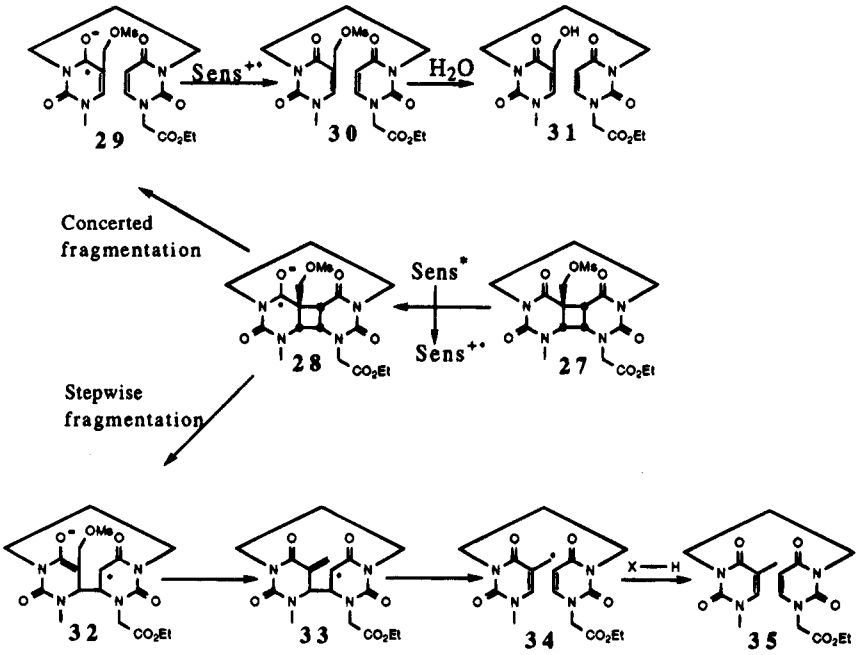

Figure 8. Strategy for trapping the putative one-bond-cleaved intermediate during the fragmentation of the photodimer radical anion.

loss of a leaving group β to a carbanion,[41] was therefore developed using photodimer **27** (Figure 8). If the fragmentation reaction proceeded by a stepwise mechanism, loss of mesylate from **32** followed by β-scission and hydrogen atom abstraction would give **35**.[42] If the fragmentation of **28** was concerted, **31** would be the only reaction product.

When **27** was irradiated in the presence of *N*-phenylpiperazine as sensitizer, **31** was formed as the major reaction product. **35** was not detected in the reaction mixture (detection limit <0.01%).[43] This result supports the interpretation of the isotope effects and also suggests that the fragmentation of the photodimer radical anion is a concerted reaction.[44]

Enzymatic Reaction

DNA photolyase catalyzes the cleavage of the pyrimidine photodimer **2** in UV-damaged DNA (Figure 1). The enzyme is widely distributed and has been purified from several organisms. The *Escherichia coli* enzyme is the best characteized system, and the discussion here will therefore focus on this enzyme.[45,46] Since there are only about 10 molecules of photolyase per cell in wild type *E. coli*, the cloning and overexpression of the photolyase gene were prerequisites to mechanistic studies, and *E. coli* strains are now available where the photolyase is among the most abundant of the cellular proteins.[47] The enzyme has a molecular mass of 54 kDa[48] and utilizes a deprotonated reduced flavin ($FADH^-$, **36**) and a folate (**37**) as cofactors.[49] The folate functions as an antenna chromophore by increasing the extinction coefficient of the enzyme.[50] The reduced flavin is directly involved in the photodimer cleavage. The enzyme can utilize light in the 300–500-nm range, and the quantum yield for the reaction is high (0.6).[51] This rules out the possibility of photodimer cleavage by direct light absorption or by direct energy transfer from the photoexcited reduced flavin to the photodimer, as the photodimer does not have appreciable absorbance at $\lambda > 220$ nm, and suggests that the cleavage reaction occurs via radical ion intermediates. Since the oxidation and reduction potentials of the photodimer (−2.0

(41) Thiophenolate expulsion from the cyanide stabilized anion of $O_2NPhS(CH_2)_2CN$ occurs with a rate constant of approximately 10^{10} s^{-1}. Fishbein, J. C.; Jencks, W. P. *J. Am. Chem. Soc.* **1988**, *110*, 5087–5095.

(42) In support of this pathway (**33** → **35**) radical **33** was independently generated by reaction of the corresponding 5-iodomethyl-substituted photodimer with tributyltin hydride and a catalytic amount of AIBN in refluxing acetonitrile. HPLC analysis of the reaction mixture indicated the formation of **35** as the only product. McMordie, R. A.; Begley, T. P. Unpublished results.

(43) McMordie, R. A.; Altmann, E.; Begley, T. P. *J. Am. Chem. Soc.* **1993**, *115*, 10370–10371.

(44) While the experimental data support a concerted mechanism for the fragmentation of the photodimer radical anion, there are still some significant uncertainties. It is possible that the 6,6 isotope effect on the fragmentation of the photodimer radical anion is due to a large β-isotope effect. This is unlikely because large β-isotope effects were not observed for the closely related fragmentation of the photodimer radical cation. In addition, while the rate of mesylate loss from **32** is expected to be very fast, the trapping experiment cannot be rigorously used to exclude the possibility of a stepwise fragmentation until this rate constant has been measured.

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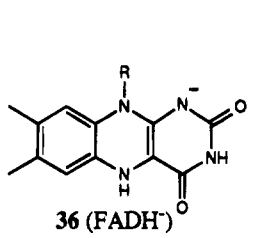
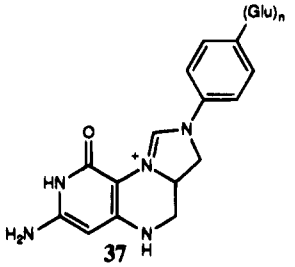
(51) Payne, G.; Sancar, A. *Biochemistry* **1990**, *29*, 7715–7727.

and −2.4 V, respectively)[52] lie well outside the physiologically accessible range, DNA photolyase has an absolute requirement for light energy to enhance the redox potential of the enzyme.



36 ($FADH^-$)

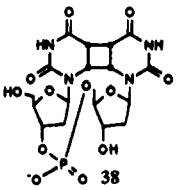


37

In principle, the cleavage reaction could occur via the pyrimidine radical cation or anion as both of these species are known to undergo facile fragmentation. Using approximate redox potentials for the photodimer[53] and the reduced flavin,[54] it is possible to estimate that the electron transfer from the photoexcited dihydroflavin to the photodimer is exergonic ($\Delta G \approx -9$ kcal mol^{-1}) while the electron transfer from the photodimer to the photoexcited dihydroflavin is endergonic ($\Delta G \approx 33$ kcal mol^{-1}). Formation of the photodimer radical anion therefore appears to be favored. However, due to the uncertainties in the estimated redox potentials, an experimental test of this prediction was warranted.[55]

On the basis of the results obtained with the model systems, an isotope effect experiment should allow differentiation between these two mechanisms.[56] If the enzymatic reaction proceeds via the photodimer radical cation, the isotope effects should be localized at the 6,6 positions, while if the reaction proceeds via the photodimer radical anion, the isotope effect should be delocalized over all four centers. Critical to the success of this experiment is the requirement that formation of the enzyme substrate complex is readily reversible under the reaction conditions. It was therefore of particular significance that the enzyme catalyzed the cleavage of the dinucleotide photodimer **38**. For this substrate, the quantum yield is essentially unaltered but the binding constant is greatly reduced compared to the binding of the enzyme to a thymine dimer in DNA (3.5×10^8 M^{-1} vs 1.8×10^4 M^{-1}, respectively).[57]

The isotope effects on the enzyme-catalyzed cleavage of **38** are shown in Figure 9 and provide support for the intermediacy of the photodimer radical anion.[58]

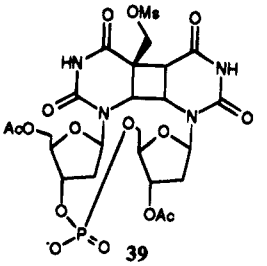


38

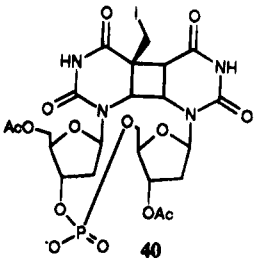
Isotope effects	
6,6-d_2	1.07±0.01
5,5-d_2	1.08±0.01
5,5,6,6-d_4	1.15±0.01

Figure 9. Isotope effects on the enzyme−catalyzed cleavage of **38**.

Trapping experiments analogous to those carried out with the model systems have been attempted with disappointing results. The mesylate **39** is not a substrate for the enzyme. Iodomethyl-substituted photodimers **40** and **41** were substrates but turned out to be ambiguous probes due to the possibility of competing reductive cleavage of the carbon−iodine bond.[59]



39



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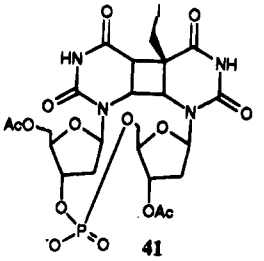


41

Photoenzymes are ideal systems for rapid kinetic studies because the enzyme−substrate complex remains unreactive until irradiated. Transient absorption spectroscopy on DNA photolyase involves irradiating the enzyme−substrate complex with a very short laser pulse and monitoring changes in the system using a second analyzing light beam. With this technique, the absorption spectra and the rates of formation and disappearance of the intermediates involved in the cleavage reaction can be readily probed.

When DNA photolyase, complexed to the deoxyuridine photodimer **38**, is irradiated with a 12-ps 340-nm laser pulse, the first intermediate to appear has a broad absorbance spanning 450−900 nm.[60] This absorbance has been assigned to the singlet excited state of the deprotonated reduced flavin ($FADH^-$).[61] This signal decays with a rate constant of 5×10^9 s^{-1}, due to electron transfer from the reduced flavin to the photodimer, and a new photodimer-derived radical, of unknown structure, absorbing at 400 nm, is observed

(52) (a) Yeh, S.-R.; Falvey, D. E. *J. Am. Chem. Soc.* **1992**, *114*, 7313−7314. (b) Pac, C.; Kubo, J.; Majima, T.; Sakurai, H. *Photochem. Photobiol.* **1982**, *36*, 273−282.

(53) $E°$ (dimer/dimer radical anion) = −2.4 V (NHE). Yeh, S.-R.; Falvey, D. E. *J. Am. Chem. Soc.* **1992**, *114*, 7313−7314. $E°$ (dimer radical cation/dimer) = 2.0 V (NHE). Pac, C.; Kubo, J.; Majima, T.; Sakurai, H. *Photochem. Photobiol.* **1982**, *36*, 273−282.

(54) $E°$ (Enz-$FlH^{\bullet}$/Enz-FlH_2) < −0.33 V (NHE), $E°$ (Enz-FlH_2/Enz-$FlH_2^{\bullet-}$) < −1.9 V (NHE). The flavin singlet energy is 57 kcal/mol. Heelis, P. F.; Deeble, D. J.; Kim, S.-T.; Sancar, A. *Int. J. Radiat. Biol.* **1992**, *62*, 137−143.

(55) The estimated dimer radical cation reduction potential is based on an irreversible half-peak potential. Pac, C.; Kubo, J.; Majima, T.; Sakurai, H. *Photochem. Photobiol.* **1982**, *36*, 273−282. The photodimer reduction potentials estimated in refs 52 and 53 are significantly different.

(56) A competitive isotope effect on an enzymatic reaction is called a V/K isotope effect. *Enzyme Mechanism from Isotope Effects*. Cooke, P. F., Ed.; CRC Press: Boca Raton, 1991.

(57) (a) Jorns, M. S.; Sancar, G. B.; Sancar, A. *Biochemistry* **1985**, *24*, 1856−1861. (b) Kim, S.-T.; Sancar, A. *Biochemistry* **1991**, *30*, 8623−8630.

(58) (a) Witmer, M.; Altmann, E.; Young, H.; Begley, T. P.; Sancar, A. *J. Am. Chem. Soc.* **1989**, *111*, 9264−9265. (b) Witmer, M. R. Ph.D. Thesis, Cornell University, 1990.

(59) (a) Burdi, D. F. Ph.D. Thesis, Cornell University, 1992. (b) Witmer, M. R. Ph.D. Thesis, Cornell University, 1990. (c) Reference 40.

(60) Okamura, T.; Sancar, A.; Heelis, P.; Begley, T. P.; Hirata, Y.; Mataga, N. *J. Am. Chem. Soc.* **1991**, *113*, 3143−3145.

(61) Heelis, P. F.; Hartman, R. F.; Rose, S. D. *Photochem. Photobiol.* **1993**, *57*, 1053−1055.

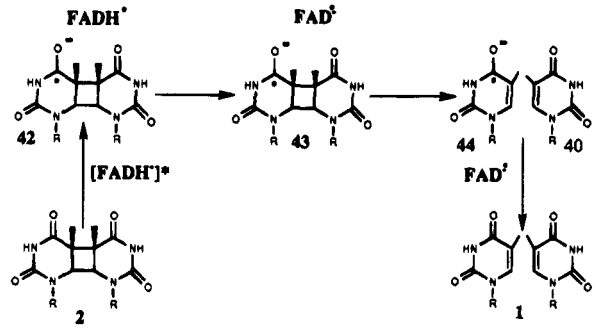


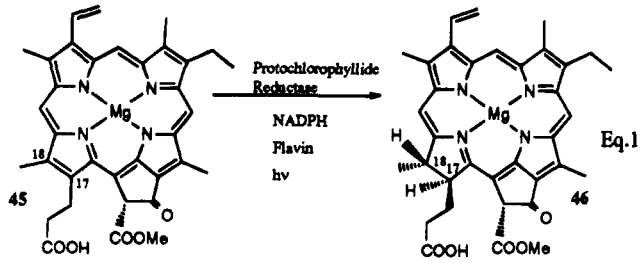
Figure 10. Mechanistic proposal for the DNA photolyase catalyzed cleavage of the thymidine cyclobutane photodimer.

to form over 2 ns.[62] The flavin semiquinone radical is not detected. This may be due to rapid proton transfer from the flavin semiquinone radical to the enzyme. Such a proton transfer would increase the lifetime of the photogenerated radicals and thus increase the efficiency of the cyclobutane cleavage.

A mechanistic proposal for the enzymatic reaction is summarized in Figure 10. Reduction of the photodimer by the photoexcited deprotonated dihydroflavin followed by rapid proton transfer generates **43**. Concerted fragmentation of this species, followed by electron and proton transfer back to the flavin, completes the cleavage reaction.

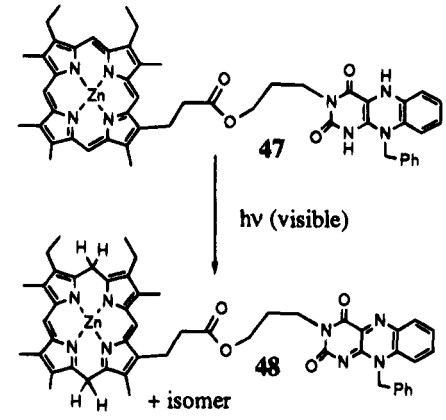
Protochlorophyllide Reductase

The light-dependent step in the biosynthesis of chlorophyll involves the reduction of the C17–C18 double bond of protochlorophyllide (**45**) to give chlorophyllide (**46**, eq 1).[63] This reaction, called the greening reaction, is catalyzed by the enzyme protochlorophyllide reductase, a 37-kDa membrane bound protein that utilizes dihydronicotinamide (NADPH) and flavin as cofactors.[64] The action spectrum for the reaction demonstrates that protochlorophyllide is the light-absorbing species.[65] In addition to its central biosynthetic role, the greening reaction serves as a light-activated trigger to initiate the development of the etioplast into the chloroplast in the flowering plants.



The regiochemistry and stereochemistry of the reduction have been determined.[66] The C18 proton is derived from water, and the C17 proton is derived from the *pro-S* face of NADPH. The formal hydride transfer from the nicotinamide to protochlorophyllide is accompanied by approximately 30% exchange of label with the solvent. This exchange has been observed in other flavoenzymes[67] and suggests that the nicotinamide first reduces the flavin and that the resulting dihydroflavin undergoes exchange with solvent in competition with photochemical reduction of the protochlorophyllide.

The mechanistically interesting question then centers on the photochemical reduction of the porphyrin by the dihydroflavin. This has been addressed using the model system **47**.[68] The appended dihydroflavin strongly quenched the porphyrin fluorescence, indicating that efficient electron transfer was occurring from the dihydroflavin to the photoexcited porphyrin. Continuous irradiation of **47** with visible light resulted in the formation of the two-electron-reduced porphyrin (**48**, eq 2).



Transient absorption spectroscopy studies on this model system demonstrated that the porphyrin singlet is rapidly reduced ($k_1 = 4 \times 10^9$ s^{-1}) by the appended dihydroflavin to give the porphyrin radical anion. This is then reduced in a slower second electron transfer step ($k_2 = 1.1 \times 10^6$ s^{-1}) to give the porphodimethene **48**. While the order of the proton transfer steps relative to the electron transfers has not yet been determined, this simple model system indicates that the photochemical reduction of porphyrins by dihydroflavin proceeds via a sequential electron transfer proton transfer mechanism.[69]

The model studies suggest the mechanistic proposal outlined in Figure 11 for the enzymatic reaction. The first step involves flavin reduction by NADPH. A one-electron reduction of the photoexcited protochlorophyllide by the dihydroflavin would give the protochlorophyllide radical anion and the dihydroflavin radical cation ion pair **50**. Proton transfer from the flavin to C17 of the protochlorophyllide, as dictated by the stereochemical study, would give the radical pair **51**.

(62) Kim, S.-T.; Volk, M.; Rousseau, G.; Heelis, P.; Sancar, A.; Michel-Beyerle, M.-E. J. *Am. Chem. Soc.* **1994**, *116*, 3115–3116.

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(68) Nayar, P.; Brun, A.; Harriman, A.; Begley, T. P. *J. Chem. Soc., Chem. Commun.* **1992**, 395–397.

(69) The corresponding protochlorophyllide/reduced flavin model has also been synthesized and shows efficient quenching of the protochlorophyllide fluorescence by the appended dihydroflavin. Nayar, P. Ph.D. Thesis, Cornell University, 1992.

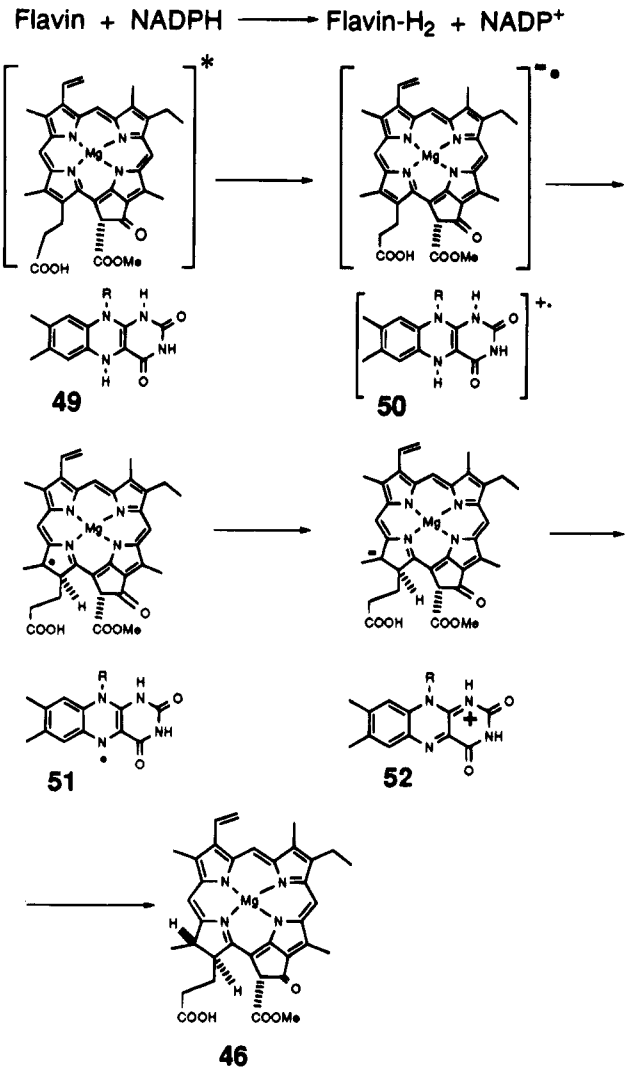

Figure 11. Mechanistic proposal for the enzymatic reduction of protochlorophyllide (**45**) to chlorophyllide (**46**).

A final electron transfer followed by a proton transfer would complete the reaction.

Evolution of Photoenzymes

In contrast to enzymes that function in the ground state, photoenzymes represent a very simple catalytic motif. The high energy content of the photoexcited enzyme–substrate complex opens the possibility that it may cascade to products without requiring much of the essential structural complementarity that exists between the enzyme and the substrate, intermediates, and transition states in ground state catalysis. On the basis of this simplicity, and the fact that photosensitized reactions represent a well-established class of chemical reactions,[70] it is likely that photoenzymes played an important role in the early biotic environment. To illustrate this idea, three examples of currently used enzymes are compared with plausible photoenzyme precursors in Figure 12. Pyruvate dehydrogenase is a very complex enzyme that utilizes flavin, thiamin, lipoic acid, coenzyme A, and nicotinamide as cofactors to catalyze the conversion of pyruvate **53** to an activated ester **54**, a reducing agent (NADH), and carbon dioxide.[71] Irradiation of keto acid **56** in the presence of oxidized flavin **55** gives a reducing agent (**57**) which is also an active acylating agent and carbon dioxide.[72] Therefore a simple light-dependent flavoenzyme is a plausible precursor to the ketoacid dehydrogenases. The second example illustrates a possible photoenzyme precursor to the enzymes that catalyze vitamin B_{12} dependent rearrangements.[73] The first step in all of these rearrangements involves the homolysis of the carbon–cobalt bond. This homolysis is accelerated 10^{12}-fold at the active site.[74] Since photoexcitation will also result in carbon–cobalt bond homolysis,[75] these B_{12}-dependent enzymes may have had a photoenzyme precursor. Finally, on the basis of the many parallels that exist between the chemistry of singlet oxygen and the chemistry of the oxygenases,[76] it is possible that primitive oxygenases may have utilized the photosensitized generation of singlet oxygen to activate oxygen before the evolution of the sophisticated transition metal complexes currently used.[77]

There are four potential disadvantages to the widespread use of photoenzymes by living systems. The first is that photoenzyme dependent organisms can only function in sunlight. Related to this, the evolution of complex multicellular organisms necessitated the replacement of photoenzymes with light independent enzymes because the fraction of cells able to absorb light energy in multicellular organisms is less than that for single-celled organisms. A third potential problem relates to the regulation of enzymatic activity. As electron transfer can occur over relatively long distances, photoenzymes may be more difficult to regulate by allosteric control. Finally, the generation of excessively reactive intermediates at the photoenzyme active site may shorten the catalytic lifetime of the enzyme.

After 3.5 billion years of evolution, several thousand different enzymatic activities have evolved. Only two of these have been identified as light requiring. Even for these, alternative light independent strategies have evolved. In humans, thymine photodimers are repaired only by an excision repair mechanism,[78] and in at least some of the photosynthetic bacteria, protochlorophyllide is reduced in a light independent process.[79] Thus, while it is possible that some additional photoenzymes remain to be discovered, it is

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(80) We propose that protochlorophyllide reductase has survived evolutionary selection because of its key role in the regulation of chloroplast morphogenesis and that photoreactivation has survived because it is a more effective strategy than excision repair for the repair of pyrimidine photodimers in bacteria.
(81) Preliminary experiments suggest that the (6–4) photoproduct (**5**) may also be repaired by a photoenzyme. Kim, S.-T.; Malhotra, K.; Smith, C. A.; Taylor, J. S.; Sancar, A. *J. Biol. Chem.* **1994**, *269*, 8535–8540.

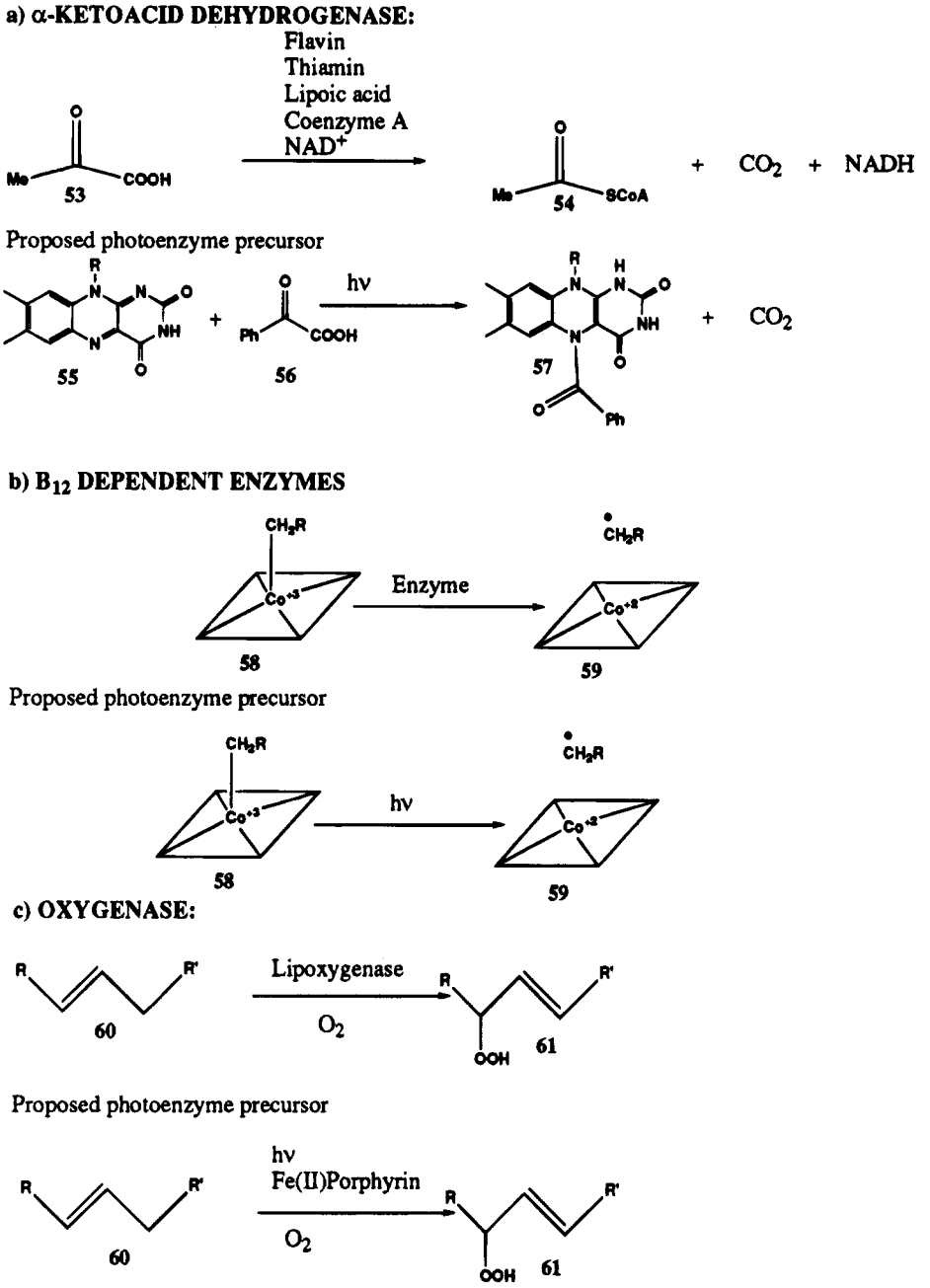

Figure 12. Three plausible photoenzyme precursors to currently used enzymes.

clear that evolution has selected against this catalytic motif.[80] DNA photolyase and protochlorophyllide reductase may well be the only survivors in a class of enzymes well advanced on the path to extinction.[81]

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