DNA-mediated electron transfer from a modified base to ethidium: π-stacking as a modulator of reactivity

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Background: The DNA double helix is composed of an array of aromatic heterocyclic base pairs and, as a molecular π-stack, represents a novel system for studying long-range electron transfer. Because many base damage and repair processes result from electron-transfer reactions, the ability of DNA to mediate charge transport holds important biological implications. Seemingly contradictory conclusions have been drawn about electron transfer in DNA from the many different studies that have been carried out. These studies must be reconciled so that this phenomenon can be understood both at a fundamental level and in the context of biological systems.

Results: The photoinduced oxidation of a modified base, 7-deazaguanine, has been examined as a function of distance, sequence, and base stacking in DNA duplexes covalently modified with ethidium. Over ethidium/deazaguanine separations of 6–27 Å, the photooxidation reaction proceeded on a subnanosecond time scale, and the quenching yield exhibited a shallow distance dependence. The efficiency of the reaction was highly sensitive to small changes in base composition. Moreover, the overall distance-dependence of the reaction is sensitive to sequence, despite the constancy of photoexcited ethidium as acceptor.

Conclusions: The remarkable efficiency of deazaguanine photooxidation by intercalated ethidium over long distances provides new evidence for fast electron-transfer pathways through DNA. By varying sequence as well as reactant separation, this work provides the first experimental demonstration of the importance of reactant stacking in the modulation of long-range DNA-mediated electron transfer.

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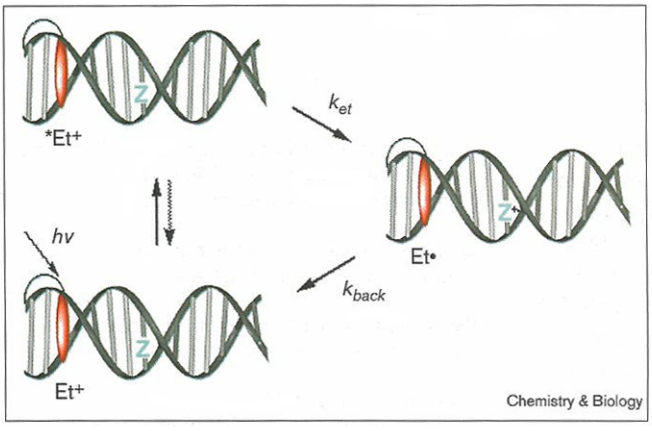
Introduction

The array of aromatic heterocycles within the DNA double helix is uniquely suited for the study of electron transfer mediated by an extended and well-defined π-stack. Reports of redox-induced base damage [1–8] and repair [9,10] highlight the importance of charge migration within DNA, and underscore the potential significance of DNA-mediated electron transfer in biology. Furthermore, radical migration and damage to DNA could have critical implications with respect to carcinogenesis and mutagenesis. It is therefore of great interest to elucidate the role of the DNA helix both as a bridge and as a reactant in electron-transfer processes.

Charge transfer through DNA has been the subject of diverse biophysical and biochemical studies [1–36]. Our work has focused on photoinduced electron-transfer reactions utilizing molecules intercalated into DNA, and has provided many examples of ultrafast, long-range charge transport through the base stack [1–4,9,11–18]. Most recently, we have investigated the distance dependence of photoinduced electron transfer through DNA using duplexes covalently modified with ethidium, an organic intercalator, and $Rh(phi)_2bpy^{3+}$ [17]. Over donor–acceptor distances spanning 17–35 Å, the ethidium excited state undergoes oxidative quenching on the subnanosecond time scale, indicating that electron transfer is remarkably efficient through the DNA double helix.

In contrast, other studies of photoinduced electron transfer in DNA employing nonintercalated or partially intercalated reactants have produced a wide range of results [21–26]. For a DNA duplex modified with two metal complexes coordinated to functionalized sugars, slower electron-transfer kinetics comparable to those observed in proteins were reported [23]. In a DNA hairpin containing a bridging stilbene unit, distance-dependent electron-transfer kinetics between photoexcited stilbene and guanine were measured and a value for β (the decay of electronic coupling with distance) [37] was calculated as 0.4–0.6 $Å^{-1}$ [24,25]. Although this value for β reflects more efficient electron transfer in DNA compared to σ-bonded systems ($\beta \sim 1\ Å^{-1}$) [38], it suggests a greater sensitivity to distance than implied by the fully

Figure 1



Schematic diagram for proposed photooxidation of deazaguanine by intercalated ethidium. Et^+, ethidium; $*Et^+$, excited-state ethidium; Z, 7-deazaguanine.

intercalated systems. Furthermore, recent studies of electron transfer between acridine covalently bound to the DNA backbone and guanine report a β value even greater than that observed in σ-bonded systems ($\beta = 1.4$ Å^{-1}) [26]. Although these systems have sought to gain information about electron transport through the same medium, it is remarkable that such different results have been obtained. Clearly, from the very broad range of experimental results, it is evident that other parameters besides distance must also affect electron transfer through the DNA helix.

To probe further the dynamics and distance dependence of electron transfer through DNA and to begin formulating a consistent picture of the double helix as a medium for charge transport, we have investigated the efficiency of the photooxidation of a novel base analogue, 7-deazaguanine (dz-G, Z), by ethidium (Et; Figure 1) as a function of donor–acceptor distance, sequence and base stacking. We have observed extraordinarily fast ($k_{et} \geq 10^{10}$ s^{-1}) electron transfer over a large range of ethidium/7-deazaguanine separations (14–30 Å). Importantly, the distance dependence of the photooxidation reaction is sensitive to sequence and also appears to be modulated by base–base interactions. The results of this study provide the first experimental demonstration that the stacking of reactants within the double helix modulates efficient, long-range electron transfer through DNA, a factor overlooked in many previous studies of this phenomenon.

Table 1

Reduction potentials for *Et, dGTP and dz-dGTP.

	Potential versus NHE*
$Et^{*/0}$	+1.2 V†
$dGTP^{+/0}$	+1.3 V‡
$dz\text{-}dGTP^{+/0}$	+1.0 V‡

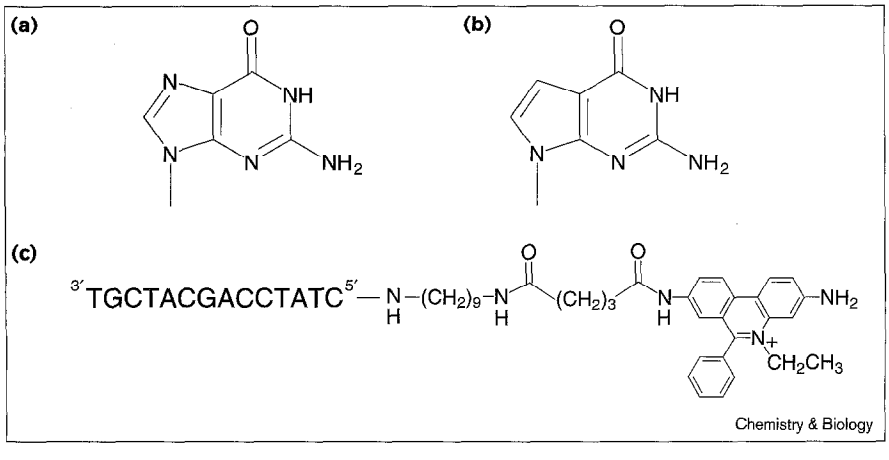
*Potentials were obtained in 100mM phosphate buffer, pH7 with a normal three-electrode configuration consisting of a glassy carbon working electrode, saturated calomel reference electrode, and platinum auxiliary electrode. †Calculated using expression $E^{o(*/0)} = E_{oo} - E^{o(+/0)}$. ‡Irreversible peak potential.

Results

Redox properties of 7-deazaguanine

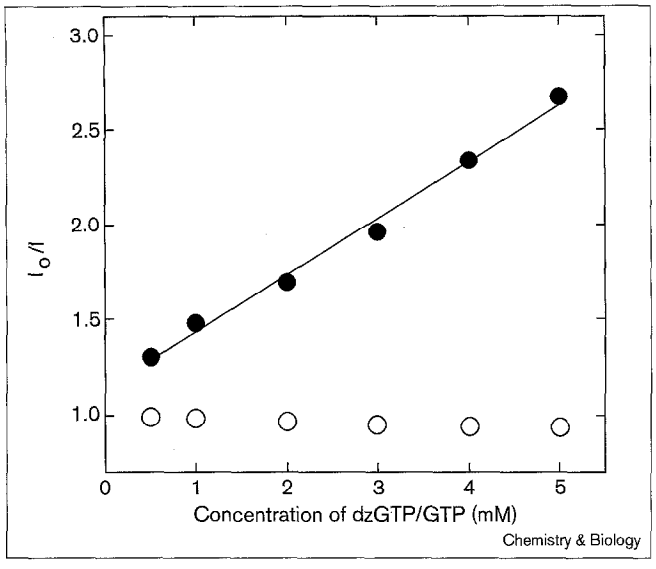
The base analogue 7-deazaguanine differs in structure from the natural guanine base by only one atom (Figure 2), a small change in structure that shifts the oxidation potential from ~ +1.3 V (versus normal hydrogen electrode [NHE]) for guanine to ~ +1.0 V for 7-deazaguanine (Table 1). The lowered potential for the modified base is apparent in the reactivity of the nucleotides

Figure 2



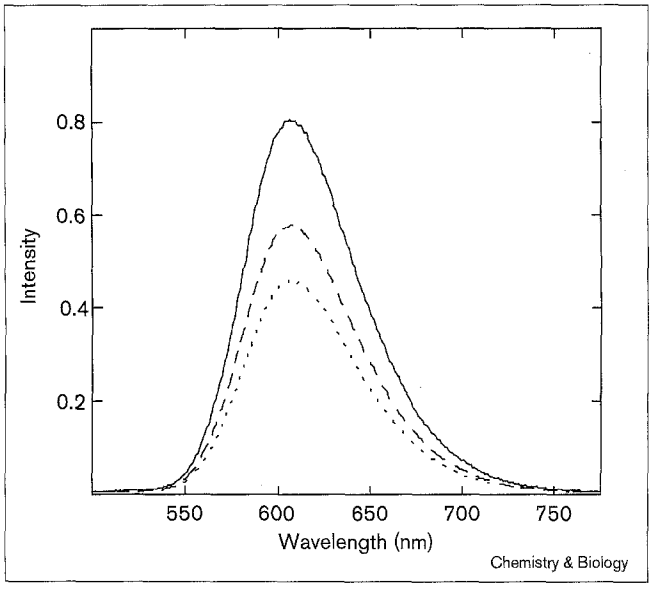
Structures of **(a)** guanine, **(b)** 7-deazaguanine, and **(c)** Et′-DNA, an ethidium-modified oligonucleotide.

Figure 3



Steady-state fluorescence quenching of 10 μM ethidium by dGTP (○) and dz-dGTP (●) in 5 mM phosphate, 50 mM NaCl, pH 7. I_0, initial intensity; I, intensity with added quencher.

Figure 4



Steady-state emission spectra for ethidium (5 μM) noncovalently bound to 5′ACACTGCTGACGGTA (solid), 5′ACACTGCTZACGGTA (long dash), and 5′ACACTZCTZACGGTA (short dash). Sequences were hybridized with appropriate complements and samples contained 5 μM duplex DNA in 5 mM phosphate, 50 mM NaCl, pH 7.

towards the fluorescent singlet excited state of ethidium in aqueous solution (Figure 3). No change in the ethidium quantum yield is observed in the presence of dGTP, whereas addition of dz-dGTP results in efficient fluorescence quenching ($K_{SV} = 3 \times 10^2\ M^{-1}$). Neither the absorption of dGTP nor of dz-dGTP shows any spectral overlap with ethidium emission. Because these molecules differ only in oxidation potential, the mechanism of the fluorescence quenching most likely proceeds by photoinduced oxidation of 7-deazaguanine by ethidium.

Photooxidation of 7-deazaguanine in DNA by noncovalently bound ethidium

When incorporated into DNA duplexes, 7-deazaguanine is also oxidized by the ethidium excited state. Significant decreases in the fluorescence quantum yield are observed for ethidium noncovalently bound to DNA duplexes containing 7-deazaguanine relative to those containing guanine (Figure 4). No changes in the shape of the spectra are evident, but there are changes in the intensity of the spectra. Parallel spectral results were obtained using covalently bound ethidium (see below). A similar decrease in ethidium fluorescence in the presence of 7-deazaguanine was found in studies in which the modified base was incorporated into large DNA polymers [39]. In that case, the decreased fluorescence of deazguanine-containing sequences, relative to natural sequences, was not recognized as electron-transfer quenching, but was instead suggested to arise from an alteration in the ethidium electronic structure upon binding at sites adjacent to 7-deazaguanine [39].

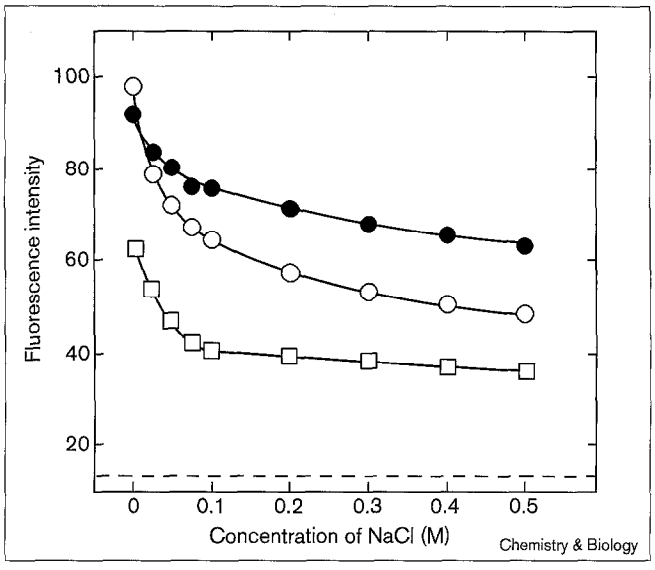
Photooxidation of deazaguanine in ethidium-modified duplexes

The redox properties of 7-deazaguanine make this base analogue a suitable reactant for studying long-range electron transfer in ethidium-modified duplexes of heterogeneous sequence. Because the excited state of ethidium is not sufficiently oxidizing to react with any of the other DNA bases, electron transfer between an intercalator and a localized component of the base stack can be evaluated in the absence of competing reactions. Moreover, because duplexes containing 7-deazaguanine differ from those containing guanine by only one out of over 800 atoms, quenching effects can cleanly be attributed to redox processes. Thus, the photooxidation reaction has been studied using fluorescence-quenching measurements as a function of distance, flanking sequence and base pairing. Because the oxidation potential for 7-deazaguanine differs from guanine by only ~300 mV and quenching is only observed with 7-deazaguanine, it should be noted that the driving force for reaction is particularly small.

Duplex characterization and linker-length optimization

In order to investigate the long-range photooxidation of 7-deazaguanine by ethidium as a function of distance it was necessary to construct a well-defined assembly. Duplexes containing covalently linked ethidium exhibit the structural properties expected for small cylindrical helices [17]. Steady-state polarization studies of ethidium-modified

Figure 5



Effect of linker and ionic strength on fluorescence intensity of ethidium covalently bound to the duplex (Et-NH-CO-$(CH_2)_3$-CO-NH-$(CH_2)_n$-NH-CO-^{5}'CTACCAGACATCGT (n=2 (□), n = 6 (○), and n = 9 (●)). The dotted line represents fluorescence for free *N*-8-glycyl ethidium.

duplexes have revealed that the covalently attached fluorophore is bound rigidly within the site of intercalation; these studies indicated little motion of the intercalator independent of the overall duplex dynamics [17].

It has been noted in many of our studies of DNA-mediated electron transfer that the integrity of intercalative interactions appears to modulate reaction efficiencies and kinetics [11–15]. In choosing a linkage between ethidium and the DNA duplex, a tether that afforded the strongest intercalative binding mode for the intercalator was sought. Therefore, ethidium–DNA linkers of differing lengths (Et-NH-CO-$(CH_2)_3$-CO-NH-$(CH_2)_n$-NH-CO-^{5}'CTACCAGACATCGT (n = 2 (C2), 6 (C6), 9 (C9)) were investigated.

Consideration of the three-dimensional structure of DNA duplexes containing tethered ethidium using CPK models revealed that only a C9 linker permitted unconstrained ethidium intercalation. These models assume that the DNA duplex remains intact and is not denatured, which is a reasonable assumption given that the covalent attachment of ethidium increases the melting temperature of the duplex by > 2°C. From these models, the preferred intercalation site for ethidium tethered either with a C9 or C6 linker was identified as the second base step; it appeared that the C6 linker would encounter greater strain in this conformation, however. The models also revealed that ethidium attached by the C2 linker was substantially constrained and could only partially intercalate at the first base step.

The fluorescence of ethidium was investigated in DNA duplexes featuring the different linkages (Figure 5). Ethidium-modified duplexes containing the C9 and C6 linkers gave the highest overall fluorescence intensities, indicating efficient protection from solvent through tight binding of the fluorophore within the hydrophobic core of the DNA helix. Ethidium tethered via the C2 linker showed significantly less fluorescence, indicating that this linker must sufficiently constrain the intercalator to limit the extent of protection from solvent. These observations are fully consistent with the predictions from the CPK models described above.

The binding mode(s) of the tethered intercalator can also be probed by examining fluorescence as a function of ionic strength (Figure 5): electrostatic associations that would stabilize a groove-bound form of ethidium are expected only at very low ionic strengths and intercalative binding is favored at higher ionic strengths [40–43]. For the duplex containing the C9 linker, only small changes in the ethidium quantum yield are evident with increasing salt (Figure 5). Similar decreases in fluorescence with increasing ionic strength are commonly observed for intercalated ethidium [41]. The binding of ethidium to the duplex containing the C9 linker, therefore, appears to reflect only a small contribution from electrostatic interactions.

Ethidium tethered by the C6 linker exhibits a much greater sensitivity to ionic strength compared with the C9 linker, consistent with intercalation being more constrained with the C6 tether and a greater electrostatic component contributing to the binding of ethidium in the context of the C6 linker. With the C2 linker, significant decreases in the already diminished quantum yield are also observed. Hence, from this line of experimentation and previous studies of the photophysical properties of duplexes modified with the linked intercalator [17], it appears that the C9 linker allows the most favorable intercalative interactions to occur between ethidium and the derivatized duplex.

The C9 linkage was, therefore, the linker chosen to probe the long-range photooxidation of 7-deazaguanine. Establishing that the ethidium tethered by the long linker is intercalated is an important issue, for if the linked fluorophore were groove-bound in a fully extended conformation, it could directly contact the first five base pairs of the duplex (~17 Å), albeit with substantial strain. The photooxidation of 7-deazaguanine was studied with the quencher located from four (14 Å) to ten bases (34 Å) from the end of the duplex. Thus, if ethidium were bound in the groove, the fluorophore would be provided direct access to the quencher only in the first duplexes of the series. But, in fact, the C9 linkage allows the tethered ethidium to intercalate very strongly with the DNA helix, probably at the first or second base steps, and, therefore,

direct contact with 7-deazaguanine is precluded in all of the assemblies we prepared. Instead, with intercalated ethidium, photoinduced reactions must occur over ethidium/7-deazaguanine separations of 6–27 Å.

Distance dependence

The ability of the DNA base stack to facilitate the oxidation of 7-deazaguanine over extended distances was investigated in 14 base-pair duplexes covalently modified with *N*-8-glycyl ethidium (Et′). By systematically replacing a guanine residue with a 7-deazaguanine residue along a DNA duplex, fluorescence-quenching experiments can be used to investigate the efficiency and time scale of the base-oxidation reaction as a function of donor/acceptor separation through the base stack.

Steady-state fluorescence measurements were employed to determine the yield of ethidium excited state quenching by the photooxidation of 7-deazaguanine in each of the duplexes shown in Table 2. In these 14 base-pair DNA duplexes containing tethered ethidium, the position of 7-deazaguanine was systematically moved across the duplex to provide a range of donor/acceptor distances. As described earlier, an ethidium-binding site located between the second and third base steps is postulated, based on the structural dimensions of the ethidium-modified duplex. Recent studies of base-damage reactions occurring within ethidium-modified duplexes upon irradiation with high-energy light indicate that the majority of binding occurs at the first base step. The assignment used here is conservative, however, as ethidium also intercalates to a much lesser extent at the second base step. It is worth noting that although a narrow distribution of possible binding sites might exist, the binding site was held constant throughout the series of duplexes used to evaluate the electron-transfer distance dependence, allowing the effect of distance on the photooxidation efficiency and kinetics to be unambiguously evaluated. Therefore, with ethidium intercalated between the second and third base steps, and with 7-deazaguanine contained within a 5′-TZG site (top half of Table 2), the different duplexes have ethidium/7-deazaguanine distances of 10–27 Å.

In the series of duplexes shown in Table 2, fluorescence quenching was observed until the donor/acceptor separation exceeded six base pairs. Over the range in which quenching was detected (10–24 Å), the amount of quenching decreased from 70% to 4%. Fluorescence quenching was also investigated as a function of 7-deazaguanine flanking sequence, 5′-GZA (bottom half of Table 2). In this set of duplexes, which differ from those described above only in the sequence flanking 7-deazaguanine, quenching yields varied from 55% to 0% over an ethidium/7-deazaguanine separation range of 6–20 Å. Despite less efficient quenching at short distances for 7-deazaguanine stacked within the 5′-GZA site, the photooxidation still proceeded over long distances.

Table 2

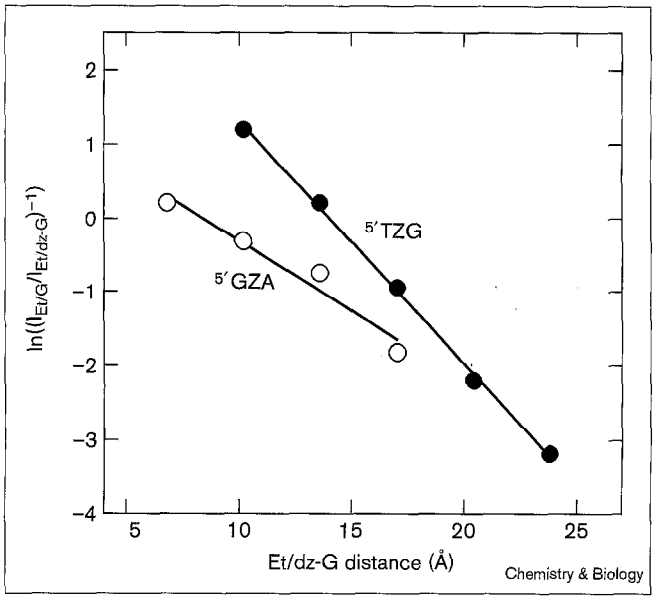
Steady-state fluorescence quenching measurements for Et/dzG duplexes.

	Et/dz-G separation (Å)*	I(Et/G)	I(Et/dzG)	Fraction quenched†
5′CT ACCAGACATCGT‡ GA TGZTCTGTAGCA	10	0.36	0.11	0.70(5)
5′CT ATCCAGCATCGT‡ GA TAGZTCGTAGCA	14	0.40	0.18	0.56(4)
5′CT AATCCAGCTCGT‡ GA TTAGZTCGAGCA	17	0.37	0.27	0.28(2)
5′CT AAGTCCAGCTCT‡ GA TTCAGZTCGACA	20	0.33	0.30	0.10(3)
5′CT AAGCTCCAGTCT‡ GA TTCGAGZTCACA	24	0.33	0.32	0.04(2)
5′CT AAGCTTCCAGCT‡ GA TTCGAAGZTCCA	27	0.36	0.37	−0.03(3)
5′CT TCCAGACATCGT§ GA AZGTCTGTAGCA	6	0.40	0.18	0.55(3)
5′CT ATCCAGCATCGT§ GA TAZGTCGTAGCA	10	0.40	0.23	0.43(4)
5′CT AATCCAGCTCGT§ GA TTAZGTCGAGCA	14	0.38	0.25	0.32(2)
5′CT AAGTCCAGCTCT§ GA TTCAZGTCGACA	17	0.37	0.30	0.16(4)
5′CT AAGCTCCAGTCT§ GA TTCGAZGTCACA	20	0.38	0.38	−0.01(2)

Steady-state fluorescence intensity (I) relative to 10 μM $Ru(bpy)_3^{2+}$(λ_{exc} = 480 nm, 20°C, 5 μM duplex, 5 mM phosphate, 50 mM NaCl, pH7). *Distances calculated with Et intercalated between second and third base step. †Fraction quenched ($F_q = 1-(I_{Et\text{-}dzG}/I_{Et\text{-}G})$) calculated from 3–4 sets of independent samples; approximate errors are listed in parentheses. Deazaguanine is contained within a 5′ TZG sequence (‡) or a 5′ GZA sequence (§).

The quenching yields (Figure 6) in both sets of duplexes exhibit a shallow exponential dependence on distance, paralleling trends reported in studies of long-distance electron transfer between intercalators [17]. Importantly, the distance dependence of the ethidium/7-deazaguanine quenching reaction is sensitive to the sequence flanking the modified base. In the 5′-TZG site, the exponential function used to evaluate the dependence of the quenching efficiency on distance yields a slope (γ) of 0.33(3) $Å^{-1}$. For a 5′-GZA site, a more shallow distance dependence is observed with $\gamma = 0.20(4)$ $Å^{-1}$. By simply changing the flanking sequence, a change in the variation of quenching yield with distance was detected. The most obvious difference between these two sites is the number of purines flanking deazaguanine, and thus, because the duplexes are otherwise identical, this effect

Figure 6



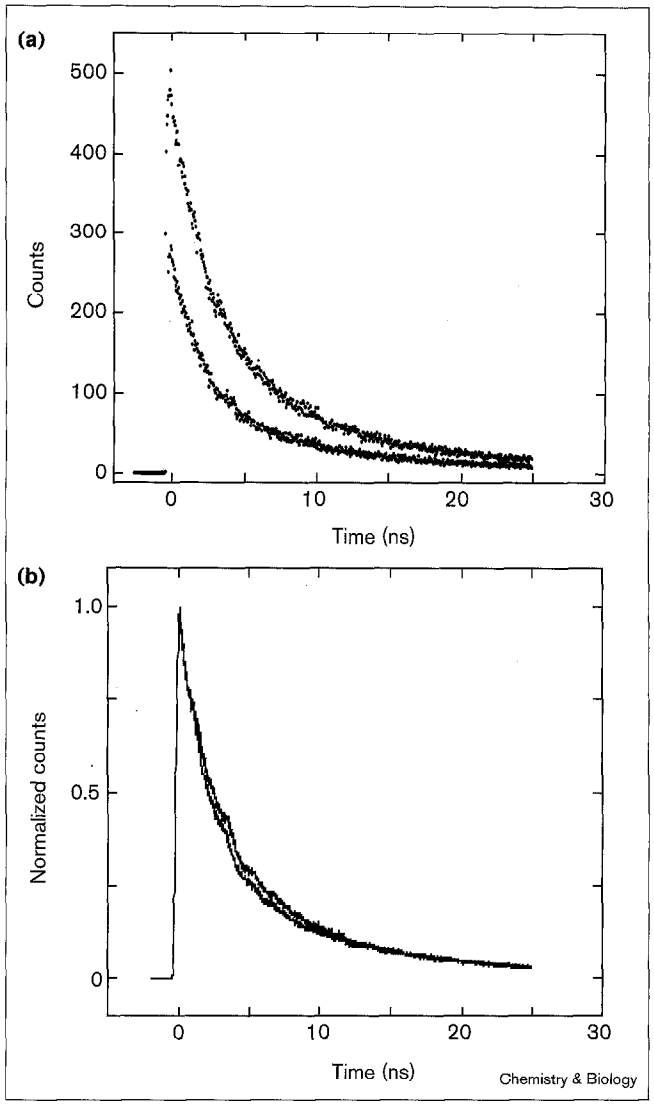
Exponential distance dependence for photoinduced oxidation of ethidium by deazaguanine. Data shown correspond to two different flanking sequences, 5' TZG (●) and 5' GZA (○).

might result from more favorable stacking in the site containing two purines [44].

It should be noted that these results are fully consistent with intramolecular processes. At the micromolar (1–15 μM) concentration range, the quenching yield is constant. If ethidium were to intercalate intermolecularly, we would expect essentially equal quenching at all distances. Indeed, when the concentration of ethidium-modified duplexes is raised above 20 μM, the observed quenching is somewhat less sensitive to the ethidium/7-deazaguanine separation. For duplexes modified only with ethidium, small amounts of self-quenching are also apparent at concentrations higher than 20 μM [17].

The kinetics of the photoinduced oxidation of 7-deazaguanine in these assemblies were investigated using time-correlated single photon counting (TCSPC; Figure 7). In all of the ethidium/7-deazaguanine duplexes, fluorescence quenching occurred on a subnanosecond time scale, that is, all of the observed quenching was manifested only as a decrease in initial intensity (static quenching), and not as a decreased ethidium excited state (*Et) lifetime. For example, in a duplex in which ethidium and the site of 7-deazaguanine modification were separated by ~ 14 Å, the ethidium excited state exhibited a biexponential decay with t1 = 8.7 ns (38%) and t2 = 2.6 ns (62%) for the duplex containing guanine. In the analogous deazaguanine-modified duplex, in which 55% quenching of excited-state ethidium emission is

Figure 7



(a) Time-correlated single photon counting measurements for Et-5'-CTATC(**C/G**)AGCATCGT (top trace) and Et-5' CTATC(**C/Z**)AGCATCGT (lower trace) illustrating static quenching on a nanosecond time scale (instrument response ~150 ps). Samples contained 5 μM duplex in 5 mM phosphate, 50 mM NaCl, pH 7; sequences indicated were hybridized to complements containing guanine or 7-deazaguanine at positions denoted in bold (see the top half of Table 2, 14 Å ethidium/7-deazaguanine separation for full duplex). **(b)** Normalized single photon counting for sequences listed above. The following lifetimes were obtained for the duplexes listed in Table 2: Et/G = 10 Å, τ_1 = 6.4 ns (30%), τ_2 = 2.7 ns (70%); Et/dz-G = 10 Å, τ_1 = 6.4 ns (25%), τ_2 = 2.3 ns (75%); Et/G = 14 Å, τ_1 = 8.7 ns (38%), τ_2 = 2.6 ns (62%); Et/dz-G = 14 Å, τ_1 = 9.4 ns (31%), τ_2 = 2.2 ns (69%); Et/G = 17 Å, τ_1 = 8.5 ns (32%), τ_2 = 2.2 ns (68%); Et/dz-G = 17 Å, τ_1 = 9.0 ns (26%), τ_2 = 2.1 ns (74%); Et/G = 24 Å, τ_1 = 8.4 ns (25%), τ_2 = 23 ns (75%); Et/dz-G = 24 Å, τ_1 = 8.4 ns (25%), τ_2 = 2.3 ns (75%). Fluorescence decay profiles for ethidium-modified single strands were multiexponential.

observed in steady-state measurements, the fluorescence decay profile is quite similar with t1 = 9.4 ns (31%) and

t2 = 2.2 ns (69%). Equivalent profiles were measured for all duplexes. Figure 7, depicting representative raw and normalized SPC data, graphically illustrates that the quenching is almost entirely static on the time scale of this experiment and is not reflected in large changes in measurable lifetimes or in the percentages of these components. As the resolution of this experiment is ~ 150 ps, these measurements indicate that the quenching process occurs faster than this time response at all donor–acceptor separations. Thus, consistent with our earlier studies, electron transfer in DNA can proceed across a range of extended distances on very fast time scales.

It is noteworthy that the excited-state decay profiles we observed are inconsistent with the presence of a significant concentration of free ethidium. *N*-8-glycyl ethidium has an excited-state lifetime of 440 ps in H_2O (which is shorter than that of ethidium because of the modification at the exocyclic amine), a decay component that would be easily detected in this experiment. The intrinsic lifetime of *N*-8-glycyl ethidium is shorter than that of underivatized ethidium (~ 2 ns), and, consistent with this observation, lifetimes for ethidium-modified duplexes are shorter than for ethidium bound to DNA. Both cases reveal similar degrees of fluorescence enhancement upon binding of the fluorophore within the hydrophobic environment of the base stack.

Sequence dependence

To explore in more detail the effect of the sequence environment surrounding 7-deazaguanine, duplexes were synthesized containing each of the four bases on either side of the modified base (Table 3). In this study, the base adjacent to 7-deazaguanine was varied in each duplex and the effect of these sequence changes on the quenching yield was monitored. Only very small perturbations in the quenching yield were observed when the adjacent base on the 5′-side was varied. Dramatic changes in the efficiency of photooxidation resulted when the 3′-sequence was altered, however. For instance, when 7-deazaguanine is located 3′ adjacent to cytosine, about 26% of the ethidium fluorescence is quenched; changing the 3′-site to guanine nearly doubled the quenching yield to 48%.

The variation in quenching with sequence observed may reflect small changes in oxidation potential of deazaguanine within the DNA helix. Theoretical studies have predicted that the ionization potentials for stacked base pairs should increase in the order 5′ GG < 5′ GA ≤ 5′ GT < 5′ GC [45]. In the series of duplexes we examined, a parallel trend in quenching efficiency was observed: 5′ dz-GG > 5′ dz-GT ≥ 5′ dz-GA > 5′ dz-GC. Interestingly, for the photoinduced electron-transfer reaction studied here, it appears that the sequence 3′ of the 7-deazaguanine is most crucial in determining the reactivity of 7-deazaguanine, consistent with theoretical prediction [45]. These results provide experimental evidence that stacked DNA bases have redox properties distinct from monomeric species in solution.

Effect of base mismatches

Single-base mutations were incorporated into ethidium/7-deazaguanine duplexes to monitor the effect of different 7-deazaguanine base pairings on the oxidation efficiency (Table 4). In a duplex featuring deazaguanine paired to its natural partner, cytosine, the quenching yield was 28%. When the modified base was paired to any other base, however, the efficiency of the oxidation reaction was significantly diminished (fraction quenched = 7–10%). These results indicate that the photoinduced reaction is mediated by the DNA base stack. If the photooxidation was occurring on the periphery of the helix, the instability of the stacking for 7-deazaguanine introduced by the presence of a mispair would surely enhance the fluorescence quenching.

Table 3

Effect of flanking sequence on Et/dzG quenching yield.

5′-variation	Fraction quenched	3′-variation	Fraction quenched
Et — 3′ — AZA — 5′	0.24(6)	Et — 3′ — TZG — 5′	0.35(3)
Et — 3′ — AZT — 5′	0.26(3)	Et — 3′ — GZG — 5′	0.48(1)
Et — 3′ — AZG — 5′	0.32(2)	Et — 3′ — CZG — 5′	0.26(1)
Et — 3′ — AZC — 5′	0.26(2)		

Quenching yields obtained in Table 2. Full sequence used in study: Et′-5′-CTAAXCYAGCTCGT, X/Y = T,A,G,C (C is base-paired with deazaguanine). All sequences displayed very similar amounts of hypochromicity (23(2)%) at 260 nm.

Table 4

Effect of base pairing on Et/dzG quenching yield.

	Fraction quenched*	T_m†	% Hypochromism (260 nm)†
Et 5′ — C — Z	0.28(3)	51.5	24
Et 5′ — T — Z	0.07(2)	41.0	19
Et 5′ — A — Z	0.09(1)	40.1	19
Et 5′ — G — Z	0.10(1)	37.8	19

Full sequence used in this study: Et′-5′-CTAATCXAGCTCGT, X = C, T, A, G (across from 7-deazaguanine). *Quenching yields calculated as described in Table 2. †Approximate error for melting temperatures (T_m) ~0.8°C. †Approximate error for % hypochromism ~1%.

An analysis of the helix melting profiles obtained via absorbance spectroscopy reveals that these mismatched duplexes have lower melting temperatures and reduced hypochromicity at 260 nm compared to the full Watson-Crick paired duplex. As hypochromicity is directly related to base–base interactions [46], it can be inferred that the presence of the mismatch causes a significant perturbation in the stacking of 7-deazaguanine within the duplex. These results, therefore, provide further evidence that the efficiency of long-range electron-transfer reactions within DNA is very sensitive to π-stacking effects.

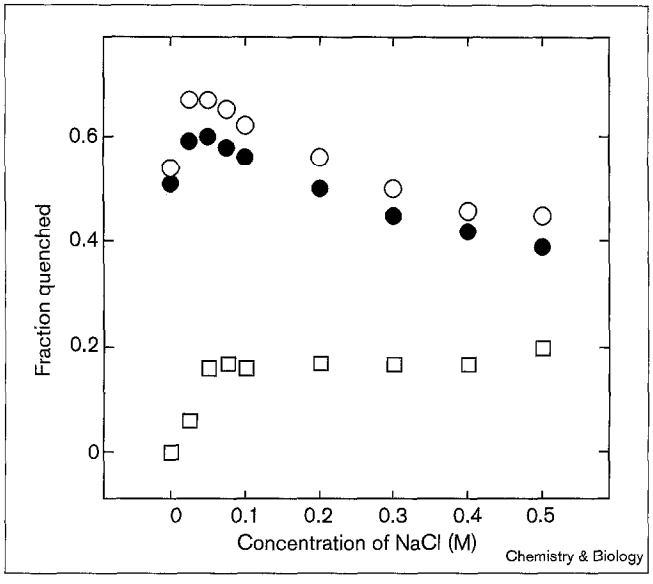
Effect of linker length on photooxidation

The remarkably fast time scale and shallow distance dependence of the described reaction necessitates a more detailed examination of the interaction of tethered ethidium with the helix. Is there any direct contact possible between the tethered ethidium and the 7-deazaguanine? It is well established that ethidium binds to DNA primarily via intercalation [40–43], and, on the basis of the physical and photophysical properties of the ethidium-modified duplexes, it appears that the ethidium is similarly intercalated when covalently tethered. If tethered ethidium were instead groove-bound, direct contact between the fluorophore and the modified base might be permitted, and hence this possibility required experimental consideration. Here again, the variation of the ethidium-DNA linker length and ionic strength provides a means to assess the contribution of a nonbase-stack-mediated pathway.

On the basis of the dimensions of the duplexes employed in this study, the C9 linker was the only tether sufficiently long to allow direct contact between ethidium and the 7-deazaguanine incorporated at the fifth base position from the tethered end. Such an interaction could only occur if the fluorophore were in a strained, extended position bound in the groove of the duplex. Even with the C9 linker, no direct contact appears possible between ethidium and any base position six or more sites from the tethered end on the complementary strand, where quenching yields up to 55% are observed. Moreover, for the C6 and C2 linkers, even if ethidium is fully extended along the DNA groove, no direct contact is permitted between ethidium and 7-deazaguanine when the modified base is located at the fifth base step (or further down the duplex). In fact, although these linkers appear to be quite long when extended, they are attached to the 5′ end of the DNA backbone, pointed away from the helix, and, in three-dimensional space, actually provide access to a very limited region of the duplex.

The quenching of ethidium by 7-deazaguanine is affected by linker length and ionic strength (Figure 8). At low ionic strengths (5 mM phosphate, 0–25 mM NaCl), smaller quenching yields are observed relative to those observed at higher ionic strengths (5 mM phosphate, 25–100 mM NaCl). Because groove binding has only been characterized for ethidium at low ionic strengths (< 0.01 M), these results appear to indicate that binding in the groove inhibits, rather than facilitates, the quenching reaction. It is interesting that in the duplexes containing C6 or C9 linkers, essentially identical levels of quenching are observed at all ionic strengths, even though the shorter C6 linker cannot directly contact the deazaguanine in the duplex (even if fully groove bound). The similar levels of quenching for these two linkers instead suggest that the fluorophore is intercalated at the same site in these two assemblies, a result also consistent with modeling. Ethidium tethered to the duplex by the C2 linker shows no quenching at very low ionic strength and is only quenched by 7-deazaguanine in the presence of sufficient ionic strengths to favor intercalation. Overall, lower levels of quenching are observed with the C2-modified duplex, probably a result of poorer ethidium stacking within the duplex, due to the constraints of the linker. The lower fluorescence of ethidium-modified duplexes containing the C2 linker supports this conclusion.

Figure 8



Effect of linker and ionic strength on fluorescence quenching by 7-deazaguanine for ethidium covalently bound to the duplex (Et-NH-CO-$(CH_2)_3$-CO-NH-$(CH_2)_n$-NH-CO-5′CTACCAGACATCGT (n=2 (□), n = 6 (○), and n = 9 (●). 7-deazaguanine is located at the fifth base step from the ethidium modified terminus.

Discussion

This study of the long-range photoinduced oxidation of deazaguanine by ethidium represents a systematic investigation of a DNA-mediated electron-transfer reaction between a tethered intercalator and a modified DNA base in mixed sequence DNA using fluorescence spectroscopy.

The dynamics, distance and sequence dependence of a base-oxidation reaction can be directly monitored in this system. We find that the reaction between ethidium and 7-deazaguanine occurs over extended distances through DNA on a remarkably fast time scale. Over distances of 6–24 Å, the reaction occurs within 150 ps, demonstrating that the DNA base stack can mediate efficient electron transport with little sensitivity to distance.

Because our probe is a modified base, we can examine electron-transfer reactions across a range of sequences using all four natural bases. We find that the efficiency of the electron-transfer reaction is remarkably sensitive to the flanking sequence surrounding 7-deazaguanine. Neighboring bases may serve to promote small changes in redox potential of the stacked deazaguanine [45], and these changes could lead to significant variations in quenching yield. Our observations agree with studies of oxidative damage to DNA that have identified correlations between base-oxidation potential as a function of sequence and preferred sites of damage [1–7]. The base pairing of 7-deazaguanine also modulates the efficiency of the photooxidation reaction. Paired to any base other than its natural mate, 7-deazaguanine is a much less efficient reductive quencher for the ethidium excited state. These observations indicate that the photooxidation reaction occurs through the base stack and that even very small changes (even a single base) in the composition of the stack can significantly alter the efficiency of the electron-transfer reaction. Perhaps most dramatically, the distance dependence of electron transfer differs depending upon the base(s) neighboring the reactant. This observation requires a more detailed consideration of the electronic coupling in double helical DNA; significant deviations arise with small, but obviously not insignificant, energetic differences within the base-pair stack.

In fact, the sensitivity of the photooxidation of 7-deazaguanine to very subtle changes in the structure of the base stack underscores the unique features of this medium. The DNA double helix is unlike any other structure through which long-distance electron transfer has been investigated. Protein biopolymers in solution provide primarily σ-bonded pathways for charge transport. Although structurally similar to the base stack of DNA, π-stacked arrays in the solid state [47] do not offer the dynamic fluctuations afforded to DNA molecules in solution. Π-stacking offers significant stabilization to the double helix [48], and depends upon both sequence and environment. The enthalpic contribution of π-stacking to the overall stability of DNA arises largely from a combination of dipolar and induced dipolar interactions between heterocyclic base pairs. Because the most direct electron-transfer pathway through DNA proceeds through the π-stacked bases, any model to explain these long-range processes must take into account how these stacking interactions influence the electronic structure of DNA.

Consideration of alternate mechanisms

Because in this study ethidium was attached to the DNA duplexes by a long and flexible tether, a thorough investigation of the binding geometry of ethidium within these duplexes was necessary to establish that the electron-transfer reaction was in fact mediated by the DNA base stack. The interactions of ethidium with DNA have been studied extensively [40–43], and the intercalation of ethidium is typically the benchmark by which the binding modes of other molecules are determined. Nonetheless, by examining the photophysics of the ethidium-modified duplexes with different linkers and as a function of ionic strength, the interactions between ethidium and the DNA helix in these assemblies can be better understood.

Direct contact between 7-deazaguanine and ethidium in these assemblies is only possible if the tethered fluorophore is not intercalated, but is instead extended into the groove of the helix. Yet, ionic strength conditions that should have maximized groove binding instead inhibited the quenching reaction. Based upon CPK modeling, direct contact is only possible with a C9 linker and even with this tether, only at the smallest donor–acceptor separation investigated in this study. Significant quenching on a fast time scale is apparent across a range of donor–acceptor separations with the C9 linker, however, and with a C6 analog (where direct contact with 7-deazaguanine is precluded even at the closest donor–acceptor separation employed) equivalent quenching is observed. Ethidium-modified duplexes containing a C2 linker show substantially lower levels of quenching in the presence of deazaguanine, but also exhibit reduced fluorescence quantum yields, indicating that the binding allowed by the C2 tether does not provide a high degree of protection from solvent. The constrained binding with this tether might also limit the coupling of the fluorophore into the DNA base stack.

In addition, if some percentage of ethidium were groove-bound, a distinct excited-state population should be detected, and this population should likewise be selectively quenched. Photoexcited ethidium bound within these duplexes exhibits a biexponential decay profile with lifetimes on the order of 7 and 2 ns. Both lifetimes reflect significant enhancements relative to free *N*-8-glycyl ethidium (400 ps), and the average ratio of τ(free)/τ(intercalated) for free ethidium (the ratio is 11) [43] parallels that observed with intercalated ethidium (τ(free)/τ(intercalated) = 12). Lifetimes intermediate between free and intercalated are typically observed for groove-bound ethidium [43] with levels of enhancement lower than either of the components described for the covalently

bound fluorophore. If the shorter of the observed lifetimes did correspond to groove-bound ethidium, then this component should be selectively quenched, but instead, both lifetimes are equally quenched on a fast time scale.

The contribution of dynamic exchange between a groove-bound and intercalated form of ethidium to the quenching reaction might also be considered as a possible explanation for the results described here. The fast kinetics of the quenching observed across the entire range of donor/acceptor distances appear to preclude such a dynamical process. On the picosecond time scale of the quenching, essentially only static conformations of ethidium within the duplexes can be considered. Moreover, previous studies of steady-state fluorescence polarization trends for ethidium tethered to DNA duplexes [17] indicated rigid binding of this species to the duplex.

Based on the experimental evidence presented, it appears that this remarkably fast electron-transfer reaction occurs between intercalated ethidium and deazaguanine and is facilitated by the base stack, not through space contacts. The significant effects of local sequence within the duplexes also indicate that the photooxidation reaction must occur through the helix, as opposed to on the periphery.

Models for distance dependence

The photooxidation of deazaguanine is relatively insensitive to distance: it proceeds up to distances of 30 Å and occurs on a subnanosecond time scale over this entire range. The ultrafast time scale of the electron-transfer kinetics observed here over all distances precludes the calculation of β. It is nonetheless clear that β must be low (≤ 0.2 Å^{-1}), because rate constants less than 10^9 s^{-1} were not observed over a large range of distances (6–24 Å). Thus, for reactants that are well-stacked within the helix, the distance dependence of electron transfer must be exceedingly shallow.

Moreover, the change in photooxidation efficiency with distance without appreciable changes in electron-transfer dynamics indicates that a mechanism unique to the double helix as a stacked array in solution is operative. In studies of electron transfer between intercalators covalently bound to DNA, the same behavior was observed [17]. In that case, the observed distance dependence was proposed to result from conformational heterogeneity arising from the internal dynamics of the DNA helix. This heterogeneity within the base stack could be envisioned to give rise to disruptions in π-stack mediated coupling and determine the yield of electron transfer at a given distance. Because the probability of having a properly stacked duplex on the time scale of the photoinduced experiment would depend exponentially on the number of intervening base pairs, this model provides a reasonable explanation for the distance dependences of the quenching yields in these DNA-mediated reactions. Thus, if such motions serve to gate electron transfer through the base-pair stack, this reaction might offer a new measurement of base-pair dynamics.

The remarkable characteristics of DNA-mediated electron transfer have often prompted discussion of DNA as a molecular 'wire' [15,24,25,49,50]. From the work described here and elsewhere [11–18], it is increasingly clear that the DNA base-pair stack can facilitate long-range, ultrafast reactions. Given the apparent sensitivity to base-pair stacking dynamics, if double-helical DNA can function as a 'wire', it does so transiently. Thus, the base-pair stack provides remarkable long-range coupling, but also limits the range over which this type of reactivity can proceed because of its dynamical nature.

Other factors may also modulate the trends observed in this study. This DNA-mediated electron-transfer reaction is slightly more sensitive to distance ($\gamma = 0.20$–0.35 Å^{-1}) than the oxidative quenching reaction observed between ethidium and $Rh(phi)_2bpy^{3+}$ ($\gamma = 0.10$ Å^{-1}) [17], but other differences between these reactions exist. The ethidium/7-deazaguanine reaction might proceed through an occupied orbital state of the DNA bridge, as ethidium is being reductively quenched, whereas the ethidium/$Rh(phi)_2bpy^{3+}$ reaction might involve an unoccupied bridge orbital state for the oxidative quenching reaction. These states have different electronic structures and different energy gaps relative to the ethidium excited state. In addition, the driving forces for these two reactions vary significantly (ethidium/7-deazaguanine: $\Delta G \sim -200$ mV, ethidium/$Rh(phi)_2bpy^{3+}$: $\Delta G \sim -800$ mV). In addition, it is worth noting that these systems employ reactants (metallointercalator versus DNA base) that might interact with the base stack differently, and this might also affect the efficiency of the reaction. More systematic studies exploring the effects of these parameters on DNA-mediated electron transfer reactions are necessary to determine the origin of the different distance dependences observed in these systems.

Certainly, how the donor and acceptor are coupled into the DNA base stack is a critical issue, and the application of a modified base as probe of long-range electron transfer has permitted us directly to monitor the effect of subtle changes in stacking on the reactivity of this molecule within the helix. The incorporation of 7-deazaguanine in a base mismatch, for example, significantly decreases the yield of fluorescence quenching. Generally base mismatches do not result in gross structural changes within the DNA helix [48], but instead are known to cause only local disruptions in stacking. This result, therefore, underscores the exquisite sensitivity of the electron-transfer phenomenon to small perturbations in stacking. Here, the presence of the mismatch also causes a measurable decrease in the

hypochromicity of these duplexes, confirming that stacking of deazaguanine within the base stack is significantly altered. Therefore, although the reactants still retain the same through-space separation, the change in stacking for 7-deazaguanine results in a dramatic decrease in the efficiency of this electron-transfer reaction.

Stacking also appears to dramatically modulate the distance dependence of DNA-mediated electron transfer. For the photooxidation of deazaguanine, the variation in the yield with donor/acceptor separation (γ) clearly depends upon the sequence surrounding the modified base. A more shallow distance dependence is observed for deazaguanine stacked within a purine–purine site, perhaps indicating that greater stacking stabilization within a given sequence provides more favorable electronic coupling for this long-range reaction. Importantly, this result provides the first demonstration that the distance dependence of a DNA-mediated electron transfer reaction is not only sensitive to the identity of donor and acceptor, but is also affected by the stacking environment of the donor and acceptor.

Reconciliation of previous studies: stacking as a parameter governing electron transfer through DNA

As evident from the above discussion, base stacking affects many aspects of the photooxidation of 7-deazaguanine by ethidium. Base stacking may determine the electron-transfer yield at a given distance, influence the ability of this modified base to serve as an electron donor within base mismatches, and also modulate the overall distance dependence for this reaction. The stacking interactions available within the DNA helix distinguish this medium structurally from any other examined in systematic studies of electron transfer.

Different studies of electron-transfer processes within DNA have reached conclusions very distinct from those discussed with respect to our system [21–26]. Much slower electron-transfer kinetics and steeper distance dependences have been observed, leading to a great deal of controversy concerning the nature of DNA as an electron-transfer medium. It is now clear that there are factors other than donor/acceptor distance that must be considered in order to make any conclusions concerning the efficiency of charge transport in DNA. The results described here underscore that point.

When intercalators or components of the base stack are used to probe DNA-mediated electron transfer, very fast reactions can be observed over extended distances. In systems employing groove-bound reactants, or partially stacked molecules, the time scales and reaction distances are diminished significantly [12,21–25].. In our own studies, we have observed very different reactivities for intercalating versus groove-bound species, and even among intercalators of different chiralities [11–15]. The electron-transfer kinetics in a stilbene-modified DNA hairpin must depend in part upon the interaction of this chromophore with the DNA bases [24,25]. As stilbene is not a molecule that naturally binds to DNA without being covalently constrained, it is likely that stilbene is not favorably stacked in a conformation analogous to intercalation; in fact, no hypochromism is associated with the interaction between stilbene and the DNA base stack. Significantly, still slower electron-transfer kinetics were observed with reactants attached to DNA only through σ-bonded linkages [23]. Most recently, a β value of 1.4 $Å^{-1}$ was obtained for a DNA-mediated electron-transfer reaction between an acridine-based fluorophore and guanine [26]. The level of electronic coupling through DNA indicated by this experiment was extremely poor, even when compared to the stilbene hairpin assembly. Biophysical studies [51] have indicated that substantial changes in fluorescence, hypochromicity, and thermal stability arise with small changes in the acridine assembly. How the acridine derivative is actually accommodated within the base stack and what local base-pair disruptions arise still need to be established for proper interpretation of these results.

Our recent investigations have not only focused on stacked reactants, but have also included tests of whether the DNA base stack represents the intervening pathway. Other studies have not addressed this issue. In fact, in cases where reactants are not directly interacting with the base stack, electron transfer might proceed through alternate pathways with higher intrinsic values of β. Indeed, even in fully stacked systems, we see here that very small sequence changes that alter local stacking interactions cause marked changes in the overall distance dependence of the reaction. It is reasonable, therefore, that reactants which do not have direct contact with the base stack, or have limited interactions, would display drastically different behavior. The results obtained in all of these systems may therefore be reconciled if the detailed interactions between the reactants and the base stack, as well as the structure of the base stack, are considered. The structural complexity of DNA, and the now apparent sensitivity of electron transfer through DNA to the stacking of reactants with the DNA bases, necessitates that careful consideration be given to all factors that might affect experimental results obtained in these types of experiments.

Significance

The base stack contained within the DNA helix is again implicated in the facilitation of long-range charge transport. This study of photooxidation of 7-deazaguanine, a modified base, by ethidium not only provides another example of a fast, long distance electron-transfer reaction mediated by the DNA helix, but also underscores the importance of another parameter besides distance: stacking. Base-stacking interactions determine the efficiency of long-range electron transfer in DNA and might

also determine the overall distance dependence of the electron-transfer process. This work establishes that the behavior of the DNA helix as a bridge and reactant in electron-transfer processes is contingent upon stacking interactions within the structure of DNA itself. Previous studies which suggest that DNA is a poor mediator of charge transport can now be reconciled within this context. Reactants that are not well-stacked within the DNA helix have repeatedly revealed much less efficient electron transfer than those that interact with the base stack via intercalation. We have demonstrated here that changing the stacking of a given reactant attenuates the reaction efficiency. The interaction of reactants with the DNA base stack, therefore, must be carefully considered when drawing conclusions about DNA-mediated electron transfer. It is becoming increasingly clear that to assess properly the nature of charge transfer in nucleic acids, and apply this knowledge to an understanding of base damage and repair mechanisms, the detailed structure of the biopolymer must be considered.

Materials and methods

Materials

Unless otherwise noted, all reagents were purchased from Aldrich or Fluka and used without further purification. Reagents for oligonucleotide synthesis were obtained from Glen Research. Triphosphate nucleotides were obtained from Pharmaicia.

Preparation of ethidium/7-deazaguanine duplexes

N-8-glycyl ethidium and ethidium-modified oligonucleotides (Et-NHCO$(CH_2)_3$CONH$(CH_2)_n$-5′-DNA, n = 2,6,9) were prepared as previously described [17]. Oligonucleotides containing deazaguanine were synthesized on a 394 ABI synthesizer using standard automated techniques [52], with the exception of a 3 min oxidation step employing 10-camphorsulfonyl oxaziridine (1g/10 ml CH_3CN). All conjugates and unmodified oligonucleotides were purified using reversed-phase high-performance liquid chromatography (HPLC). For the hybridization of duplex samples, appropriate amounts of complementary materials based on the extinction coefficient for ethidium-modified sequences ($\varepsilon_{484} = 4000\ M^{-1}\,cm^{-1}$) and calculated extinction coefficients for unmodified sequences (ε_{260} ($M^{-1}\,cm^{-1}$): $dC = 7.4 \times 10^3$; $dG = 12.3 \times 10^3$; $dzG = 10.5 \times 10^3$; $dT = 6.7 \times 10^3$; $dA = 15.0 \times 10^3$), were combined at 1:1 stoichiometry and dissolved in 5 mM phosphate, 50 mM NaCl (pH 7) to yield a final duplex concentration of 5 μM. The resulting solutions were heated to 90°C and slowly cooled to ambient temperature over 2–3 h to anneal the duplex.

Characterization of modified duplexes

As previously described [17], ethidium-modified sequences were characterized as single strands using mass spectrometry, base-digestion analysis, phosphate analysis, and ultraviolet–visible absorbance. In double-stranded form, thermal-denaturation measurements monitored by absorbance at 260 nm were used to confirm the integrity of modified duplexes (see above). The fluorescence intensities of the 14 base-pair duplexes used in this study were comparable to those observed for the variable-length series previously reported [17].

Electrochemical measurements

The reduction potential of ethidium was measured via cyclic voltammetry on a BAS CV-50W potentiostat in 0.1 M TBAH/CH_3CN. Potentials for 2′-deoxyguanosine triphosphate (dGTP; Pharmacia) and 7-deaza-2′-deoxyguanosine triphosphate (dz-dGTP; Pharmacia) were obtained on the same apparatus in 0.1 M phosphate buffer, pH 7. Cyclic voltammetry was carried out at 20°C with a normal three-electrode configuration consisting of a glassy carbon working electrode, saturated calomel reference electrode, and platinum auxiliary electrode. The ethidium excited-state reduction potential ($E^o(^{*/0})$) was calculated using the expression $E^o(^{*/0}) = E_{oo} - E^o(^{+/0})$. Values of E_{oo} were approximated both by averaging absorption and emission maxima and examining intersection points of absorption and emission spectra. Both methods yielded equivalent results for ethidium. Potentials are reported versus NHE.

Photophysical measurements

Steady-state fluorescence experiments. Steady-state fluorescence measurements were made at 20°C with excitation at 480 nm on an SLM 8000 spectrofluorimeter. Fluorescence intensities were integrated from 520 to 800 nm. Samples with identical absorbance at the wavelength of excitation were measured at a duplex concentration of 5 μM in 5 mM phosphate, 50 mM NaCl, pH 7. Quenching yields for modified duplexes obtained by steady-state fluorescence measurements were calculated from 3–4 samples sets.

Time-correlated single photon counting (TCSPC). TCSPC was carried out using facilities described previously [16] with $\lambda_{exc} = 335$ nm obtained by doubling the 670 nm fundamental line; data fitting was accomplished using least-squares methods in a commercial software program (Axum). Data sets contained 5,000–10,000 counts, except in cases when small time windows were necessary to ensure constant laser power. Steady-state quenching yields obtained with $\lambda_{exc} = 480$ nm were identical to those observed with $\lambda_{exc} = 335$ nm. Sample conditions and concentrations were identical in time-resolved and steady-state measurements. No photodegradation was observed, as confirmed by UV–Vis absorption spectra taken before and after irradiation.

Melting profiles

Thermal denaturation experiments were performed on a HP8452A diode-array spectrophotometer with samples at a duplex concentration of 5 μM in 5 mM phosphate, 50 mM NaCl (pH 7). Absorbance at 260 nm was monitored every 2°C with 3 min equilibration times.

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