appear to be much better isoelectronic isosteres of sulfate esters than phosphonates are of phosphate esters. This suggests that, in contrast to phosphonates which are generally poor phosphatase inhibitors, sulfonates should be potent sulfatase inhibitors. In addition, because of the resistance of the sulfonate group toward hydrolysis, sulfonated steroids should be very useful agents for probing the metabolism, transport, and biological function of the analogous sulfated steroids. Such studies are currently being pursued.

Supplementary Material Available: Experimental details and ^1H and ^{13}C NMR spectra for the compounds mentioned in the text (22 pages). Ordering information is given on any current masthead page.

Model Studies of DNA Photorepair: Energetic Requirements for the Radical Anion Mechanism Determined by Fluorescence Quenching

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The most significant form of photochemical damage to DNA is the [2 + 2] cyclodimerization of pyrimidine bases.[1] Direct reversal of this damage is mediated by photolyase enzymes, which use a photon to initiate the catalytic step.[2] The successful cloning and overexpression of *Escherichia coli* photolyase by Sancar[3] has renewed interest in the mechanism of these photochemical enzymes. Studies of model repair reactions have suggested a reductive SET (single electron transfer) mechanism.[4] In this mechanism, the enzyme excited state transfers a single electron to the pyrimidine dimer. The dimer anion radical then fragments to yield a monomer and a monomer anion radical. The latter re-reduces the enzyme, and the DNA bases are reverted to their normal form. Recently, we demonstrated that thymine dimer anion radicals cleave very rapidly (2×10^6 s^{-1}) in free solution.[5] Evidence for the corresponding cation radical mechanism also exists.[6] The experiments reported here were undertaken in order to evaluate the energetic requirements for the anion radical mechanism. The results support that mechanism.

$$S + Q \xrightarrow{h\nu} S^* + Q \underset{}{\overset{k_{dif}}{\rightleftharpoons}} [S\cdots Q]^* \overset{k_\alpha}{\rightleftharpoons} S^{\cdot+} + Q^{\cdot-} \quad (1)$$

The most common test for photoinitiated single electron transfer (SET) mechanisms is to calculate the free energy change for the charge-transfer step (ΔG_{ct}).[7] This is usually done by measuring the oxidation potential of the donor (E_{ox}) and the reduction potential of the acceptor (E_{red}) and applying eq 2. Generally, if

$$\Delta G_{ct} = 23.06\left(E_{ox} - E_{red} - \frac{e^2}{R\epsilon}\right) - E_{oo} \quad (2)$$

the charge transfer is more than ca. 5 kcal/mol endergonic, it does not compete effectively with relaxation of the sensitizer excited state. On the other hand, if the charge-transfer step is exergonic, then SET can be considered a feasible pathway. Despite the growing interest in the DNA photorepair mechanism and the large number of model reactions studied, no reduction potential for any pyrimidine dimer is known.[8]

In order to assess the proposed mechanism, we sought to determine E_{red} for thymine dimers. Because our attempts to measure this quantity by cyclic voltammetry were unsuccessful, an alternative technique was used. This approach is based on the principle that the rate constant for electron transfer (k_{ct}) is related to ΔG_{ct}. For the bimolecular systems illustrated in eq 1, k_{ct} can be determined from the rate constant for fluorescence quenching, k_q. In the present case, the sensitizer (S) is an excited-state electron donor and the quencher (Q) is the pyrimidine dimer.

A series of sensitizers was examined where the excited-state oxidation potential, E_{ox}^* (= $E_{ox} - E_{oo}/23.06$), was systematically

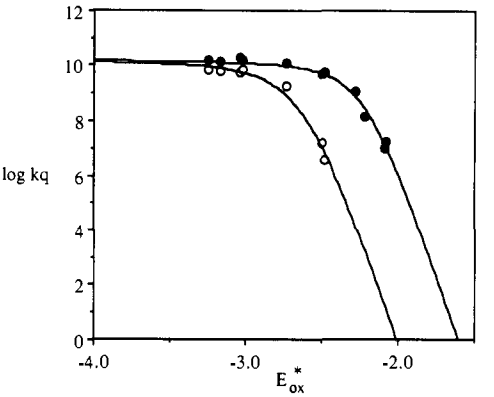

Figure 1. Effect of sensitizer excited-state oxidation potential, E_{ox}^*, on the rate constant for fluorescence quenching (k_q). The substrates were dimethylthymine (●) and its cis,syn dimer (○). The solid lines are calculated on the basis of the diffusion-limited Rehm-Weller relationship using $E_{red} = -2.60$ V, $\lambda = 15.7$ kcal/mol (dimer) and $E_{red} = -2.21$ V, $\lambda = 13.7$ kcal/mol (monomer). The solvent is CH_3CN.

Table I. Sensitizer Excited-State Oxidation Potentials (E_{ox}^*) and Quenching Rate Constants (k_q) for Dimethylthymine and Dimethylthymine Dimer

sensitizer	E_{ox}^*, V	k_q, M^{-1} s^{-1} (monomer)	k_q, M^{-1} s^{-1} (dimer)
N,N,N',N'-tetramethylphenylenediamine	−3.25	1.54×10^{10}	6.54×10^9
N,N,N',N'-tetramethylbenzidine	−3.17	1.39×10^{10}	5.91×10^9
N,N-dimethylaniline	−3.04	1.79×10^{10}	5.47×10^9
aniline	−3.02	1.55×10^{10}	7.06×10^9
p-dimethoxybenzene	−2.73	1.17×10^{10}	1.80×10^9
acenaphthene	−2.50	5.04×10^9	1.52×10^7
naphthalene	−2.48	5.53×10^9	3.65×10^6
2-methoxynaphthalene	−2.28	1.09×10^9	$<1 \times 10^6$ [a]
anthracene	−2.22	1.42×19^8	$<1 \times 10^6$ [a]
phenanthrene	−2.09	9.84×10^6	$<1 \times 10^6$ [a]
chrysene	−2.08	1.72×10^7	$<1 \times 10^6$ [a]

[a] No quenching was measurable at 0.2 M quencher concentration.

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varied. Acetonitrile solutions containing the sensitizer and the quencher (either dimethylthymine[9] or its cis,syn dimer[10]) were prepared in sealed cuvettes and purged with dry nitrogen. The fluorescence intensity was measured and compared with the fluorescence of an identical solution which contained no quencher. The resulting Stern-Volmer plots[11] showed good linearity and gave correlation coefficients of 0.98 or greater. The k_q values were calculated using literature values for the sensitizer singlet lifetimes (τ).

The effect of E_{ox}* on k_q is shown in Figure 1 and Table I. As E_{ox}* becomes increasingly negative, k_q increases, reaching the diffusion limit near -2.7 V. Without assuming any particular quantitative model, two qualitative generalizations can be made. First, the clear correlation of k_q with E_{ox}* is consistent with the proposed anion radical mechanism and establishes its generality.[12] A plot of k_q vs the sensitizer singlet energy, E_{oo}, for example, showed no clear correlation. The latter indicates that charge transfer is the only significant quenching mechanism. Second, as the sensitizer potentials become more positive than -2.5 V (vs SCE), the rate drops off significantly. Therefore, sensitizers with potentials more positive than ca. -2.4 V would probably not be effective at initiating cleavage via the SET mechanism.

For systems which behave as eq 1, k_q can be quantitatively related to ΔG_{ct} by the Rehm-Weller relationship:[13]

$$k_q = \frac{k_{dif}}{1 + 0.25\{\exp(\Delta G_{ct}^*/RT) + \exp(\Delta G_{ct}/RT)\}} \quad (3)$$

where

$$\Delta G_{ct}^* = \left[\left(\frac{\Delta G_{ct}}{2}\right)^2 + \left(\frac{\lambda}{4}\right)^2\right]^{1/2} + \frac{\Delta G_{ct}}{2} \quad (4)$$

The data in the present work were fit to this relationship using λ (the solvent reorganization energy) and E_{red} (= E_{ox}* - ΔG_{ct}/23.06) as adjustable parameters. The values thus derived are E_{red} = -2.60 V (vs SCE), λ = 15.7 kcal/mol for dimethylthymine dimer and E_{red} = -2.21 V, λ = 13.7 kcal/mol for the monomer. To test the validity of this method, it was applied to a quencher with a known reduction potential, methyl benzoate. The value obtained by our method, -2.26 V, is in reasonable agreement with the value reported from electrochemical measurements (-2.3 V).[14]

One approximation in this analysis is the structural difference between the model compound and the biological substrate (thymidine) which is unsubstituted at N-3. (Thymine, 1-alkylated thymine, and their dimers are not sufficiently soluble for this study.) Methyl groups are weakly electron donating. Therefore, it is possible that E_{red} for the biological substrate is slightly more positive. However, a difference of more than 0.10 V would be very surprising.[15]

Electron transfer to the dimer alters the thermodynamics of the cleavage step. Analysis of a simple thermodynamic cycle gives eq 5. The extra potential required to reduce the dimer adds to the driving force of the cleavage step. The free energy change of the anion radical cleavage step (ΔG_{anion}) depends on the reduction potentials of the dimer and the monomer along with the $\Delta G_{neutral}$ (cleavage of the neutral dimer to neutral monomer):

$$\Delta G_{anion} - \Delta G_{neutral} = 23.06(E_{red}^{dimer} - E_{red}^{monomer}) \quad (5)$$

Our results show that cleavage of the anion is 9 kcal/mol more exergonic than cleavage of the neutral.[16]

The data here are consistent with the proposed reductive SET mechanism for photolyase.[17] The relevant enzymic chromophore is a 1,5-dihydroflavin. In free solution, these species have E_{ox}* of -2.6 V.[18] Therefore the SET step in the enzymatic reaction is approximately thermoneutral. This is in agreement with the picosecond measurements of Okamura et al.[19] These workers showed that the excited-state flavin interacts with the damaged DNA very rapidly, with a rate constant of 5.5×10^9 s^{-1}. This requires that ΔG_{ct} in the enzyme be <1 kcal/mol.[20]

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Registry No. DNA photolyase, 37290-70-3; dimethylthymine, 4401-71-2; *cis,syn*-dimer, 3660-32-0.

Supplementary Material Available: A table of sensitizers, $k_q\tau$ values, and the parameters used in the data analysis (1 page). Ordering information is given on any current masthead page.

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Cross-linked Enzyme Crystals as Robust Biocatalysts

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Chemical cross-linking of enzyme crystals stabilizes the crystalline lattice and its constituent enzyme molecules, forming highly concentrated immobilized enzyme particles which can be lyophilized and stored indefinitely at room temperature. Cross-linked enzyme crystals (CLCs) retain catalytic activity in harsh conditions, including temperature and pH extremes, exogenous proteases, and exposure to organic or aqueous solvents and aqueous-organic mixtures. Lyophilized CLCs can be reconstituted easily in these solvents as active, monodisperse suspensions. We present data comparing free enzyme vs CLCs of thermolysin, which is used in the manufacture of the artificial sweetener aspartame.[1,2] Results with other enzymes suggest that the CLC process may be broadly applicable.

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