Model Studies of DNA Photorepair: Reduction Potentials of Thymine and Cytosine Cyclobutane Dimers Measured by Fluorescence Quenching

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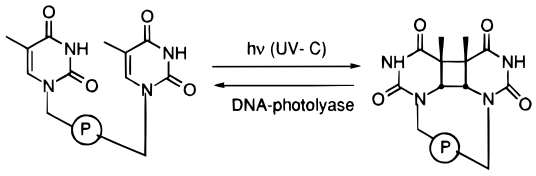
Abstract: The interactions of various pyrimidines (1,3-dimethylthymine, DMT, 1,3-bis(N^4,N^4-dimethylcytosin-1-yl)propane, DMC) and their corresponding *cis-syn* cyclobutane dimers (DMTD and DMCD) with a series of excited-state electron donors were examined with the goal of understanding the energetics and mechanism of UV repair by DNA photolyase. For each substrate there is a good correlation between the excited state oxidation potential (E_{ox}*) and the quenching rate constant (k_q). The value for k_q increases as E_{ox}* becomes more negative, asymptotically approaching a value that is at or below the solvent diffusion limit. These data all showed good fits to the Rehm−Weller equation. Reduction potentials for each of the substrates could be extracted from this analysis: −2.20 V (vs SCE) for DMTD; −2.14 V for DMT; −2.17 V for DMCD; and −2.16 for DMC. These values show that the initial electron transfer step in the photolyase mechanism is exergonic by ca. 10−15 kcal/mol. Thus these data support the reductive electron transfer mechanism for DNA photolyases proposed by Jorns et al. (*J. Biol. Chem.* **1987**, *262*, 486−491).

Introduction

Photoenzymes are a class of proteins that harness UV (ultraviolet) and/or visible light energy in order to effect specific chemical transformations.[1] The best characterized example of these are the *cis-syn* DNA photolyases.[2,3] These are monomeric proteins, found in a wide variety of organisms, that mediate the photoreversal of *cis-syn* pyrimidine cyclobutane dimers (eq 1). The dimers are formed as a consequence of UV light



(1)

damage to the DNA molecule.[4−6] The repair mechanism involves two distinct stages. The first is a light-independent binding to the damage site; the second is a light-dependent catalytic step in which the C5−C5′ and C6−C6′ carbon−carbon bonds are broken.

There has been considerable interest in elucidating the detailed mechanism of the photoenzymatic repair process. Site-directed mutagenesis,[7] substrate specificity studies,[8,9] kinetic isotope competition experiments,[10] time-resolved EPR,[11] and laser flash photolysis[12−14] have been employed. Catalytic antibodies that mimic the functions of the *cis-syn* pyrimidine dimer photolyase have been characterized.[15] Recently a crystal structure of the photolyase from *E. coli*, resolved to 2.3 Å, has been reported.[16] While many details of the mechanism remain controversial, it is becoming increasingly clear that the splitting step (i.e. scission of the C5−C5′ and C6−C6′ bonds) is initiated by transfer of a single electron between the a $FADH^-$ cofactor on the enzyme and the substrate.

Model studies indicate that the electron flow occurs from the $FADH^-$ to the dimer substrate (Scheme 1). Electron donors, such as indoles, have long been known to sensitize the splitting of thymine and uracil dimers.[17,18] Recent studies[19] have extended these observations to the cytosine dimers and cytosine−thymine heterodimers. Interestingly, pyrimidine dimer splitting reactions were discovered to occur with higher efficiency in nonpolar media.[20] $FADH_2$ is most effective as a photosensitizer when it is in its conjugate base form (i.e. $FADH^-$).[21] Laser flash photolysis studies from this laboratory,[22]

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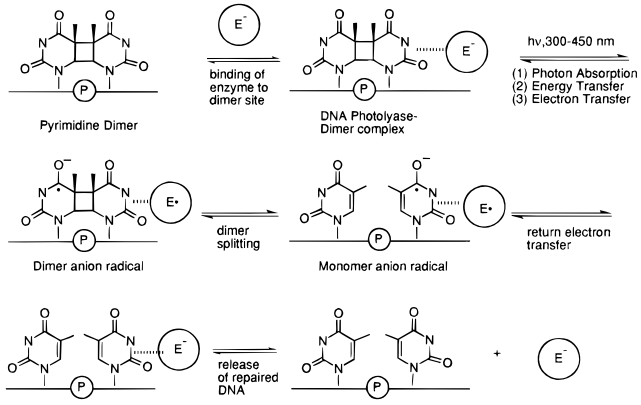
Scheme 1 Mechanism for the Photochemical Splitting of Pyrimidine Dimers Mediated by DNA Photolyase



along with CIDNP results reported by others,[23] indicate that *cis-syn* pyrimidine dimer anion radicals cleave very rapidly—with rate constants on the order of 10^6 s^{-1}.

$$S + Q \xrightarrow{h\nu} S^* + Q \rightleftharpoons (SQ)^* \rightleftharpoons S^{+\cdot} + Q^{-\cdot} \qquad (2)$$

Equation 2 shows a general scheme[24,25] for photochemical electron transfer where a sensitizer *S* absorbs a photon and then transfers an electron to a quencher *Q*. A key consideration in evaluating any proposed photochemical electron transfer mechanism is the free energy change in the charge transfer step (ΔG_{ct}). Generally speaking, photochemical electron transfer reactions occur only when the charge transfer step is either exergonic or <5 kcal/mol endergonic.[26] In this case charge transfer is fast enough to compete with nonradiative deactivation of the excited state sensitizer molecule. The value of ΔG_{ct} (in kcal/mol) can be determined from the oxidation potential of the donor (E_{ox}, in V), the reduction potential of the acceptor, (E_{red}, in V), the excited state energy of the sensitizers (E_{oo}, in kcal/mol), along with a term that accounts for desolvation and Coulombic interactions in the ion pair $q^2/\epsilon r$ as described in eq 3.

$$\Delta G_{ct} = 23.03\left(E_{ox} - E_{red} - \left(\frac{q^2}{\epsilon r}\right)\right) - E_{oo} \qquad (3)$$

Very little is known about the reduction potentials (E_{red}) of the pyrimidine dimers, or indeed even of monomeric pyrimidines. The functional groups present in these species (imido groups, enamines, etc.) are not generally considered to be electrochemically reactive. This consideration, along with a lack of knowledge about the precise nature of the enzymic chromophore, caused early workers to exclude the reductive single electron pathway.

We recently reported fluorescence quenching measurements with dimethylthymine dimer and a series of excited state electron donors having varying reduction potentials.[27] Based on this it was possible to estimate the E_{red}. The findings demonstrated that the proposed electron transfer step is thermodynamically feasible. This provided some quantitative support for the proposed mechanism. Here we provide a full account of the work in that preliminary communication which refines our original estimates, and extends our observations to cytosine-containing dimers.

Chart 1. Pyrimidines and Pyrimidine Dimers Used in This Study

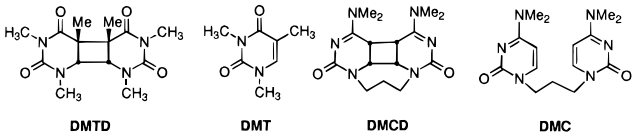


Table 1. Sensitized Splitting of Pyrimidine Cyclobutane Dimers

sensitizer	reactant (mM)	time (min)	concentration (mM) monomer	concentration (mM) dimer
TMPD[a]	DMTD (9.75)	40	12.20	3.41
	DMCD (0.66)	30	0.57	0.00
TMB[b]	DMTD (10.71)	300	10.93	5.75
naphthalene	DMTD (1.11)	300	0.80	0.62
pyrene	DMTD (14.80)	1080	2.60	13.45
	DMCD (0.66)	840	0.86	0.00
chrysene	DMTD (8.40)	2160	0.56	8.02
	DMCD (0.66)	840	0.91	0.00

[a] *N,N,N′,N′*-Tetramethylphenylenediamine. [b] *N,N,N′,N′*-Tetramethylbenzidine.

Results and Discussion

1. Synthesis of Pyrimidine Dimers. Four substrates were employed in this study, the *cis-syn* cyclobutane dimer of dimethylthymine DMTD along with its monomer DMT, and a trimethylene linked *cis-syn* cyclobutane dimer of dimethylcytosine DMCD along with its "monomeric" isomer DMC. Dimer DMTD was prepared from the irradiation of **DMT** frozen in ice according to the classical procedure.[28,29] Dimer DMCD and monomer DMC were synthesized starting with 1,3-(1-uracilyl)-propane according our previously reported procedure.[19] Purity of samples was determined by ^{1}H NMR and HPLC. The substrates used in this study are illustrated in Chart 1.

2. Dimer Splitting Experiments. Previous work demonstrated that excited state electron donors photosensitize the splitting of pyrimidine dimers. Sensitizers employed include aromatic amines,[17,30] indoles,[31] tryptophan,[18] and dimethoxybenzene.[20,32] We have also demonstrated that *N,N*,-dimethylaniline sensitizes the splitting of DMTD.[22] Laser flash photolysis experiments confirmed the intermediacy of ion radical intermediates in this latter reaction.[22] Despite this earlier work it seemed worthwhile to re-examine this photochemistry using some of the sensitizers employed here to ensure that the fluorescence quenching events observed below resulted in the expected chemical reactions.

Each of the sensitizers listed in Table 1 was irradiated in the presence of 1−10 mM of either DMTD or DMCD. Pyrene, naphthalene, and TMPD were chosen as representative excited state electron donors. All samples were purged with Ar, irradiated for the times indicated, and analyzed by HPLC to determine the amount of monomers formed and the amount of dimer remaining. Irradiations were carried out using cutoff filters to ensure that the light was absorbed by the sensitizer and not the dimers. The results are compiled in Table 1.

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The efficiency and ability of the sensitizers to effect dimer splitting is qualitatively related to their excited-state oxidation potentials, E_{ox}*, which are listed in Table 2.[33] E_{ox}* is determined from literature values for the oxidation potential (E_{ox}) and the singlet state energy, E_{oo}, using eq 4 where E_{ox} and E_{ox}* are in volts (vs SCE) and E_{oo} is in kcal/mol.

$$E_{ox}^* = E_{ox} - \frac{E_{oo}}{23.06} \quad (4)$$

Aromatic amines, TMB, and TMPD have give the cleanest and most efficient splitting of both dimers. Naphthalene was also effective at splitting the thymine dimer DMTD; however, much longer photolysis times were employed and even then the conversion was low. Naphthalene could not be tested with DMCD because their UV absorption bands overlapped and sensitized photolysis would not be distinguished from direct photolysis. Chrysene also sensitizes dimer splitting, but as with naphthalene, much longer photolysis times are required. Cytosine dimer DMCD was split to completion in 14 h, whereas with DMTD less than 5% conversion was detected after 36 h.

It should be noted that the photolysis rates provide only a semiquantitative indication of the electron transfer efficiency of the sensitizers. These rates also reflect the spectral overlap of the sensitizers with the medium-pressure Hg lamp, the lifetime of the excited state sensitizer, the efficiency of the initial electron transfer, and the ability of the splitting reaction of the dimers to compete with back electron transfer. For example, below it is shown that pyrene is not quenched particularly efficiently by any of the substrates. That it does cause a splitting reaction can be attributed to its relatively long lifetime and broader absorption spectrum.

3. Fluorescence Quenching Experiments. To better understand the photosensitized splitting reaction mechanisms, fluorescence quenching experiments were carried out. A series of sensitizers with varying redox properties and singlet energies were examined using dimers DMTD and DMCD and monomers DMT and DMC as quenchers. It was reasoned that if the splitting occurred via the proposed ion radical intermediates (Scheme 1), then a correlation between the excited state oxidation potential (E_{ox}*) and the quenching efficiency would be observed. In any case, we anticipated that comparing the quenching efficiencies with sensitizer properties would help identify the minimal requirements for an enzymatic photorepair system. Our results, outlined below, support the electron transfer mechanism.

The quantum yield of fluorescence without quencher, Φ_o, relative to that with quencher added, Φ, is given by the rate constant for the reaction of the excited sensitizer with the quencher (k_q), the lifetime of the sensitizer's excited state (τ), and the concentration of the quencher, [Q], according to the Stern−Volmer equation (eq 5).

$$\frac{\Phi_o}{\Phi} = 1 + k_q\tau[Q] \quad (5)$$

The pyrimidine dimers DMTD and DMCD quench the fluorescence of various sensitizers. Figure 1 shows typical examples where CH_3CN solutions of the electron donor sensitizer, *N*,*N*,*N*′,*N*′-tetramethylbenzidine (TMB), were irradiated in the presence of various concentrations of cytosine dimer DMCD. In this case the fluorescence decreases and no new emission bands are observed. Similar behavior was observed with the other sensitizers.

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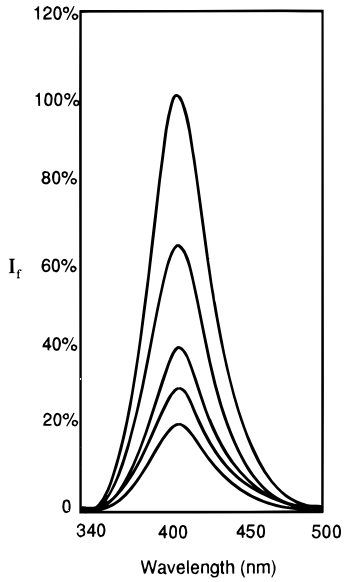

Figure 1. Fluorescence spectrum of *N*,*N*,*N*′,*N*′-tetramethylbenzidine in Ar-purged CH_3CN. The fluorescence intensity decreases as increasing amounts (0−13 mM) of DMC are added.

Table 2. Properties of Various Sensitizers Used in This Study

sensitizers	τ (ns)	E_{ox}* (V vs SCE)	E_{oo} (eV)	E_{ox} (V vs SCE)
tetramethyl-1,4 phenylene-diamine	7.1[e]	−3.25	3.45[a]	0.20[g]
tetramethyl- benzidine	10.0[c]	−3.17	3.60[f]	0.43[f]
dimethylaniline	2.78[a]	−3.04	3.87[a]	0.83[g]
aniline	3.10[a]	−3.02	3.97[a]	0.95[h]
acenaphthene	46.0[a]	−2.66	3.91[a]	1.41[h]
1-methoxy naphthalene	17.2[e]	−2.49	3.85[e]	1.36[e]
naphthalene	96.0[a]	−2.48	4.02[a]	1.54[d]
9-methylanthracene	5.80[e]	−2.46	3.42[e]	0.96[e]
2-methoxynaphthalene	15.00[b]	−2.28	3.70[b]	1.42[c]
1-acetamidopyrene	12.9[i]	−2.23	3.56[i]	1.33[i]
anthracene	5.30[c]	−2.22	3.31[c]	1.09[d]
pyrene	322[e]	−2.17	3.34[e]	1.16[e]
phenanthrene	61.0[c]	−2.09	3.59[c]	1.50[d]
chrysene	43.0[c]	−2.08	3.43[c]	1.35[d]

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The fluorescence intensities at various concentrations were fit to the Stern−Volmer relationship (eq 5). The rate constants for fluorescence quenching, k_q, were determined from our measured $k_q\tau$ values and literature data for the various sensitizer τ values. Studies of DMCD and DMC with aniline and *N*,*N*-dimethylaniline could not be carried because the long-wavelength absorption band of the quenchers overlapped the absorption bands of these sensitizers making it difficult to distinguish quenching from inner filter effects.

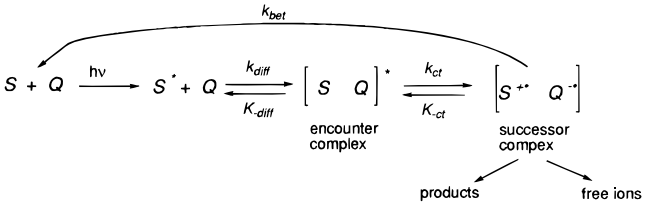
The monomeric bases DMT and DMC quench fluorescence of the sensitizers. The k_q values for these are listed in Table 3. The quenching rate constants for both substrates increase as the E_{ox}* of the sensitizer becomes increasingly negative. In both cases limiting k_q values of ca. 1.9×10^{10} M^{-1} s^{-1} (the diffusion limit) are reached as E_{ox} becomes more negative than −2.4 V.

The dimeric substrates, DMTD and DMCD, show qualitatively similar behavior. For both dimers the limit is approached

Table 3. Fluorescence Quenching Rate Constants, k_q ($\times 10^{-9}$ M^{-1} s^{-1}), for Excited State Electron Donors with Pyrimidines and Their Corresponding *cis-syn* Cyclobutane Dimers

sensitizers	DMTD	DMCD	DMT	DMC
tetramethyl-1,4-phenylenediamine	6.54	11.5	14.2	20.1
tetramethylbenzidine	5.91	10.7	14.3	20.4
dimethylaniline	5.47		12.8	
aniline	5.06		12.3	
acenaphthene	3.31	9.04	8.08	9.80
1-methoxynaphthalene	3.34	7.01	7.51	7.23
naphthalene	2.48	6.32	2.05	6.87
2-methoxynaphthalene	0.821	6.17	1.61	0.652
9-methylanthracene	0.532	2.52	0.686	0.604
1-acetamidopyrene	0.623	2.34	0.528	0.521
anthracene	0.362	2.09	0.568	0.443
pyrene	0.152	1.20	0.228	
phenanthrene	0.103	0.201	0.0717	0.0420
chrysene	0.287	0.178	0.0589	0.0366

Scheme 2. Kinetic Model for Fluorescence Quenching by Electron Transfer



near $E_{ox}^* = -2.5$ V. It is interesting that both of the dimeric substrates give asymptotic k_q values that are clearly lower than the diffusion limit[34] of 1.9×10^{10} M^{-1} s^{-1} predicted by the Smoluchowski and Stokes−Einstein equations.[35] This is particularly pronounced in the case of DMTD which limits at 6×10^9 M^{-1} s^{-1}. This effect is most clearly seen in Figures 2 and 3 which compare the k_q for DMT and DMC respectively with their corresponding dimers. The origins of this behavior are considered in the following section.

The correlation of the k_q values with E_{ox}^* supports the reductive single electron transfer mechanism for both thymine and cytosine dimers. Comparing the k_q values with other substrate parameters such as the singlet energy, E_{oo}, showed no discernible correlation. Even without fitting these data to a quantitative model, it is clear that any enzymatic sensitizer must possess an E_{ox}^* more negative than ca. −2.4 V in order to effect efficient splitting.

4. Rehm−Weller Analysis of Quenching Rate Constants. The fluorescence quenching behavior was also analyzed in a more quantitative way. The model of Rehm and Weller[36,37] divides the process into the three steps shown in Scheme 2. There is an initial diffusive encounter (k_{diff}) of the excited state molecule (S^*) with the ground state molecule Q forming an encounter complex. The latter undergoes charge transfer (k_{ct}) to form a successor complex (also known as a contact ion pair). The successor complex decays through a number of pathways including solvent relaxation and back electron transfer leading to ground state reactants, S and Q. The latter are grouped under rate constant k_3.

The quenching rate constant is the overall rate constant for the loss of S^* due to reaction with Q. Equation 6 follows from applying the steady-state approximation to the encounter complex and the contact ion pair, with the further assumption[38] that $k_3 \gg k_{-ct}$ and substituting k_{ct} with the Eyring expression ($k_{ct} = k_{max} \exp(-\Delta G_n^{\ddagger}/RT)$). K_{diff} is the diffusional equilibrium constant ($=k_{diff}/k_{-diff}$) and k_{max} is the so-called frequency factor. The diffusion rate constant (k_{diff}) has been determined to be 1.9×10^{10} M^{-1} s^{-1}.[34]

$$k_q = \frac{k_{diff}}{1 + \frac{k_{diff}}{K_{diff} k_{max}} \exp\left(\frac{\Delta G_{ct}^{\ddagger}}{RT}\right)} \qquad (6)$$

The free energy barrier for the charge transfer step, $\Delta G_{ct}^{\ddagger}$, can be predicted from the driving force of the electron transfer reaction, $-\Delta G_{ct}$, along with the reorganization energy, λ. There are a number of treatments of this relationship,[26] the most widely known being the Marcus theory (see eq 8, below). The latter is an extrathermodynamic treatment of reaction barriers which assumes a quadratic dependence of the barrier on the driving force. This predicts the so-called inverted region where the barrier begins to increase with increasing driving force. This treatment has been highly successful in predicting the rates of electron transfer in rigid systems[39−41] and back electron transfer in photochemical systems (e.g. k_3).[42−44] However, for photoinduced electron transfer reactions, inverted behavior has been observed only in a few special systems.[45,46] More typical is behavior where the k_q increases with driving force and then saturates at the diffusion limit.[47−49] Rehm and Weller[36,37] demonstrated that the following monotonic relationship between $\Delta G_{ct}^{\ddagger}$ and ΔG_{ct} was successful at predicting rate constants for the latter types of reactions:

$$\Delta G_{ct}^{\ddagger} = \left[\left(\frac{\Delta G_{ct}}{RT}\right)^2 + \left(\frac{\lambda}{4}\right)^2\right]^{1/2} + \frac{\Delta G_{ct}}{2} \qquad (7)$$

The values of E_{ox}^* and the experimentally derived k_q in Tables 2 and 3 were analyzed using eqs 3, 6, and 7. The two adjustable parameters were λ and E_{red}. The desolvation term in eq 3 was estimated at 1.34 kcal/mol assuming a 700 pm separation distance for each of the sensitizer quencher pairs. The diffusion rate constant, k_{diff}, was set at 1.9×10^{10} M^{-1} s^{-1}.

The appropriate value for the preexponential term, $k_{max}K_{diff}$, has been the subject of some recent discussion. It was originally assumed to be 10^{11} M^{-1} s^{-1}.[36,37] Subsequently it has been shown that certain fluorescence data can be made to conform to the Marcus theory by revising this factor upward.[50−52]

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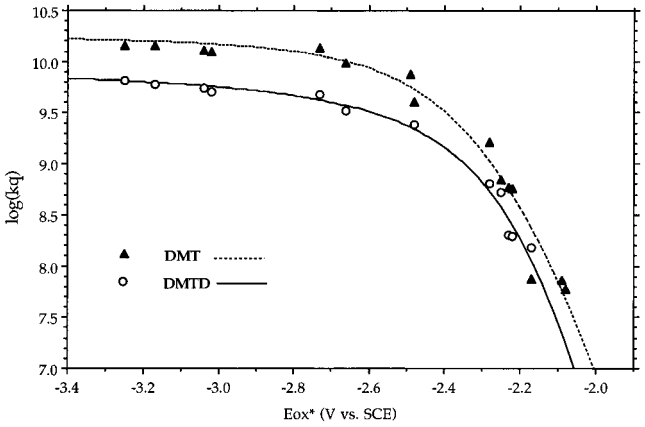

Figure 2. Rehm−Weller analysis of the dependence of fluorescence quenching rate constants (k_q in M^{-1} s^{-1}) for DMTD (filled triangles) and DMT (open circles) on the excited state oxidation potentials (E_{ox}* in V vs SCE) of various sensitizers in N_2-purged CH_3CN. Curves show fits calculated for DMTD (E_{red} = −2.20 V, broken line) and DMT (E_{red} = −2.14 V solid line).

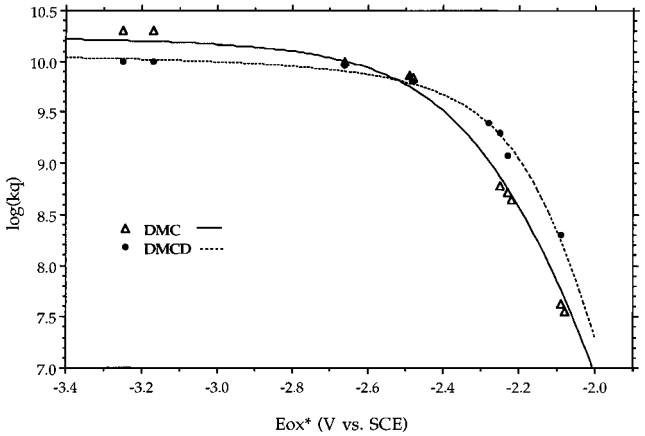

Figure 3. Rehm−Weller analysis of the dependence of fluorescence quenching rate constants (k_q in M^{-1} s^{-1}) for DMCD (filled circles) and DMC (open triangles) on the excited state oxidation potentials (E_{ox}* in V vs SCE) of various sensitizers in N_2-purged CH_3CN. Curves show fits calculated for DMCD (E_{red} = −2.17 V, broken line) and DMC (E_{red} = −2.16 V solid line).

Marcus[53] and Weaver[54] have analyzed the k_{max} factor part of this and have argued that in CH_3CN, k_{max} should take a value of 10^{12} to 10^{13} s^{-1}. The other factor in the preexponential term, K_{diff}, has apparently not been subjected to the same level of scrutiny. Of course, measurements of the sort reported here are sensitive only to the product of these two parameters and are incapable of resolving the individual contributions.

In view of the above considerations, the value for $k_{max}K_{diff}$ was not fixed. Instead, 10 to 20 fits were undertaken for each quencher as this parameter was systematically varied from 10^{10} to 10^{14} M^{-1} s^{-1}. While the quality of the fits varied significantly over this range, the E_{red} were relatively insensitive to large changes in $k_{max}K_{diff}$. For example with DMC, E_{red} ranged only from −2.25 to −2.14 V, as $k_{max}K_{diff}$ was varied from 1×10^{10} to 1×10^{13} M^{-1} s^{-1}.

Adjusting the $k_{max}K_{diff}$ term significantly improves the fits for the dimers DMTD and DMCD. Only through this consideration is it possible to capture the fact that the asymptotic k_q values fall below the diffusion limit for these substrates. The best fit values $k_{max}K_{diff}$ were found to be 2×10^{10} M^{-1} s^{-1} for DMTD and 4×10^{10} for DMCD. We suggest that these low values are due to differences in the K_{diff} term. On the basis of Fuoss' model,[55] K_{diff} is often taken to be 0.86 M^{-1}. However, this is based upon the assumption of spherical and isotropic reactants. In this case all relative orientations of the quencher and the excited state sensitizer would be presumed to be equally reactive. In cases such as the present, where the reactants are non-isotropic, K_{diff} is the product of the diffusional equilibrium constant and any orientational equilibrium constants that lead to the reactive orientation. This lower value for K_{diff} found in the dimer experiments causes us to assume that the precursor complexes involving the dimers and sensitizers must adopt rather specific relative orientations in order to acheive productive electron transfer. The geometries of these "productive" orientations are not clear at this time. Further experimental and/or computational investigations into this issue would be interesting as a knowledge of geometric constraints on electron transfer to pyrimidine dimers is of obvious relevance to the enzymatic system.

Table 4. Parameters for Rehm−Weller Fits of Fluorescence Quenching Data

	DMTD	DMT	DMCD	DMC	$PhCO_2Me$
E_{red} (V)[a]	−2.20	−2.14	−2.17	−2.16	−2.28
λ (kcal/mol)[b]	13	22	12	27	28
$k_{max}k_{diff}$(M^{-1} s^{-1}) × 10^{-10}	2	64	4	510	640

[a] ±0.08 V. [b] ±10 kcal/mol.

The reduction potentials (E_{red}) for all of the substrates were estimated from the k_q values they each showed with the various sensitizers. The k_q data sets from Tables 2 and 3 were then compared with theoretical curves determined using eqs 6 and 7. A simplex algorithm was used to minimize the sum of the squares of the residuals as the parameters λ and E_{red} were varied. The optimized plots along with the experimental data are presented in Figure 2 (DMTD and DMT) and Figure 3 (DMC and DMCD). Table 4 lists the best fit values.

It was of interest to determine the uniqueness of the fits and to estimate uncertainties in the best-fit parameters. To this end, the procedure described above for the $k_{max}K_{diff}$ parameter was applied to the remaining parameters, λ and E_{red}. A series of fits were undertaken as λ was held at 100 values between 1 and 50 kcal/mol while both E_{red} and λ were optimized. Likewise an additional series of fits was undertaken where E_{red} was held at 100 different values between −1.90 and −2.30 V. Each of these three procedures converged on the same best fit parameters to within the stated uncertainties. We estimate the uncertainty in λ as ±10 kcal/mol and the uncertainty in E_{red} as ±0.08 V.

To further test the validity of this approach, the E_{red} of methyl benzoate ($PhCO_2Me$) was determined in the same fashion. Figure 4 shows experimental data for this substrate as well as the optimized curve calculated from eqs 6 and 7. The value of E_{red} for this compound (−2.28 V) compares favorably with a previously reported polarographic measurement of −2.3 V.[56] Fits to the classical Marcus theory were also undertaken. For these eq 7 was replaced with eq 8. In these cases the theoretical curves did not match the experimental data as well, but similar values for E_{red} and λ were extracted from the best fits.

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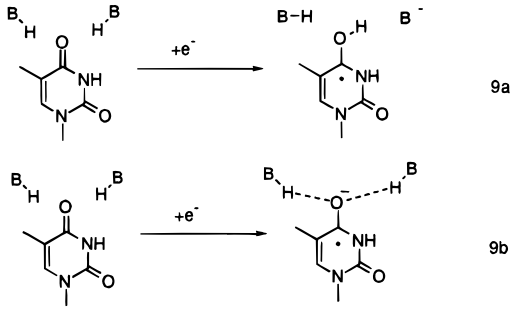
$$\Delta G_{ct}^{\ddagger} = \frac{(\lambda + \Delta G_{ct})^2}{4\lambda} \quad (8)$$

Rehm−Weller analysis of DMTD yields $E_{red} = -2.20$ V and $\lambda = 13$ kcal/mol. In the preliminary communication, we estimated a somewhat more negative value of −2.6 V. However in that experiment, the Rehm−Weller plot had only two sensitizers whose k_q values fell below the asymptotic limit. The E_{red} and λ thus extracted were highly dependent on the accuracy of these values. In this work we have repeated these determinations now using five sensitizers whose k_q values are below the asymptotic limit. This along with an improved fitting procedure permits a more accurate analysis.

The E_{red} values derived from these experiments compare well with previous reduction potentials measured for similar systems in aprotic solvents. Aromatic amides have reduction potentials in ethanol that range from −2.0 to −2.4 V.[57] Cyclic voltammetry of cytosine in DMSO gives a value of $E_{red} = -2.36$ V.[58] This agrees reasonably with our fluorescence quenching value of $E_{red} = -2.2$ V for DMC in CH_3CN. It is interesting to note that earlier polarographic experiments on cytosine in aqueous solution showed an irreversible reduction wave near −1.4 V−a potential considerably less negative than that measured in DMSO.[59] The electrochemical behavior of cytosine, and indeed all pyrimidines, in aqueous solution is complex. In the case of cytosine, the electron transfer is coupled with a fast and exothermic proton transfer. In aqueous solution, the equilibrium process, and thus electrochemically measured reduction potential, reflect a net H-atom transfer reaction (eq 9a). In contrast, the reduction potentials measured in the aprotic solvents, CH_3CN and DMSO, reflect only the electron transfer process.



There appears to be little electrochemical information on thymine. In aqueous solution this base does not give a polarographic wave distinct from the solvent discharge due to reduction of H^+.[60] Pulse radiolysis studies of thymine in aqueous solution have shown that its anion radical reacts with various *N*-methylpyridinium salts.[61] The yields of these reactions were used to derive equilibrium constants. Based on these experiments E_{red} was estimated at ca. −1.1 V. Interestingly, the same study gives a $E_{red} = -1.09$ for cytosine, in contrast to the electrochemical value of $E_{red} = -2.36$ in DMSO.[58]

One obvious source of discrepancy in these values is the nature of the solvent. Water and other protic solvents can provide significant stabilization to the anion radical through H-bonding. This is the case even in the absence of an explicit proton transfer such as that depicted in eq 9a.

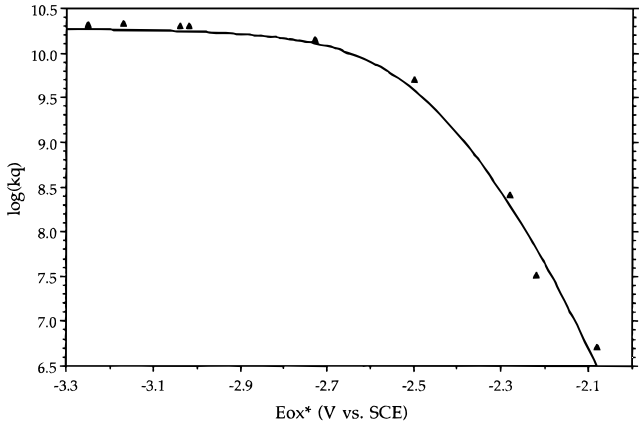


Figure 4. Rehm−Weller analysis of the dependence of fluorescence quenching rate constants (k_q in M^{-1} s^{-1}) for $PhCO_2CH_3$ on the excited state oxidation potentials (E_{ox}* in V vs SCE) of various sensitizers in N_2-purged CH_3CN.

Another estimate for E_{red} of thymine dimers comes from the pulse radiolysis studies of Heelis' et al.[62] It was demonstrated that dimer splitting could be induced by $CO_2^{\bullet-}$ although with only moderate efficiency. On this basis it was concluded that the E_{red} for thymine dimers in aqueous solution was near that of CO_2, placing the value at ca. −1.9 V. We regard this as quite reasonable agreement with the values determined here considering the differences in solvent and method of determination.

The E_{red} values here are consistent with behavior from previous model studies. Rose et al.[20,21,63] reported that pyrimidine dimers undergo dihydroflavin-sensitized decomposition through a radical anion chain mechanism. Such a mechanism requires that the pyrimidine anion radical generated in the splitting reaction be capable of transferring an electron to the dimer. This chain propagation step is plausible only if the electron transfer is exothermic or weakly endothermic. The values derived from this study predict that the propagation step should be slightly endothermic and are thus consistent with the proposed mechanism.

The values for λ extracted from the fitting procedures ranged from 12 to 28 kcal/mol. These fall into a range that is typical for organic sensitizer and quenchers in CH_3CN. For example, Rehm and Weller[37] obtained a λ value of 9.6 kcal/mol for their series of aromatic compounds. On the other hand, Chen et al.[64] report λ values of 23.5 kcal/mol for phenanthrene quenching by various amines. It is notable that the dimers give λ values that are roughly half that of the corresponding monomers. The λ is known to be inversely proportional to the radii of the reacting partners.[26] The larger effective radii of the dimers could therefore account for at least part of this difference.

5. Energetics of Enzymatic Photorepair. The enzymatic chromophore responsible for electron transfer to the dimer is a reduced flavin ($FADH^-$). Anderson's[65] pulse radiolysis studies provide a redox potential of −0.124 V for the flavin radical to reduced flavin transition. The fluorescence spectrum of the $FADH^-$ chromophore in DNA photolyase[66] shows an apparent 0−0 band at 450 nm. This corresponds to a singlet energy, $E_{oo} = 63.6$ kcal/mol. Application of eq 4, gives $E_{ox}* = -2.8$

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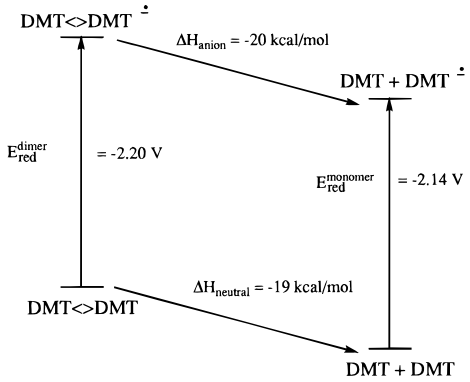

Figure 5. Thermodynamic cycle used to estimate the enthalpy change for the splitting of the dimethylthymine dimer anion radical (ΔH_{anion}). The reduction potentials of the dimer DMTD (= DMT⟨⟩DMT in the figure) and monomer DMT are taken from this work (Table 4). The enthalpy for neutral splitting, $\Delta H_{neutral}$, is taken from ref 67.

V for the proximate enzymic photosensitizer. This means it is a slightly more effective photosensitizer than 1,4-dimethoxybenzene. These values can be used to estimate the exergonicity of the charge transfer step in the enzymatic reaction. For both pyrimidine dimers, the initial charge transfer would be exergonic with $\Delta G_{ct} \approx -10$ to -15 kcal/mol. The uncertainty in this estimate is largely due to differing environmental effects in the model system and the photolyase active site.

The thermodynamic cycle illustrated in Figure 5 can be used to calculate the enthalpy change of the splitting reaction. Using a photothermal technique, we have previously estimated the neutral-to-neutral enthalpy of dimer splitting ($\Delta H_{neutral}$) at -19 kcal/mol for DMTD.[67] (Diogo et al.[68] obtained a value of -26 kcal/mol using a derivative of DMTD where the two bases were linked at their respective N3 positions with a trimethylene bridge.) The reduction potentials of the DMT and DMTD determined here provide the energy difference between the neutral forms and the anion radicals. It is further assumed that the entropy change for the electron transfer to the dimer and monomer is equal. On the basis of these considerations, $\Delta G = -20$ kcal/mol is predicted for the splitting reaction.

Experimental Section

Synthesis. Dimethylthymine dimer **1** and monomer **3** were synthesized as previously reported.[29,69] Dimethylcytosine dimer **2** and monomer **4** were also synthesized as previously reported.[19]

Product Studies. A quartz test tube was charged with the monomer or dimer being studied in CH_3CN. An excess of sensitizer of interest was added (if used) and the tube was sealed with a septum. The solution was purged with Ar for 10 min to remove oxygen. The solution was irradiated with a 450-W medium-pressure Hg-vapor lamp. A filter for the experiment (Corex, flint, uranium, or none) was used so all the light was absorbed by the sensitizer and none by the dimer (monomer).

After irradiation the samples were analyzed by HPLC. The peak areas of the products were compared to the peak areas of authentic samples. For the cytosine system, an analytical amino-modified phase silica gel column (Microsorb-MV) was used with a MeOH:Et_2O mobile phase (2:3 until the monomer elutes, then 9:1). For the thymine system, an analytical C_{18} reversed phase column was used with a H_2O:CH_3CN (8.5:1.5) mobile phase. Products were detected by a UV detector set at 222 (**2** and **4**) and 246 (**1** and **3**).

Fluorescence Quenching. A stock solution of the fluorescent sensitizer was prepared by sonicating 1−3 mg of sensitizer in 100 mL of spectroscopic grade acetonitrile for 30 min. In general, the stock solutions had sensitizer concentrations of about 0.01 mM. Several quencher samples were prepared by sonicating various concentrations of the quencher being studied (monomers or dimers) in the stock sensitizer solution for 10 min. This resulted in several samples with quencher concentrations varying from 2 to 20 mM and constant sensitizer concentrations. The samples were each placed in a quartz cuvette and fitted with a septa lined with Teflon tape (to prevent leeching of any impurities present in the septa). Each sample was then purged for 15 min with argon and the fluorescence was measured and recorded.

Acknowledgment. We are grateful to the National Institutes of Heath for the support of this work (GM45856).

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