Mechanistic Studies on DNA Photolyase. 1. Secondary Deuterium Isotope Effects on the Cleavage of 2'-Deoxyuridine Dinucleotide Photodimers

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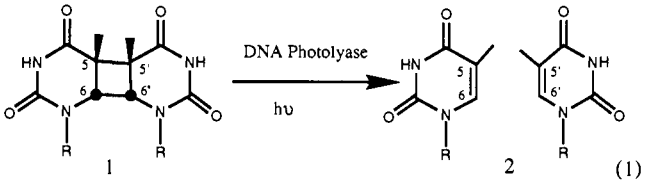
Received September 5, 1989

The lethal and mutagenic effects of ultraviolet light on bacteria can be reversed by exposure to near-UV-visible light. The biochemical basis of this remarkable phenomenon has been extensively studied.[1] Pyrimidine dimerization is the highest quantum yield lethal damage caused to cells by long wavelength ultraviolet light.[2] One of the repair pathways involves DNA photolyase,[3] an enzyme that requires visible light to catalyze the monomerization reaction (eq 1). The enzyme from *Escherichia coli*, used in these studies, has a molecular weight of 54 000 Da (daltons)[4] and requires reduced flavin ($FADH_2$)[5] and 5,10-methenyltetrahydropteroylpolyglutamate[6] as cofactors. The mechanism of this intriguing reaction is poorly understood.



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On the basis of model photosensitized cleavage reactions with quinones and indoles, two mechanisms are suggested for the enzymatic reaction (Scheme I).[7] The first mechanism involves initial reduction of the dimer, by the photoexcited flavin ($FADH_2$*), to give a dimer radical anion intermediate **4**, which then undergoes sequential fragmentation of the 5,5' and the 6,6' bonds. Oxidation of the resulting uracil radical anion by the sensitizer radical cation completes the reaction.[7d] Alternatively, cleavage could be achieved by initial photooxidation of the dimer to give **8**, followed by sequential cleavage of the 6,6' bond and the 5,5' bond and back electron transfer.[7a] No experimental evidence exists to differentiate between these two possible pathways for the enzymatic reaction.

Scheme I

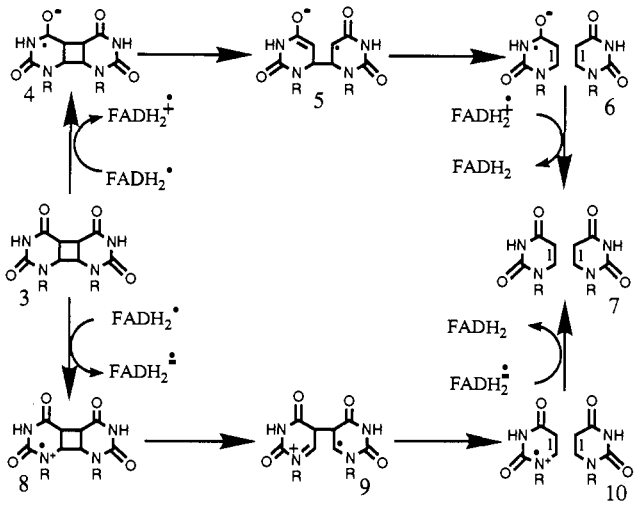

Table I. V/K Isotope Effects

substrates	$^D(V/K)$
H_4-[U=U] + 6,6',5,5'-D_4-[U=U]	1.150 ± 0.014[a,b]
H_4-[U=U] + 5,5'-D_2-[U=U]	1.082 ± 0.011[a,c]
H_4-[U=U] + 6,6'-D_2-[U=U]	1.071 ± 0.012[a,d]

[a] Confidence coefficient = 99%. All isotope effects were corrected to 100% deuteriation. [b] Based on 15 determinations. [c] Based on 12 determinations. [d] Based on 12 determinations.

2'-Deoxyuridine photodimer cleavage results in the conversion of four sp^3 centers to four sp^2 centers. It should be possible to distinguish between the two mechanisms by measuring the secondary deuterium V/K isotope effects on the enzymatic cleavage of suitably deuteriated 2'-deoxyuridine photodimers.[8] A deuterium (V/K) isotope effect would reflect any hybridization changes occurring up to and including the first irreversible enzymatic step in the reaction sequence. If it is assumed that the first C-C bond cleavage is the first irreversible step,[9] then a V/K isotope effect would be expected for the cleavage of the 5,5'-dideuterio photodimer if the reaction proceeded via the radical anion intermediate, and for the 6,6'-dideuterio photodimer if the reaction proceeded via the radical cation intermediate. In this communication, we report the secondary deuterium V/K isotope effects on the enzymatic reaction.

We have previously found that the DNA photolyase from *E. coli* catalyzes the monomerization of the 2'-deoxyuridine dinucleotide photodimer.[10-13] This observation is important both in the context of maximizing the observed V/K isotope effects and in the increased facility with which substrate derivatives can be synthesized for further mechanistic studies.

Deuteriated nucleosides[14] were elaborated to the corresponding deuteriated photodimers using the phosphotriester approach.[15,16]

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To facilitate quantitation, all deuteriated photodimers were labeled with [^{14}C]acetic anhydride at the 3'- and 5'-hydroxyl groups.[17] The protio photodimer was similarly labeled with [^{3}H]acetic anhydride.

Mixtures of protio and deuterio photodimers[18] were irradiated in the presence of the enzyme in a Rayonet photochemical reactor.[19] Products and unreacted substrates were purified by HPLC. Standard liquid scintillation counting techniques were used to accurately determine the percent conversion and the ^{3}H/^{14}C ratio in products and substrates. The secondary deuterium isotope effects, calculated using the integrated rate equations described by Cleland,[8] are summarized in Table I.

The enzymatic cleavage of the tetradeuterio photodimer shows a V/K isotope effect of 1.150. In contrast to our prediction, this effect is not primarily associated with either the cleavage of the 5,5' bond [$^{D}(V/K)$ = 1.082] or the 6,6' bond [$^{D}(V/K)$ = 1.071] of the photodimer but is almost equally distributed between the two bonds. Furthermore, the observation that the product of the V/K isotope effects for the 6,6'-dideuterio photodimer and the 5,5'-dideuterio photodimer equals the isotope effect for the tetradeuterio photodimer suggests that all four C-H bonds of the cyclobutane ring undergo a simultaneous hybridization change in a single transition state.

The simplest explanation is that the reaction proceeds via the dimer radical anion or cation and shows a large β-secondary isotope effect in addition to the α-secondary isotope effect on the first C-C bond cleavage. However, the β-isotope effects on radicals described in the literature are considerably smaller than the α-effects.[20] In addition, the magnitude of the β-effect is predicted to depend on $\cos^2[\phi]$ where ϕ is the dihedral angle between the bond undergoing cleavage and the β-C-H bond.[21] For the photodimer, this angle is 94°, suggesting that the β-effect will be small.

An alternative explanation is that the cleavage of the two C-C bonds from the dimer radical anion or cation is a concerted process. Radical anion **4** and radical cation **8** are both delocalized radicals. It is therefore not unreasonable that both the 5,5' and the 6,6' bonds are weakened by reduction/oxidation of the photodimer. Quasi-concerted [2 + 1] cycloadditions of a variety of alkene radical cations have been proposed on the basis of stereochemical[22] and theoretical[23] considerations. The cycloreversion of the *trans*-anethole cyclobutane radical cation has also been proposed to occur via a concerted pathway.[22b,c] While there is no precedent for analogous chemistry with alkene radical anions, the dimer radical anion cannot be excluded as a possible intermediate. Experiments are currently in progress, using model systems, to determine if the surprising pattern of isotope effects observed for the enzymatic reaction is characteristic of photodimer cleavage from either a radical cation or a radical anion intermediate.

Acknowledgment. We thank Doug Burdi for insightful discussions. This work was supported by grants from the National Institutes of Health (CA 45251 and GM 40498 to T. P. B. and GM 31082 to A.S.) and the Cornell Biotechnology Program, including a predoctoral fellowship to M.R.W., which is sponsored by the New York State Science and Technology Foundation, a consortium of industries, the U.S. Army Research Office, and the National Science Foundation.

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(16) For the 5,5',6,6'-tetradeuterio photodimer, the levels of deuteriation at the 5- and the 6-positions, as determined by NMR analysis, were 95.3 ± 0.7% and 93.9 ± 0.7%, respectively. For the 5,5'-dideuterio photodimer, the level of deuteriation at the 5-positions was 95.9 ± 0.8%. For the 6,6'-dideuterio photodimer, the level of deuteriation at the 6-positions was 84.1 ± 0.6%.

(17) Control experiments demonstrated that the esters were stable under our reaction conditions and that acetylation of the photodimer had a negligible effect on the enzymatic reaction.

(18) Mixtures of protio and deuterio photodimers were copurified by HPLC to >99% purity and to constant ^{3}H/^{14}C.

(19) λ_{max} = 350 nm; the reaction mixture (300 μL) consisted of 30 mM Tris, pH 7.2, 6.6 mM NaCl, 0.6 mM EDTA, 1 mM DTT, 1.7 mM substrates, and 7.4 μM enzyme.

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Synthesis and Characterization of Phenalenyl Cations, Radicals, and Anions Having Donor and Acceptor Substituents: Three Redox States of Modified Odd Alternant Systems

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Received July 7, 1989

Three redox states of an odd alternant hydrocarbon, phenalenyl cation, radical, and anion ($\mathbf{1}^+$, $\mathbf{1}^\bullet$, $\mathbf{1}^-$), have already been isolated or generated.[2,3] The importance of this skeleton[4-6] has been renewed from recent growing interest in the multistage redox systems to explore new organic materials.[7-9] Extension of the

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