Interaction of Triplet Photosensitizers with Nucleotides and DNA in Aqueous Solution at Room Temperature

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Abstract: The study of triplet excited state behavior of nucleic acids and component mononucleotides is hampered by the very small yields produced by direct photolysis. We have used high energy triplet sensitizers to generate these species in high yield, thus facilitating the study of their photophysical and photochemical behavior. Acetone-sensitized triplet formation of all triplet state nucleotides allowed nucleotide triplet−triplet absorption spectra to be measured. Triplet−triplet absorption coefficients were determined using comparative actinometry. Self-quenching of the nucleotide triplet states was found to occur efficiently with rate constants, $k_{sq} > 10^7$ M^{-1} s^{-1}. The interaction of a variety of ketone triplet sensitizers with mononucleotides has been studied as a function of the relative energies of the sensitizer−nucleotide pair. In all cases, the triplet states of the sensitizers were efficiently quenched by the nucleotides, although different reaction mechanisms were observed depending on the reaction pair under study. Acetone, the sensitizer with the highest triplet energy, sensitized all triplet state nucleotides. Sensitizers with triplet energies, $E_T > 74$ kcal mol^{-1}, sensitized TMP and those with $E_T < 74$ kcal mol^{-1} did not exhibit any triplet sensitization, although an efficient quenching reaction ($k_q > 10^8$ M^{-1} s^{-1}) was observed. Where *energy* transfer did not take place, sensitizers were quenched by *electron* transfer from the purines. The quantum yield for this process was determined as 0.31 for GMP and 0.09 for AMP. In DNA, triplet energy transfer from the same sensitizers was probed by determining the relative efficiency of pyrimidine dimer formation in pBR322, an exclusively triplet-mediated reaction under sensitized conditions. Our results allow some conclusions to be drawn on triplet properties and intramolecular energy transfer in DNA. Base triplet energy levels appear to be lower in DNA than in the isolated mononucleotides. In any system where ketone triplet states are generated, electron transfer from a purine should be considered as a significant reaction pathway.

Introduction

Photochemical characterization of reaction processes in nucleic acids remains a very difficult proposition due to the formation of a variety of photoproducts, such as cyclobutylpyrimidine dimers, strand breaks,[1] 6−4 photoadducts[2,3] photohydrates, and alkali-labile sites[1] which are formed on direct excitation. In addition to the large number of possible products, each formed by a distinct reaction mechanism, the product distribution itself can be complex in nature and may be influenced by such factors as nucleic acid composition,[4] wavelength,[5] and power of exciting light through monophotonic and biphotonic excitation.[3,6−10] Studies of photoprocesses in nucleic acids and model systems are rendered difficult as the photoproduct quantum yields are also very small.[1]

Nucleic acids and constituent nucleotides are adept at dispersing their absorbed photon energy through nonradiative processes (internal conversion). This results in very low quantum yields of observable photophysical processes such as fluorescence[1,11] and intersystem crossing[11−13] and ensures a degree of self photoprotection to UV light. Much of what is known or inferred about primary photoprocesses in nucleic acids has been accrued from low temperature studies in the solid phase where the quantum yields of emission (fluorescence and phosphorescence), although still very small, are detectable.[1] This problem is further compounded at room or physiological temperatures in aqueous solution which is the medium of biological relevance.[1]

Flash photolysis studies utilizing direct excitation of nucleic acids or nucleotides in solution have demonstrated the formation of low yields of transient intermediates such as triplet states[8,14−16] and radical species.[16−20] Such studies are complicated by sensitivity problems (low signal amplitudes) and the need for

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very high laser pulse energies which can also lead to nonlinear effects in DNA systems.[6−10] Mechanistic information is difficult to obtain and many questions in DNA photochemistry remain unresolved. Even such fundamental considerations as the multiplicity of the excited state mediators of the various photoproducts (*e.g.* cyclobutane photodimers) are not conclusively known.[21,22]

We have adopted a simplifying experimental approach. By exciting a sensitizer molecule in the presence of nucleic acids and nucleotides at a wavelength where absorption by the latter is negligible, the triplet state of the sensitizer is generated in high yields and, through subsequent triplet energy transfer, similar yields of triplet states of the nucleotide acceptors are obtained. In the present study, mononucleotides were employed as simple models for the behavior of the component bases in nucleic acids. This behavior is easily followed by laser flash photolysis to yield kinetic data from which mechanistic information can be inferred. Similarly, in product studies, the formation of photoproducts which are formed solely through triplet state mediation was studied, since the excited singlet state cannot be involved under our conditions.

In the course of this sensitization work, fundamental issues such as the nature of the sensitizer−nucleotide interaction, the triplet energy levels in mononucleotides and in nucleic acids, and also triplet-mediated energy and electron transfer were addressed. Comparison of the mononucleotides with nucleic acids allows some conclusions on the effects of the macromolecular structure on the photophysical behavior of the individual bases which make up the total structure to be drawn. In addition to energy transfer sensitization, efficient electron transfer was observed between triplet state sensitizers and purine mononucleotides and DNA which operate *via* different mechanisms. These processes could be relevant to other types of DNA photodamage involving sensitizers which are located in proximity to DNA in living systems.

Experimental Section

Chemicals. Uridine 5′-monophosphate (UMP), thymidine 5′-monophosphate (TMP), cytidine 5′-monophosphate (CMP), adenosine 5′-monophosphate (AMP), guanosine 5′-monophosphate (GMP), ribose 5′-phosphate and ethidium bromide were obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Acetone (AC), acetophenone (AP), propiophenone (PR), 3-methoxyacetophenone (3MAP), 4-methoxyacetophenone (4MAP), xanthone (XAN), and benzophenone (BP) were of the highest purity available from Aldrich (Milwaukee, WI) and used without further purification. α-Tetralone (αT) and 1-indanone (IN) were obtained from Aldrich and were distilled and sublimed, respectively, prior to use. pBR322 DNA obtained from Boehringer Mannheim Biochemical (Indianapolis, IN), was 92−95% supercoiled (sc DNA) as received. Concentrations of pBR322 DNA were calculated using a molecular weight of 2.88×10^6 Da. *Micrococcus luteus* dimer-specific, UV-endonuclease was purchased from Applied Genetics (Freeport, NY). All electrophoresis supplies were from Bio-Rad (Rockville, NY).

Laser Flash Photolysis. The laser flash photolysis apparatus was essentially as previously described.[23,24] The excitation source for laser flash photolysis was a Lambda Physik EMG 103 MSC XeCl excimer laser emitting 8 ns duration pulses at 308 nm. The laser energies were attenuated to < 10 mJ pulse^{-1} by neutral density filters. Data acquisition was controlled by a Macintosh Quadra 630 computer using programs written using the LabView software package (National Instruments, TX) in conjunction with NB-GPIB and LAB-NB boards (National Instruments, TX). Fast shutters (Uniblitz) were placed in the path of both laser and lamp beams in order to minimize sample exposure to both light sources. Experiments were carried out in nitrogen-purged, unbuffered, aqueous solution (*vide infra*), unless otherwise stated.[25] Time-dependent transient absorption spectra were recorded using a point-by-point approach in which an average of ∼5 shots was obtained at successive wavelength increments across a range and the resulting spectrum constructed by extraction of absorption values at chosen time windows from the individual kinetic traces recorded at each wavelength.

Triplet−Triplet Absorption Coefficient (ϵ_T) Determinations. Triplet−triplet molar absorption coefficients (ϵ_T) for the mononucleotides were determined using the comparative actinometry method,[26] as described below. Transient absorptions resulting from irradiation of nitrogen-purged, aqueous solutions of acetone containing 2−5 mM mononucleotide were compared with a solution of benzophenone in benzene (optically matched with absorbance ∼0.5 at 308 nm), used as the reference. It should be pointed out that the maximum observed absorbance (A_{obs}) measured for each sensitized nucleotide triplet was corrected for underestimation due to incomplete (<100%) quenching of acetone triplet states by the mononucleotide by

$$A_{cor} = A_{obs}k_2/(k_2 - k_1) \quad (1)$$

and then for decay of the mononucleotide convolved with its formation[27] by

$$A_{cor}' = A_{cor}/\exp[-(\ln k_2/k_3)/((k_2/k_3) - 1)) \quad (2)$$

$$k_2 = k_1 + k_{ET}[N] \quad (3)$$

where k_1 is the rate constant for decay of acetone triplet (measured at 310 nm) in the absence of nucleotide, k_2 is the decay of the sensitizer triplet in the presence of the nucleotide (given by eq 3 where k_{ET} is the bimolecular rate constant for triplet energy transfer from acetone to the acceptor nucleotide), and k_3 is the rate constant for the decay of nucleotide under these conditions.

In the case of the purines, radical formation competed with triplet energy transfer (*vide infra*) and an additional correction was required. By determining the quantum yield of radical formation, Φ_{rad}, it is possible to determine the actual quantum yield of nucleotide triplet formation ($\Phi_T^N = 1 - \Phi_{rad}$ since Φ_T (acetone) is unity[28]), and hence the discrepancy in ϵ_T. As the lifetime of the radical is very long compared to that of the triplet state, its absorption (ΔA_{rad}) exhibits negligible decay on the timescale of triplet decay (Figure 1a, insert). Φ_{rad} can be determined by comparative actinometry (using the same benzophenone actinometer) from eq 4 where slope^{rad} and slope^{Bp} are the slopes of the energy dependences of ΔA_{rad} and the triplet absorption of the benzophenone actinometer, respectively, under optically matched conditions. Hence, Φ_{rad} was determined using absorption coefficients

$$\Phi_{rad} = (\text{slope}^{rad}/\text{slope}^{Bp})(\epsilon_T{}^{Bp}/\epsilon_T{}^{rad})\Phi_T{}^{Bp} \quad (4)$$

for the neutral radicals of 2300 M^{-1} cm^{-1} (GMP; 380 nm) and 770 M^{-1} cm^{-1} (AMP; 470 nm), estimated from the data of Candeias and Steenken,[19] a quantum yield of intersystem crossing of unity for both acetone and benzophenone, and a triplet absorption coefficient of 7640 M^{-1} cm^{-1} for benzophenone triplet in benzene at the detection wavelength of 525 nm.[29]

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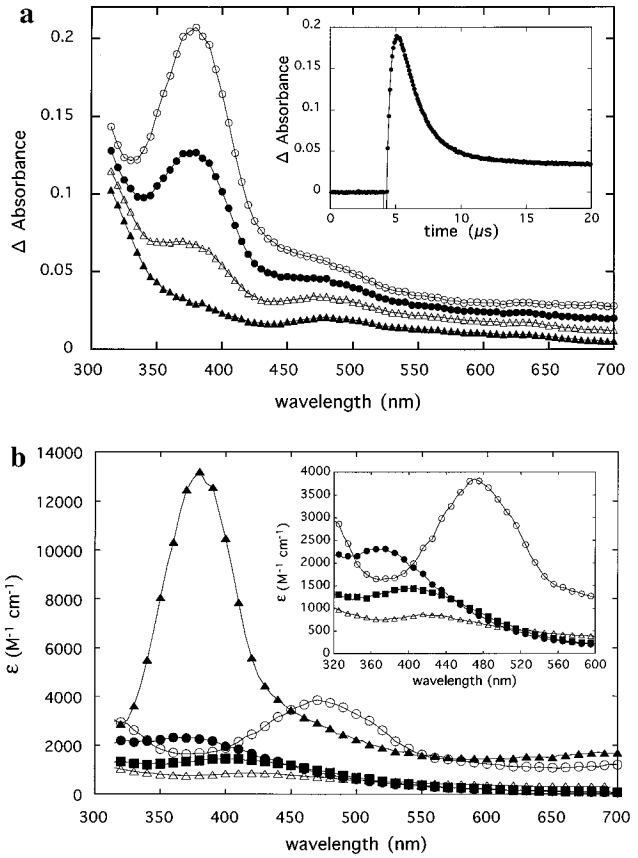

Figure 1. (a) Transient absorption spectra obtained on 308 nm excitation of a deaerated aqueous solution of acetone containing 1.7 mM GMP at delays of (○) 1.6 μs, (●) 2.8 μs, (△) 4.6 μs and (▲) 15.6 μs following the laser pulse. Inset shows corresponding transient decay at 380 nm. (b) Corrected triplet−triplet absorption spectra for (▲) GMP, (○) AMP, (△) CMP, (●) TMP, and (■) UMP.

The ϵ_T^N values were obtained from eq 5. The *corrected* triplet absorption was measured as a function of laser pulse energy to give slope^N. Total correction factors to the A_{obs} values were never greater than 25%.

$$\epsilon_T^N = \epsilon_T^{Bp}(\text{slope}^N/\text{slope}^{Bp})(\Phi_T^{Bp}/\Phi_T^N) \quad (5)$$

Product Studies. Radiation (308 nm) from a XeCl excimer laser was attenuated with microscope slides to <0.25 mJ cm^{-2} for the induction of pyrimidine dimers and <0.75 mJ cm^{-2} for induction of strand breaks and alkali labile sites. Samples were irradiated in a 1.5 mm diameter, 1 cm path length cuvette. The absorbances of the sensitizer solutions at the laser wavelength were matched to ~0.10. A variable number of pulses of constant pulse energy were delivered at 10 Hz to achieve the total doses indicated. The laser intensities used (~10^5 W cm^{-2}) were well below that required for two-photon absorption in DNA (~5×10^6 W cm^{-2}).[30] The DNA was present in the samples at a concentration of ~10^{-9} M (or 5×10^{-5} M in bases) in 1 mM Tris/HCl, 0.1 mM EDTA buffers. Samples were aerated in order to make all sensitizer triplet state lifetimes approximately equal, thus, eliminating errors due to a variation in kinetic conditions for quenching by DNA.

Assays for Cyclobutylpyrimidine Dimers.[31] Cyclobutylpyrimidine dimers were measured by dividing aliquots of each sample in two and incubating one half with *M. luteus* UV endonuclease for 30 min at 37 °C. Treated and untreated samples were then analyzed by neutral agarose gel electrophoresis. Neutral electrophoresis was performed on 1% agarose gels (Type II agarose) at 40 V for 30 min on ice using a Bio-Rad Mini-Sub Cell apparatus. Gels were stained with ethidium bromide, fluorescence was excited using a Foto-UV transilluminator (Fotodyne Inc., WI), and the gel was photographed with a Polaroid MPF camera equipped with UV and color correction filters. Gel photographs were scanned in duplicate using a Camag TLC scanner II (Camag Scientific, NC) in reflectance mode at 550 nm. The gels and the photos were analyzed in the linear response range of the film and the densitometer.[31]

To quantify the number of pyrimidine dimers, the fraction of supercoiled pBR322 DNA (F_{sc}) was calculated from the integrated areas under the peaks for supercoiled and relaxed pBR322 DNA in the densitometer trace. A factor (α) of 1.66 was used to correct for the greater fluorescence of ethidium bromide when bound to the relaxed form compared to the supercoiled form under our conditions.[32] The results were plotted according to first order kinetics, ln(F_{sc}) versus photons absorbed. The background amount of cyclobutylpyrimidine dimers present in the starting material (apparent as F_{sc} values less than unity for the unirradiated samples) were subtracted from the values for irradiated samples.

Relative quantum yield calculations were based on single exponential fits of the data, F_{sc} versus photons absorbed (x).

$$F_{SC} = e^{-bx} \quad (6)$$

The decrease in F_{sc} per pulse will vary with the extent of the irradiation, as the overall amount of sc DNA decreases. However, the relative quantum yield, Φ, for the process is directly proportional to the slope, b, under our conditions of limited conversion of sc DNA (<30%).

Results

The sensitizers chosen exhibit similar characteristics in that they all possess high quantum yields of intersystem crossing (Φ_{isc}) to form triplet states of high energy content (>69 kcal mol^{-1}).[33] The sensitizers were chosen to give a range of energy donors encompassing the expected range for the nucleotides in solution.[34] The interaction of each possible sensitizer−nucleotide combination was investigated using laser flash photolysis in unbuffered, aqueous solution. Comparative experiments were also carried out in aqueous, buffered solution (5×10^{-2} M potassium phosphate/HCl; pH = 7.5). No significant difference in triplet state properties was observed between buffered and unbuffered solutions. The results are given below.

Acetone Sensitization. As expected from the very high triplet energy of acetone,[33] this compound was capable of sensitizing the triplet states of all the nucleotides allowing the measurement of the triplet spectra of the nucleotides. A residual absorption was observed in the case of the purines, GMP and AMP, as exemplified by GMP in Figure 1a. The residual absorptions did not decay on the timescale of triplet decay and have been subtracted from the initial absorptions to give the true triplet spectra as shown in Figure 1b. Experiments indicated that triplet absorption spectra show negligible qualitative variation on going from base to nucleoside to nucleotide; monophosphate salts were used for the current study because of their good solubility in water and the fact that they represent the best model of the DNA monomer unit. Spectra have previously been published[8,15,35,36] for some bases and related compounds. However, we have obtained data for all five mononucleotides under identical experimental conditions, thus

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Table 1. Triplet−Triplet Absorption Coefficients for Nucleotides

mononucleotide	ϵ_T, M^{-1} cm^{-1} (λ/nm)	literature values
AMP	3850 (470)	−
CMP	850 (420)	400 (450)[14]
GMP	13200 (380)	−
TMP	2300 (370)	4000 (370)[15]
UMP	1450 (400)	9000 (390)[15]

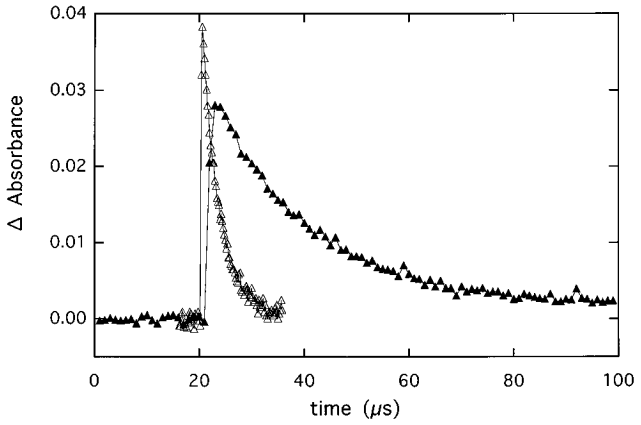

Figure 2. Decay at 370 nm of the triplet state of TMP sensitized by acetone in deaerated aqueous solution containing (▲) 1 mM and (△) 10 mM TMP.

allowing direct comparison of the respective absorptions for the first time. Furthermore, the spectra obtained in this study are well resolved due to the fact that triplet sensitization allows generation of the triplet species in yields far greater than are accessible through direct UV irradiation which is hampered by the low Φ_{isc} for these compounds in water ($\Phi_{isc} < 0.01$).[8,11,15] The spectra are shown in terms of triplet−triplet molar absorption coefficients which were determined, with appropriate corrections, as described (see Experimental Section) and are summarized in Table 1. In the case of the purines, radical formation competed with triplet energy transfer (*vide infra*). The quantum yields for this process were determined for GMP and AMP as 0.31 and 0.09, respectively.

To our knowledge, the ϵ_T values for GMP and AMP are the first to be reported. Previously determined values for the pyrimidine monophosphates are given in Table 1 for comparison. The values are in reasonable agreement with the exception of UMP for which there is 1 order of magnitude difference between the values. This is surprising as the reported triplet absorption spectrum was in qualitative agreement with that obtained in this study. The reasons for such a large discrepancy are not obvious.

In addition to spectral data, we obtained information regarding various kinetic parameters. The observed decay rate of the nucleotide triplet (N^3) absorption was dependent on the nucleotide concentration *i.e.* a self-quenching process exists.

$$N^3 \xrightarrow{k_0} N \tag{7}$$

$$N^3 + N \xrightarrow{k_{SQ}} N + N \tag{8}$$

Figure 2 illustrates this behavior for the decay of triplet TMP sensitized by acetone in the presence of 1 and 10 mM nucleotide. Such behavior was observed for all nucleotides and the self-quenching rate constants (k_{sq}) obtained from linear fits of the observed decay rate constants *vs.* nucleotide concentration are listed in Table 2. Additionally, the rate constants for the energy transfer processes were determined from the linear fits of the observed rate constants for the growths of the nucleotide triplet

Table 2. Rate Constants (k_{sq}) for Self-Quenching of Triplet Nucleotides

nucleotide	k_{sq} (10^8 M^{-1} s^{-1})	literature values
CMP	4.1	1.8[14]
AMP	3.3	3.6[42]
UMP	0.83	10[15]
GMP	0.62	−
TMP	0.27	2.0[15]

Table 3. Bimolecular Rate Constants for Sensitizer−Nucleotide Interactions ($k \times 10^{-9}$ M^{-1} s^{-1})

sensitizer	TMP	CMP	UMP	AMP	GMP
AC	1.6 (ET)[a]	2.3 (ET)	1.1 (ET)	2.6 (ET)	1.2 (ET)
IN	1.6 (ET)	1.3	1.3	1.8	3.1
PR	−	1.4	0.8	0.9	2.1
AP	1.8 (ET)	1.5	0.7	1.3	2.4
3MAP	0.01	<0.05	<0.05	<0.05	1.3
4MAP	−	<0.05	<0.05	0.05	2.1
αT	1.8 (ET)	1.6	1.0	1.1	3.2
XAN	0.16	0.01	0.03	1.5	2.3
BP	1.4	0.04	0.3	2.3	2.0

[a] ET = confirmed triplet energy transfer.

absorptions as a function of concentration. These values are shown in Table 3.

The bimolecular interactions of all possible combinations of sensitizer and nucleotide were studied in detail. The observed rate constants for triplet decay of the sensitizers (or growth of nucleotide triplet where appropriate) were plotted as a function of nucleotide concentration, allowing determination of the rate constant from the slope of the linear plot. Efficient quenching was observed in all cases but the *mechanism* of reaction was seen to vary depending on the reaction partners involved. Two types of mechanism could be identified: (i) energy transfer, as determined by the detection of nucleotide triplet absorption following sensitizer triplet decay. This behavior is aptly illustrated by the example of the acetone−TMP system in Figure 3a, where only one species, ^{3}TMP, is observed; (ii) chemical quenching: following sensitizer triplet decay residual absorptions were detected, the spectra of which were in agreement with the sensitizer ketyl radicals (*e.g.* for benzophenone−GMP in Figure 3b) and in some cases, nucleotide-derived radical species (*vide infra*), *e.g.* acetone−GMP (Figure 1a).

The reaction rate constants and types of reaction pertaining to selected sensitizer−nucleotide pairings are listed in Table 3. Various trends can be observed in the data. (a) The ability to transfer triplet energy to the base is a function of the sensitizer triplet energy, *i.e.*, (1) acetone sensitized the triplet states of all nucleotides; (2) sensitizers with triplet energies > 73 kcal mol^{-1} (AP, PR, IN[33]) sensitized TMP triplet; (3) sensitizers with triplet energies <73 kcal mol^{-1} (αT, 3MAP, 4MAP, BP[33]) did not sensitize any nucleotide triplet states. Xanthone did not sensitize formation of TMP triplet. In this respect, it exhibited behavior which is consistent with it having a triplet energy nearer to a literature value of 70.9 kcal mol^{-1} [37] than another published value of 74 kcal mol^{-1} [33] (see Discussion). These results allow us to construct the relative energy level diagram shown in Figure 4. Here, the triplet energy levels indicated in the current study are compared to those obtained[38] from blue edge phosphorescence measurements at 77 K. The data contained herein do not permit any distinction between the mononucleotide triplet energies other than thymine clearly being the lowest in energy. However, preliminary data (not shown) obtained with solutions

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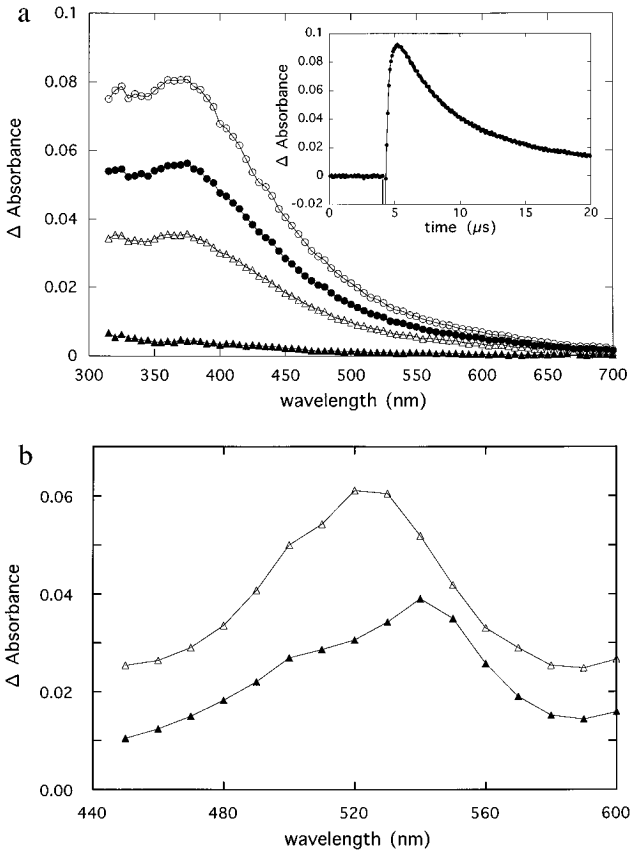

Figure 3. (a) Transient absorption spectra obtained on 308 nm excitation of a deaerated aqueous solution of acetone containing 1.7 mM TMP at delays of (○) 1.4 μs, (●) 2.8 μs, (△) 4.6 μs, and (▲) 15.6 μs following the laser pulse. Inset shows corresponding transient decay at 370 nm. (b) Transient absorption spectra obtained on 308 nm excitation of a deaerated aqueous solution of benzophenone containing 2.0 mM GMP at delays of (△) 200 ns and (▲) 3.5 μs following the laser pulse.

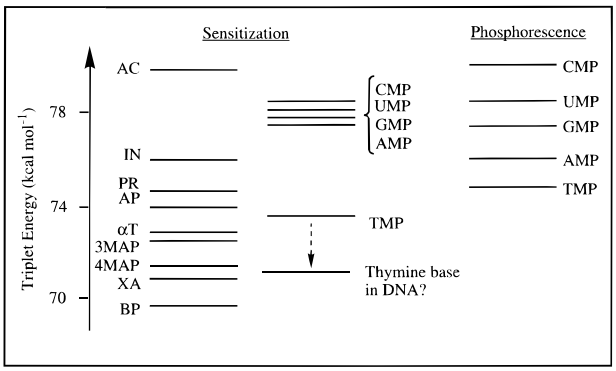

Figure 4. Schematic diagram showing relative triplet energies for photosensitizers and mononucleotides obtained in the current study compared to values obtained from low temperature phosphorescence spectra.[38]

of mixed pairs of mononucleotides suggests the order given in Figure 4 which is clearly in agreement with the phosphorescence data. Work is in progress aimed at confirming this order and determining the absolute energies.[39] (b) For sensitizer−nucleotide pairings where energy transfer is "uphill", the nature of the interaction is dependent on the nucleotide, with purines being particularly susceptible to chemical reaction, *ultimately*

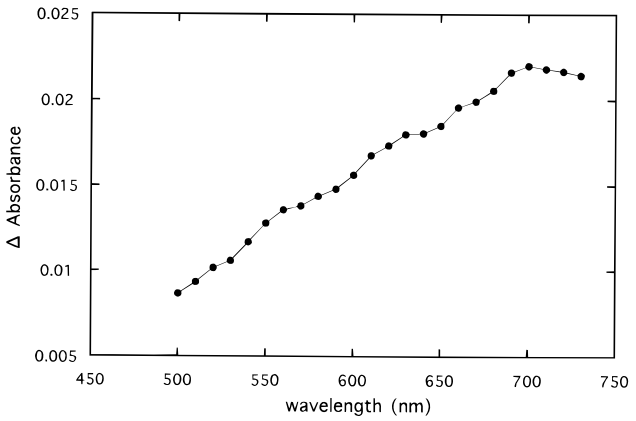

Figure 5. Transient absorption spectra obtained on 308 nm excitation of a deaerated solution of benzophenone in DMSO containing 5.0 mM adenosine.

resulting in hydrogen abstraction by the sensitizer to form the corresponding ketyl radical (*vide infra*).

Further work was carried out to investigate the chemical quenching reaction in more detail. Considering the lack of an obviously labile hydrogen atom on the nucleotide structure, the formation of a sensitizer ketyl radical would not be expected to occur by a direct hydrogen atom abstraction mechanism. We also considered a possible involvement of the sugar moiety as a hydrogen donor, but experiments involving sensitizer excitation in the presence of only D-ribose did not result in ketyl radical formation, and no quenching of the sensitizer triplet (either acetone or acetophenone) by the sugar was observed. In order to further probe the reaction mechanism, similar experiments were carried out in the nonprotic solvent, dimethyl sulfoxide (DMSO). Due to solubility problems, the nucleosides rather than nucleotides were used in the presence of benzophenone (as sensitizer) in DMSO. Figure 5 shows the transient absorption spectrum observed following excitation of benzophenone in the presence of adenosine (5.0 mM). The maximum at around 720 nm is in good agreement[36,37] with the spectrum of the benzophenone radical anion, $BP^{\bullet -}$. Since previous work involving $BP^{\bullet -}$ had been carried out in solvents such as methanol or acetonitrile,[40,41] we generated this species unequivocally by quenching triplet BP with the electron donor diazabicyclooctane (DABCO) in DMSO and showed that $BP^{\bullet -}$ has λ_{max} at 720 nm in DMSO. The spectra obtained in the two systems were in good agreement. Thus, we believe the initial step in the chemical quenching mechanism to be a one-electron transfer from the nucleotide to the sensitizer. This behavior is particularly evident for the purine derivatives.

Triplet-Sensitized Damage in DNA. For these experiments optically-matched sensitizer−DNA solutions were irradiated at 308 nm as described. Solutions were air-saturated to bring all triplet lifetimes to a similar value such that kinetic corrections for variation in triplet lifetime were unnecessary. The solutions were then assayed for pyrimidine dimer formation as a function of cumulative light dose, *i.e.* number of laser pulses. Figure 6 shows the increase in dimer formation with dose. The slope of these plots can be taken as a direct measure of the efficiency of dimer formation since all sensitizers exhibit triplet quantum yields approaching unity. The plots display a variation in sensitized dimer efficiency with sensitizer, *e.g.*, benzophenone (BP) and xanthone (XAN) show negligible difference from the control solution (containing no sensitizer) which reports the

(39) Experiments have been carried out using acetone sensitization of mixed mononucleotide solutions. Knowledge of the absorption coefficients in combination with the spectral characteristics observed for these systems allow the direction of triplet energy transfer to be elucidated.

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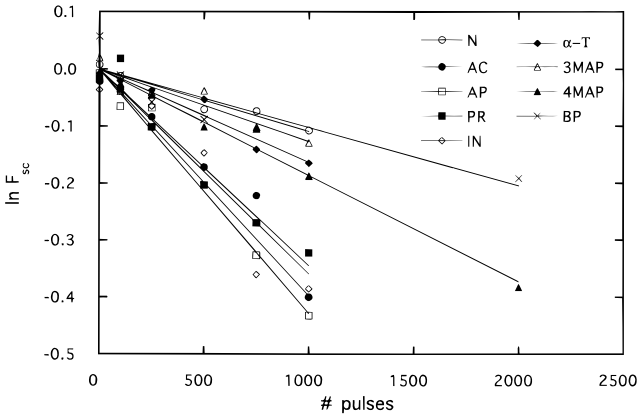

Figure 6. Plot of the dependence of cyclobutylpyrimidine dimer formation as a function of accumulated dose (see Experimental Section) for each sensitizer in the presence of 10^{-9} M pBR322 DNA in aerated aqueous solution.

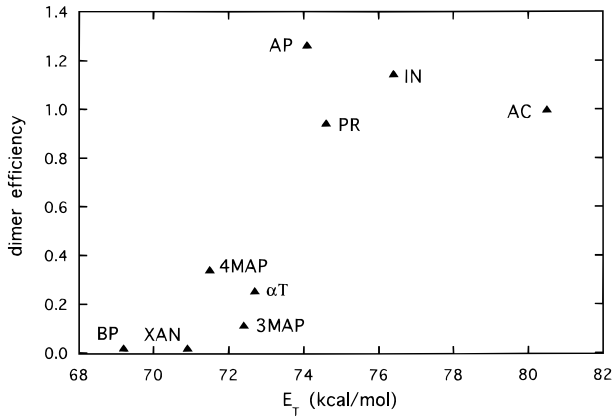

Figure 7. Plot of the relative cyclobutylpyrimidine dimer sensitizing efficiency as a function of the triplet energy of the photosensitizer.

background dimer concentration in the sample. The data is better presented by subtraction of the slope of the control from all sensitizer solutions and then plotting the relative values as a function of sensitizer triplet energy, as shown in Figure 7. This figure illustrates the differentiation of the sensitizers into three groups with regard to their pyrimidine dimer sensitization efficiency. Group 1: BP and XAN. These compounds show negligible sensitization of pyrimidine dimers. Group 2: 3MAP, 4MAP, and αT. These compounds exhibit some sensitization but only around 25% of that shown by group 3 sensitizers. Group 3: AC, AP, IN, and PR exhibit the maximum efficiency and are equally efficient within experimental error.

In our sensitizer−nucleotide flash photolysis work, efficient chemical quenching of sensitizer triplet states by nucleotides was observed *even* when energy transfer was not observed. This finding prompted us to investigate other possible mechanisms of photodamage to DNA (in addition to dimer formation) as a consequence of these other reactions. We were also concerned that even when energy transfer was observed we may also have contributions from other pathways to the quenching of the sensitizer triplet states. In most cases, significant strand breaks and alkali labile sites were observed in addition to dimer formation showing that alternative reaction mechanisms were in operation.

Discussion

The triplet sensitization approach generated the excited triplet nucleotides in sufficient yields that spectral and kinetic information was easily obtained. Spectra in the literature have been published for UMP and TMP under conditions where the nucleotide triplet state was generated by direct excitation at 266 nm, where the quantum yields in aqueous solution are reported to be ≈0.01.[15] For comparison, the quantum yields of generation of these species under our sensitized conditions were around two orders of magnitude greater, thus facilitating their study. The triplet−triplet absorption spectra shown in Figure 1b display excellent resolution and are in qualitative agreement with previously published spectra. Having obtained the current spectra under identical experimental conditions, we are in a position to make direct comparison of all five nucleotides. The energy transfer approach used in the determination of triplet−triplet absorption coefficients gives a greater accuracy, even taking into account the corrections applied, due to the higher signal sensitivity and the fact that much lower laser pulse energies were employed than in the direct excitation studies. This ensures that the energy dependencies for triplet absorption of both nucleotide and the reference, benzophenone, were in the linear range.

Kinetic studies indicated that the lifetimes of the sensitized triplet nucleotides were dependent on mononucleotide concentration, indicative of a self-quenching process. The self-quenching rate constants (k_{sq}) vary over one order of magnitude depending on the nucleotide. Good agreement with recent literature values is seen for AMP[42] and CMP[14] whereas previously published values for TMP and UMP[15] differ significantly. There was no apparent dependence on the type of base, *i.e.* purine or pyrimidine. Self-quenching of the triplet states of thymine and uracil were previously observed in direct excitation studies and were rationalized as being due to the well-known cyclobutylpyrimidine dimerization reaction.[8] Clearly this reaction cannot be applicable to the other bases where structural differences prevent this type of dimerization reaction. No new transient absorptions were evident following self-quenching for any of the mononucleotides. In the absence of product studies and spectral evidence we propose a physical mechanism of this quenching reaction. Base−base quenching reactions may be important in DNA in short base sequences where energy transfer cannot occur, and harmless dissipation of energy from absorbed photons takes place.

In our studies of the sensitizer−nucleotide systems, we observed very efficient quenching of sensitizers by nucleotides, even when triplet energy transfer is not energetically feasible: (a) where chemical reaction has occurred as determined by transient absorption spectral changes (see Figure 3b) and (b) where no chemical mechanism is visible. Mechanism (a) is very interesting as we see apparent formation of sensitizer ketyl radicals as a result of the quenching process. Such radicals are generally formed by a well-understood, direct hydrogen abstraction process, involving a suitable hydrogen donor site. Also, for sensitizers such as acetone which possess little absorption in the spectral range studied, residual absorption due to nucleotide-derived radicals were observed, these being especially evident in the case of purines. The lack of a readily apparent site for a labile hydrogen atom on the nucleic acid bases and the lack of reactivity of the sugar moiety as a hydrogen donor preclude a direct hydrogen abstraction reaction in the ketyl radical formation. Results obtained using sensitizer−nucleoside combinations in the aprotic solvent DMSO, where direct detection of the radical anion of benzophenone has been demonstrated, suggest a more complex mechanism where more than one step is involved. A likely explanation is that of electron transfer between the sensitizer and nucleotide resulting in the

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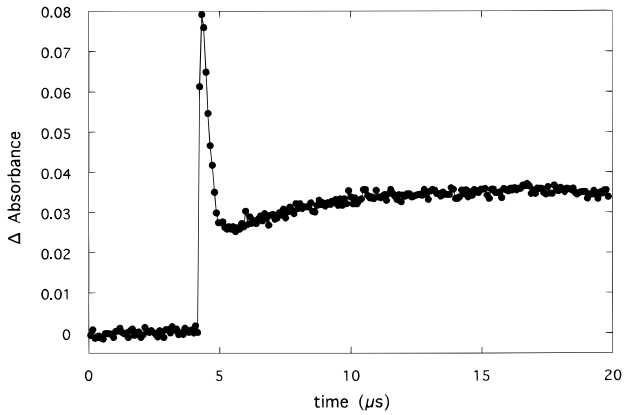

Figure 8. Transient absorption profile at 330 nm showing enhanced decay of GMP triplet state (0.5 mM), followed by growth of GMP neutral radical absorption (due to deprotonation of the GMP radical cation), in aqueous solution which has been bubbled with oxygen.

initial formation of a radical ion pair as shown in eq 9. The

$$S^3 + Pur \rightarrow S^{\bullet -} + Pur^{\bullet +} \quad (9)$$

$$S^{\bullet -} + H^+ \rightarrow SH^{\bullet} \quad (10)$$

$$Pur^{\bullet +} \rightarrow Pur(-H)^{\bullet} + H^+ \quad (11)$$

sensitizer radical anion can be converted into the ketyl radical by protonation (eq 10). The fate of the radical cation of the nucleotide may be explained in the case of purines, where strong residual absorptions are observed, by conversion to the free radical by deprotonation of the radical cation under these conditions (eq 11)[19,43] It was possible to observe the latter process by bubbling a solution of GMP with sufficient oxygen to enhance the decay of the GMP triplet without completely quenching the acetone triplet precursor. Under such conditions, we see the growth at 350 nm (Figure 8) of the neutral radical with a first-order rate constant, $k = 2.9 \times 10^5\ s^{-1}$, in good agreement with the value of $3.5 \times 10^5\ s^{-1}$ obtained by Candeias and Steenken.[19] During the preparation of this manuscript, a similar scheme of electron transfer has been proposed for AMP by Li *et al*.[42]

The radical spectra are in good agreement with those obtained following pulse radiolysis under exclusive one-electron oxidation conditions.[19,43] Furthermore, 3MAP and 4MAP quenching by AMP and especially GMP results in a residual absorption spectrum in the UV which agrees qualitatively with those obtained in previous studies *via* either direct excitation[17,18,44] or photosensitization.[42]

Reaction 10 is believed to be a rapid process since no transient absorption of the benzophenone radical anion ($BP^{\bullet -}$), detectable with maximum absorption at 720 nm, was observed in aqueous solution. This mechanism is supported by the relative oxidation potentials for the different bases where the purines are more easily oxidized,[34,43] as is the case here. Furthermore, it can be seen that the k_q values (Table 3) for systems in which no energy transfer can occur are much higher for the purines.

The quantum yields for purine radical formation have been determined as 0.31 for GMP and 0.09 for AMP. From these experiments and their mechanistic implications, we expect electron transfer from purine bases to excited triplet ketones to be a significant reaction in *any* system in which ketones are used as photosensitizers. Epe *et al*.[45] used acetone and acetophenone as photosensitizers to induce modifications in DNA. Comparison with direct excitation of DNA, in photoproduct studies, led to electron transfer being postulated as contributing to the base modifications in the sensitized system. Our results confirm this theory. Clearly electron transfer must be borne in mind when studying such systems.

Our rationale of using both sensitizers and nucleotides of varying triplet energies, and studying the kinetics and nature of the reaction mechanism, allows us to construct the relative energy diagram shown in Figure 4. Mononucleotides are good model systems for the respective base residues in nucleic acids. However, the simple systems lack the important base pair interstrand hydrogen bonding and intrastrand neighbor interactions (*e.g*., energy transfer along the strand) that are present in the macromolecules. By studying both systems we can draw some conclusions as to the effects of the latter.

Intramolecular triplet−triplet energy transfer was demonstrated earlier by studies of emission from nucleic acids in low temperature glasses. This was concluded initially from the observation of emission typical of the thymine residue, suggesting that energy was transferred along the strand and became localized at thymine residues.[46,47] Thymine, having the lowest triplet energy of the bases, acted as a sink for the absorbed energy, *i.e*., excitation energy always arrives at thymine irrespective of which base is actually responsible for the absorption of the photon. Our work using a range of triplet energy sensitizers in solution also confirms that TMP has the lowest triplet state energy of all bases. The efficiency of triplet energy transfer in nucleic acids at low temperature has been a subject of discussion in the literature. Estimates of energy transfer over a few bases[48] to tens of bases[49,50] have been given, but such higher values are less likely at physiological temperatures. At low temperature, little thermal activation energy is available to allow the "uphill" back energy transfers to compete. Therefore, although the base triplet energies vary over only a few kcal mol^{-1} the energy transfer will be quantitatively in the "downhill" direction. In solution at higher temperatures, the thermal energy available may allow the "uphill" reactions to compete to some extent, thus reducing the efficiency and range of the energy transfer process.[12]

We have been able to garner some ideas as to the nature of the energy transfer processes under more biologically relevant solution conditions. From our nucleotide experiments (Figure 4), we can see that acetone is the only sensitizer capable of sensitizing all bases in the nucleic acid, whereas others such as acetophenone can only sensitize thymine residues. Under conditions of quantitative internal energy transfer to thymine, we would expect a factor of four difference in the efficiency of sensitizing thymine triplet states using acetone and acetophenone. This can be studied using cyclobutylpyrimidine dimer formation, a photoproduct known to be accessible through the triplet state, as a probe for activated thymine residues in DNA. Figure 7 shows the distribution of relative efficiency of dimer induction as a function of the triplet energy of the sensitizer. Sensitizers with triplet energies less than around 72 kcal mol^{-1}

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(*i.e.*, BP) show negligible induction of dimers as compared to the background level. The group of sensitizers lying between 72 and 74 kcal mol^{-1} sensitize dimer formation but only in the range of 25% of the efficiency exhibited by the group of sensitizers with triplet energies >74 kcal mol^{-1}. The only exception to this behavior could be xanthone which did not sensitize dimer formation. Literature values for xanthone triplet energy vary between 70.9 kcal mol^{-1} [37] and 74 kcal mol^{-1}.[33] Xanthone photochemistry is known to be highly solvent-dependent[51] and in the current study, xanthone consistently behaved as if its triplet energy is nearer to the lower value (which was determined in alcohol−ether at 77 K). Additional evidence comes from the order of magnitude difference between the rate constants for energy transfer to TMP from acetophenone and xanthone (Table 3) despite a reported[33] triplet energy of 74 kcal mol^{-1} for acetophenone. If these rate constants are substituted into the Sandros equation,[52] xanthone triplet energy is predicted to be less than 73 kcal mol^{-1}.

An intriguing observation is that no difference in efficiency is observed between acetone and the lower energy sensitizers, acetophenone, propiophenone, and indanone. From our flash photolysis work we would expect a factor of four increase in efficiency of acetone over the others if intramolecular energy transfer was an efficient process under these conditions. There would appear to be two possible explanations for this behavior: (1) Energy transfer is inefficient. Only directly excited thymine results in dimer generation. (2) Energy transfer is efficient. However, the triplet energies of all bases are lowered with respect to the mononucleotides such that the sensitizers with E_T > 74 kcal mol^{-1} are in fact capable of transferring triplet energy to other bases in addition to thymine and hence, no difference in efficiency exists between this group and acetone.

Some experimental evidence exists to support the latter possibility. In DNA quenching of acetone, acetophenone, and propiophenone, the only observable transient absorption spectrum is similar to that of TMP, suggesting that the energy does become localized on thymine. If energy transfer were not occurring efficiently then we would expect to see a composite spectrum of all four base triplets approximately equally weighted. The large variation in triplet absorption spectra, particularly for the purines in the visible spectrum, should facilitate the detection of these species, if formed. Additionally, there is the group of lower energy sensitizers which sensitize dimer formation to a level of ∼25% of that of the higher group. These sensitizers clearly differ from the background level exhibited in the absence of sensitizer or in the presence of the slightly lower energy benzophenone and xanthone. A possible explanation is that these sensitizers are sufficiently high in energy to sensitize formation of thymine triplet but not any of the other bases, and hence, no triplet energy transfer is possible. Such an explanation would account for the factor of four in efficiency seen between group 1 and group 2 sensitizers (*vide supra*). However, it must also be borne in mind that the lowering of singlet excited state energy, which occurs on going from mononucleotide to polynucleotide to double stranded DNA,[53,54] will be accompanied by a lowering of the triplet state energy, the extent of which is not yet known.

Conclusions

This work has provided some valuable insights into the behavior of individual base triplet states in solution. The study of these simple systems is the first step toward the long-term goal of a full understanding of triplet state interactions in DNA and the consequences for DNA photochemistry. The information herein provides a basis for the rationalization of successively more complex systems currently being studied in our laboratory, *viz.* dinucleotides and oligonucleotides. Correlation of these systems with the mononucleotides, will reveal how energy is distributed between bases in a DNA strand as a function of base sequence. Given that information, it may be possible to predict successfully the photoproduct distribution as a function of base sequence.

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